SULPHOBROMOPHTHALEIN EXCRETION IN PREMATURE INFANTS

BY

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It is probable that 'physiological' jaundice of the newborn is due primarily to the inability of certain immature and fullterm infants to synthesize glucuronides efficiently. Glucuronides are formed by the transfer of glucuronic acid from uridine di-phosphate glucuronic acid (U.D.P.G.A.) to acceptor substances such as bilirubin. This reaction is enzymic and is carried out largely by the liver, although other organs may participate. It has been shown by experiments *in vitro*, using both human and animal liver tissue, that the activity of the liver in performing glucuronide synthesis is absent until about mid-gestation, rises slowly up to birth and then increases quite rapidly until adult values are reached at 14-20 days of post-natal life.

It is of fundamental interest to know whether the development of these enzyme systems is a function of chronological maturity or whether it is largely an adaptive process brought about by the necessities and opportunities of extra-uterine life. In the premature infant failure to acquire bilirubin homeostasis may result in kernikterus which renders the problem of more than academic concern. It seemed worth while, therefore, to examine the ability of the premature infant to clear sulphobromophthalein (B.S.P.), a substance which is believed to undergo excretion by the liver as a glucuronide. This dye is in general use as a test of 'liver function' and has been applied previously to infants, although not in an endeavour to obtain evidence concerning glucuronide synthesis.

Materials and Methods

The B.S.P. excretion curve was obtained in 34 premature infants. Three groups were selected: (1) Infants aged 0-24 hours (14 infants); (2) infants aged 5 days

(10 infants); (3) Infants aged 14 days (10 infants). Apart from post-natal age no special selection took place and infants were tested as available. The test was not performed on babies whose low birth weight might have been due to placental insufficiency nor were ill infants investigated. Jaundice was not regarded as a contra-indication if there was no evidence of haemolytic disease. Some tests were attempted but failed for technical reasons, and these are not included in the results. The infants received routine premature care which included the administration of potassium menaphthosulphate (Vikastab) 2 mg. soon after birth. In order to ensure that this vitamin K analogue did not affect the B.S.P. test, it was not given to three of the 1-day-old babies.

The B.S.P. excretion test was modified in order to reduce venepuncture to a minimum and to allow estimation of B.S.P. on small samples of blood. The following procedure was adopted. At the beginning of the test at least 1 ml. of whole blood was drawn by heel prick to provide a serum blank and for micro-estimation of serum bilirubin (Evelyn and Malloy). The test dose of sulphobromophthalein, 5 mg. per kg. body weight diluted in 1 ml. of sterile normal saline, was injected slowly into an antecubital or superficial ankle vein. Following the injection of B.S.P., 0.5 ml. samples of capillary blood were obtained by heel prick at five, 15, 30 and 45 minutes and at one hour. These times were accurately recorded. No local or general reactions were observed in any of the infants.

Estimation of B.S.P. The blood was collected into small centrifuge tubes without anti-coagulant. The samples were incubated at 37° C. for one to two hours before centrifuging in order to aid clot retraction. The serum was drawn off and the test solutions made up as follows: Serum 0.1 ml. Buffer 0.1 ml. (Sorénson glycine buffer (pH 10.48) made up to molar strength). Water 0.8 ml. Each test solution was read in the spectrophotometer against its own blank which comprised serum 0.1 ml. and 0.9 ml. isotonic Na₂SO₄. The optical density readings were obtained with the Hilgar 'Spekker' instrument with filter No. 606 (corresponding to 580 mμ), and the 1 cm. micro cell was used for the solutions.

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A calibrations curve was made using a standard solution of B.S.P., 10 mg. per 100 ml. in serial dilutions as follows:

Standard Solution of B.S.P. (ml.)	Serum (ml.)	Buffer (ml.)	Water (ml.)	B.S.P. (mg./100 ml.)
0.1	0.1	0.1	0.7	2
0.2	0.1	0.1	0.6	4
0.3	0.1	0.1	0.5	6
0.4	0.1	0.1	0.4	8
0.5	0.1	0.1	0.3	10

These solutions were read in the same instrument against a blank solution containing serum 0.1 ml.; isotonic Na_2SO_4 0.9 ml.

The B.S.P. excretion curve was drawn for each infant by plotting the amount of B.S.P. in mg. % remaining in the serum against time after injection of B.S.P. At least five points were obtained for each infant. If the amount of B.S.P. remaining in the serum is converted into logarithms the B.S.P. excretion is transformed into two straight lines whose slopes can be measured. The first rapid phase of B.S.P. removal we have termed Slope I, the second slower phase, Slope II. As we have modified the standard B.S.P. excretion test in order to use capillary blood, it was necessary to perform our test on adults. Altogether 23 adult volunteers without liver disease were tested. In six, parallel samples of venous and capillary blood were used for estimation of B.S.P. We were satisfied that the method used with infants was comparable with standard methods.

Results

The results are most conveniently expressed in terms of the two phases of B.S.P. excretion as measured by Slopes I and II of the semi-log plot: and also conventionally as the percentage of B.S.P. retained in the blood at 45 minutes. These results for individual babies are given in Tables 1, 2 and 3, together with correlations for sex, birth weight and incidence of jaundice. It should be noted that the 1-day-old babies (Table 1) have been classified according to the presence or absence of hypoxia. Although no infant was sick at the time the estimation was performed, some infants had undergone a period of respiratory difficulty marked by dyspnoea, apnoeic attacks or intermittent cyanotic spells. Except for Case 12, this was not serious, but it nevertheless appears to have markedly affected the results of the B.S.P. excretion test in the 1-day-old babies.

Jaundice was marked present or absent on clinical grounds. It applied to the neonatal course of the baby as well as to conditions existing at the time of the B.S.P. test. A baby was classified as 'jaundiced' if the icterus was sufficient to justify estimating the serum bilirubin. In the cases so classified serum bilirubin values of more than 10 mg. % (Malloy and Evelyn) were found. It can

TABLE 1
FOURTEEN PREMATURE INFANTS UNDER 1 DAY OF AGE WITH AND WITHOUT RESPIRATORY DISTRESS

No.	Sex	Gestational Age (weeks)	Weight (g.)	Slope I	Slope II	% B.S.P. Retention at 45 min.	Jaundice
1	F	39	2,240	0.02	—	18.6	—
2	M	38	1,814	0.04	0.013	11.5	—
3	F	35	2,353	0.05	0.004	11.2	—
4	F	34	1,729	0.03	0.006	17.4	—
5	F	34	1,588	0.04	0.002	20.8	+
6	F	37	2,410	0.04	0.008	17.0	—
7	M	37	2,041	0.02	0.011	10.7	—
Means			2,025	0.03	0.006	15.3	
		Infants 4-23 hours age		No respiratory distress			
8	M	38	2,410	0.03	0.001	14.6	—
9	M	38	2,155	0.02	0.001	18.6	+
10	M	37	1,724	0.02	0.002	23.9	—
11	F	36	2,155	0.06	0.005	8.1	+
12	F	38	2,126	0.02	0.001	33.1	Died
13	M	35	1,588	0.02	0.009	23.9	—
14	M	36	1,899	0.01	0.006	36.3	—
Means			2,008	0.03	0.004	22.6	
		Infants 4-23 hours age		Respiratory distress

FIG. 1.—Mean B.S.P. excretion curves in three groups of babies after intravenous injection of B.S.P. 5 mg. per kg. body weight.

TABLE 2
TEN PREMATURE INFANTS 5 DAYS OF AGE

No.	Sex	Gestational Age (weeks)	Weight (g.)	Slope I	Slope II	% B.S.P. Retention at 45 min.	Jaundice
15	M	34	2,013	0.03	0.012	12.3	+
16	M	36	2,183	0.03	0.006	9.3	+
17	M	32	1,871	0.02	0.015	7.1	+
18	F	36	1,814	0.04	0.020	5.6	+
19	M	37	1,928	0.02	0.001	18.6	+
20	M	38	2,410	0.04	0.010	10.9	+
21	M	37	2,182	0.02	0.013	13.2	—
22	M	37	1,984	0.04	0.010	15.8	—
23	F	38	2,353	0.03	0.008	13.8	+
24	M	35	2,296	0.04	0.010	15.5	—
Means			2,103	0.03	0.011	12.2	

be seen by inspection of the tables that jaundice did not correlate with poor ability to excrete B.S.P.

Mean excretion curves for the three groups of infants are shown in Fig. 1. It is apparent that there is a progressive trend toward adult excretion of B.S.P. from the 1-day to the 14-day-old infants. Although both stages of B.S.P. excretion are slower in the youngest infants, the most marked differences are in the excretory phase (Slope II) and in the 45-minute retention

values. The difference in 45-minute retention between the 1-day and 14-day-old babies is

FIG. 2.—B.S.P. excretion curve in infant with atresia of bile ducts.

FIG. 3.—B.S.P. excretion curve of infant aged 4 days with 'inlissated bile syndrome'.

statistically highly significant ($P = 0.005$).

As an aid to the interpretation of the excretion curves the opportunity was taken to perform the test on infants known to have obstructive jaundice. The curves of two such infants, a case of congenital atresia of the bile ducts (Fig. 2) and a case of haemolytic disease with obstructive jaundice ('inlissated bile syndrome') (Fig. 3) are shown. In the case of the latter infant it is noteworthy that the abnormal curve is due to the biliary obstruction not to the haemolytic disease as two cases of severe haemolytic disease without direct reacting bilirubin in the serum gave normal curves.

TABLE 3
TEN PREMATURE INFANTS 14 DAYS OF AGE

No.	Sex	Gestational Age (weeks)	Weight (g.)	Slope I	Slope II	% B.S.P. Retention at 45 min.	Jaundice
25	F	37	1,871	0.06	0.031	2.1	—
26	F	38	2,381	0.06	0.009	6.5	+
27	M	34	1,474	0.06	0.009	7.5	+
28	F	36	2,155	0.04	0.012	13.3	+
29	M	33	1,474	0.03	0.020	7.9	+
30	M	38	2,265	0.04	0.014	9.8	—
31	M	37	2,070	0.05	0.009	10.0	—
32	M	38	1,474	0.04	0.020	5.1	—
33	F	36	1,304	0.04	0.008	10.0	—
34	M	36	1,389	0.03	0.020	8.9	—
Means			1,786	0.05	0.015	8.1	

Discussion

Sulphobromophthalein (B.S.P.) has been used by several investigators to test 'liver function' in newborn infants. There is general agreement that during the first few days of life B.S.P. is inadequately cleared by both fullterm (Mollison and Cutbush, 1949; Yudkin, Gellis and Lappen, 1949) and premature infants (Obrinsky, Denley and Brauer, 1952). Direct comparison of our results with those of previous workers is not possible because of differences in method, and selection of cases. However, it is clear that a progressive improvement in the ability of infants to remove B.S.P. from the circulation takes place during the first 21 days of post-natal life. The impairment of B.S.P. clearance noted in the first few days of life is not clearly related to gestational age, birth weight or the incidence of jaundice. It seems likely, therefore, that the development of the excretory mechanism for B.S.P. is part of the physiological process of adaptation to extra-uterine life rather than a function only of maturity. We have, in addition to confirming these facts, showed that perinatal hypoxia of even mild degree can result in demonstrable further impairment of B.S.P. clearance.

Sulphobromophthalein is an unphysiological substance which is excreted almost exclusively in the bile (Rosenthal and White, 1925). Following intravenous injection it disappears rapidly from the blood at a rate which is influenced by several factors, including hepatic blood flow, metabolism within the liver cell and the patency of the bile ducts. Although the metabolism of B.S.P. is incompletely understood, its chemical relationship with other phenolic compounds, such as phenolphthalein, leads one to expect it to be excreted as a glucuronide. There is *in vitro* evidence that this is so (Brown and Burnett, 1957). For this reason it is permissible to regard the metabolism of B.S.P. as similar in some respects to that of bilirubin which is also excreted in the bile as a glucuronide (Schmid, 1956).

There is now much direct experimental evidence that the enzyme systems concerned with glucuronide formation have only limited activity in foetal and early neonatal life. Lathe and Walker (1957) demonstrated poor bilirubin conjugation by newborn human liver slices and Dutton (1958) has shown only negligible amounts of glucuronide formation in human foetal liver and kidney. Brown and Zuelzer (1958), working with the microsomal fractions of guinea pig liver, have demonstrated increasing conjugating activity from almost zero at mid-gestation to near adult at 14-20 days of post-natal life. At least two enzymes, uridine-diphosphate-glucose dehydrogenase and glucuronyl

transferase, are concerned in this reaction and it is the diminished activity of these enzymes which is responsible for the restricted conjugation ability of foetal and neonatal tissues. It is of interest to note that other enzyme systems essential in post-natal life, but not required in foetal life, also show negligible activity in foetal and early neonatal life, as has been demonstrated by Kenney, Reem and Kretchmer (1958) for phenylalanine hydroxylase and glucose-6-phosphatase.

It is tempting to ascribe the defect in B.S.P. excretion, which we have shown, to a conjugation deficiency. Were this so it is clear that the events of post-natal life are more important than gestational age and birth weight in bringing the process to maturity. This suggests strongly that during foetal life the conjugation mechanism is dormant and is either excited by the stimulus of birth or released from inhibition. Whether birth acts as a stimulus because of the cessation of placental function forcing the baby to conjugate its own bilirubin and other substances or whether the change in the *milieu interieur* of the liver in regard to temperature and oxygenation is the main factor is uncertain. Both factors may be of consequence. Stempfel, Broman, Escardó and Zetterström (1956) have shown that infants with severe haemolytic disease of the newborn may be born with significant amounts of conjugated (direct-reacting) bilirubin in the cord blood. This suggests that hepatic conjugation takes place during intra-uterine life if massive haemolysis creates a large enough bilirubin overload. On the other hand, our results showing the effects of even mild hypoxia in post-natal life suggest that changes in the internal environment of the liver are of equal importance. Some of the effects of anoxia on liver blood flow and function have been reviewed by Andrews (1957) and it is clear that the metabolism of the liver is extremely susceptible to changes in oxygenation.

However, we cannot assume with confidence that B.S.P. excretion is identical with that of bilirubin. There is no direct relationship between poor B.S.P. elimination and the presence of jaundice. Some babies who clear B.S.P. rapidly become jaundiced and some of the poor B.S.P. excretors maintain effective bilirubin homeostasis. Furthermore, it has been reported that humans suffering from congenital hyperbilirubinaemia due to defective conjugation have normal B.S.P. excretion (Crigler and Najjar, 1952); the same is true of congenitally jaundiced rats (Axelrod, Schmid and Hammaker, 1957). While we have not succeeded in showing that the degree of chronological maturity is of importance in determining B.S.P. clearance, there is

abundant evidence that prematurity is of importance in relation to hyperbilirubinaemia (Billing, Cole and Lathe, 1954; Meyer, 1956). In this connexion it is noteworthy that environmental factors, such as aceto-menaphthone (Synkayvit) (Bound and Telfer, 1956; Meyer and Angus, 1956); and sulphafurazole (Gantrisin) (Silverman, Andersen, Blanc and Crozier, 1956), do exert a profound effect on the incidence and severity of neonatal jaundice. In spite of these inconsistencies in the parallel between bilirubin and B.S.P. metabolism we feel that the demonstrated impairment of B.S.P. clearance is related to a conjugation defect, but other explanations must be discussed.

It is possible that some form of biliary obstruction might explain the abnormal B.S.P. curves and it is therefore of interest to compare the infants in the first days of life with those with obstructive jaundice. In both instances the initial phase of B.S.P. removal (Slope I) is rapidly accomplished. This is probably related to liver blood flow. The most marked changes are in the second phase. The completely obstructed infant, P.D., showed a flat Stage II slope (Fig. 2) while an infant with neonatal 'hepatitis' and the infant with 'inspissated bile syndrome' (Fig. 3) showed curves not dissimilar to the normal first-day infants. We reject, however, the interpretation that the first day curves are due to biliary obstruction. If retention or regurgitation of bile occurred regularly in the first few days of life evidence of conjugated bilirubin would be expected in the blood and urine, but none is found. If this absence of conjugated bilirubin is ascribed to lack of conjugation, then hyperbilirubinaemia should always occur. Most often it does not. Biliary obstruction cannot explain the impaired B.S.P. clearance in non-jaundiced infants. Obrinsky *et al.* (1952) thought that post-natal circulatory adjustments consequent upon birth and affecting the blood flow to the liver were an important factor. If this were so, alterations in the first phase of B.S.P. removal would be prominent. We were unable to show marked alterations in Slope I and in this are at variance with Obrinsky and his colleagues. However, their data do not show that complete excretion curves were obtained on each baby and it is only by obtaining adequate early samples that deductions as to the first stage can be made. The clearance of B.S.P.

is related to its concentration in the hepatic arteries and the metabolic activity of the bile ducts (Andrews, 1955). We can offer no evidence on these points, although they may well be of significance in regard to the interpretation of our findings.

Summary

The B.S.P. excretion test has been applied to three groups of premature infants aged 1 day, 5 days and 14 days respectively.

The curves obtained show a progressive change towards adult values related to post-natal age but not gestational age, birth weight or jaundice.

Mild hypoxia produces a further impairment of B.S.P. excretion in the infants less than 24 hours old.

The relationship between these findings and hyperbilirubinaemia of prematurity is discussed.

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