Papers and Originals

Liquor Bilirubin Levels in Normal Pregnancy: A Reassessment of Early Prediction of Haemolytic Disease

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Summary: A comparison was made between chemically and spectrophotometrically determined concentrations of total bile pigment in the liquor amnii. For the period 16 to 26 weeks' gestation the upper limit of normal bile pigment to protein ratio was found to be 0.4. Levels above this would be required as an indication for intra-uterine transfusion. In 110 cases of isoimmunization of pregnancy these criteria were applied in the diagnosis of severity of the condition. The ratio of the bile pigment to protein was used rather than a simple bile pigment measurement, and found to be valuable.

Nevertheless, it is important to emphasize that fallacious high bilirubin readings may arise by using whatever method, particularly owing to bilirubin and other breakdown products of blood contaminating the liquor.

Introduction

In spite of the wide use that is now made of liquor analysis in assessing the severity of haemolytic disease (Bevis, 1956, 1967; Walker, 1957; Mackay, 1961; Liley, 1963; Walker *et al.*, 1964; Freda, 1965), opinions still differ on the merits of chemical (Wild, 1961; Watson, 1962; Robertson, 1964; Watson *et al.*, 1965; Bower and Swale, 1966) compared with spectrophotometric (Liley, 1961; Alvey, 1964; Knox *et al.*, 1965; Fleming and Woolf, 1965; Savage *et al.*, 1966) methods of estimating bile pigment and on the formulation of diagnostic criteria likely to give the most accurate prognosis.

We have found that the number of correct predictions of the degree of severity of the disease increased when these were based on the ratio of bile pigment to protein in the liquor (Morris *et al.*, 1967). On the other hand, Walker *et al.* (1969), using their optical transmittance ratio (Knox *et al.*, 1965) to measure pigment, found that the number of correct predictions decreased when the transmittance/protein ratio was used. We therefore decided to examine a further series of Rh-isoimmunized pregnancies, five times as large as the last (Morris *et al.*, 1967).

During the past three years the advancing efficiency of intrauterine transfusion technique, together with development of highly sophisticated equipment, has meant that increasing numbers of cases have begun treatment soon after 20 weeks' gestation. The aim has been to try to save those babies which otherwise would have died in utero long before 28 weeks.

It became clear to us that our earlier findings (Morris *et al.*, 1967) on liquor from normal Rh-positive mothers between 30 and 34 weeks might not provide a satisfactory basis for diagnosis of severity of haemolytic disease 10 weeks earlier.

We therefore decided to examine a further group of liquors from normal pregnancies between 16 and 26 weeks.

Spectrophotometric methods for estimating bile pigment in liquor are usually devised so that they are relatively unaffected by traces of fresh blood, while frank haemolysis can be seen readily and the specimen rejected. What is not as widely appreciated is that serious error in prediction can arise from a placental bleed some days before amniocentesis. To stress this danger we have singled out a case from the series of isoimmunized mothers. Though delivering an Rh-negative fetus, this patient would have been predicted initially as having severe haemolytic disease owing to unsuspected contamination of the liquor with altered blood, whichever of the current methods was used for liquor bilirubin analysis.

Patients and Methods

Specimens of liquor amnii from 22 normal mothers were examined between 16 and 26 weeks' gestation. A few very early specimens were obtained from amniotic sacs of fetuses removed during termination of normal pregnancies. Those from 19 weeks onwards were from Rh-positive volunteers who were informed that the test was in no way part of their treatment but was only for research purposes. It is our practice to determine the position of the placenta by using ultrasound before performing amniocentesis. Consequently a number of the volunteers who were found to have anterior placentae were excluded from the investigation. We also examined 110 samples of liquor from Rh-negative mothers with histories of isoimmunization where a diagnosis of the severity of the disease was needed to help decide the line of treatment. Specimens were obtained from 63 mothers before the 27th week of gestation. These cases were thought to be severely affected and likely to require intrauterine transfusion. The remaining 47 less severely affected cases were tested between 27 and 36 weeks.

Liquor amnii was withdrawn by abdominal paracentesis (Walker and Jennison, 1962). Any red cells were removed by centrifugation in a clinical centrifuge (2,500 r.p.m.). The hazy supernatant was then clarified by centrifugation (2,500 r.p.m.; M.S.E. Major) through a cellulose pad (Carlson-Ford EKS/2 11/16 in. (1.7 cm.) diameter) in a Hemmings Seitz filter.

The optical density extinction (E) of the clarified liquor was measured against water as a blank in a 1-cm. cuvette at 454, 490, 520, and 574 nm. with a Unicam SP 500 spectrophotometer. The optical extinction was also recorded graphically and continuously between 325 and 700 nm. with a Unicam SP 800 spectrophotometer. Total bile pigment was estimated in the Seitz-filtered liquor by diazo-coupling and protein by the biuret reaction, as previously described (Morris *et al.*, 1967).

Group, Rh type, Coombs and albumin tests, and haemoglobin determination were made on cord blood. Mother's blood was grouped and Rh-typed, and the antibody

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titre was followed throughout pregnancy by means of the indirect Coombs test.

Results

Data obtained from the analysis of liquor from two groups of Rh-positive normal pregnancies, collected between 16 and 26 weeks and 30 and 34 weeks respectively, are shown in Table I. The values from the latter group are the same as those given previously by Morris *et al.* (1967) for 30 to 32 weeks. It has since been discovered that the range covered in this series was from 30 to 34 weeks. One subject in the former group (16 to 26 weeks) has been excluded as the liquor bile pigment to protein ratio exceeded the mean by more than three standard deviations. The observations in this case are therefore considered suspect. The mean protein values and mean pigment levels are shown in Table I. The mean pigment levels were determined chemically by diazo-coupling and spectrophotometrically from differences in optical extinction (ΔE) between 454 and 574 nm. or between 490 and 520 nm., as described by Alvey (1964) and Knox *et al.* (1965) respectively. The mean and standard deviation and standard error of the mean for the single measurements, bile pigment, $\Delta E(454-574 \text{ nm.})$, and $\Delta E(490-520 \text{ nm.})$ are compared with the respective ratios obtained when these quantities are divided by protein concentration.

The mean total liquor pigment level, whether determined chemically or spectrophotometrically, was higher in the earlier gestational group. The mean bile pigment to protein ratio was also higher, in spite of the fact that the mean protein concentration was also higher early in pregnancy. In all cases the standard deviations of ratios expressed as percentages of means were less than the equivalent values for undivided levels. The differences (*t* values) between corresponding means in the two gestational groups calculated by Student's *t* test were all highly significant ($P < 0.001$) (Table I). Though not analysed statistically the observed ranges for all measurements in the earlier group differed considerably from those in the later, with only a relatively small overlap in values (Table I).

Linear regressions were computed between various measurements in the normal series and the resulting correlations recorded. Total liquor bile pigment levels determined chemically were very highly correlated with the levels determined spectrophotometrically in both gestational groups and by both optical methods, that of Alvey (1964) and that of Knox *et al.* (1965) (Table II). A similarly high correlation was found for these relationships in the series of Rh-negative isoimmunized mothers. The bile pigment level was also significantly correlated with protein concentration, rather better in the later (30-34 weeks) than in the earlier (16-26 weeks) series (Table II). Separate statistical analysis of the groups showed that protein concentration was not significantly correlated with gestation time between 16 and 26 weeks but was at about 2% level during 30 to 34 weeks (Table II). As might be expected from the mean and *t* values given in Table I, however, the correlation between protein concentration and gestation time over the whole period (16-34 weeks) was very highly significant (Table II). Similarly, though the respective mean total bile pigment levels and mean bile pigment to protein ratios differed significantly between earlier and later groups (Table I), within the groups there was no significant correlation between these values and gestation time (Table II) except for bile pigment in the 30 to 34 weeks series.

Statistically significant correlations between the ultimate cord haemoglobin and total bile pigment and its ratio with protein were obtained in first specimens of liquor amnii from Rh-negative mothers whose babies survived and were not transfused in utero (Table III, Fig. 1). Correlations with cord haemoglobin and chemically determined total bile pigment levels were not in general as high as the values obtained with cord haemoglobin and total bile pigment determined by the spectrophotometric methods of Knox *et al.* (1965) ($\Delta E(490-520 \text{ nm.})$ or Alvey (1964) ($\Delta E(454-574 \text{ nm.})$). This was also true when the respective spectrophotometrically and chemically determined ratios with protein were similarly compared. The effect of gestation time on these correlations was assessed by statistical analysis of the results in two groups, early (20-26 weeks) and late (27-36 weeks), as well as in combination. Apart from the chemically determined bile pigment levels in

TABLE I.—Liquor Analysis on 48 Normal Rh-positive Mothers Showing Higher Bile Pigment Levels and Total Bile Pigment to Protein Ratios in the Earlier Group

Mode of Expression	Mean $\pm$ S.D.		Observed Range		Significance of Difference between Means 16-26 and 30-34 Weeks <i>t</i> Value
	16-26 Weeks (n = 23)	30-34 Weeks (n = 25)	16-26 Weeks (n = 23)	30-34 Weeks (n = 25)	
Protein (mg./ml.)	6.06 $\pm$ 1.46	3.74 $\pm$ 0.94	3.6-10.3	2.4-5.9	6.616*
Total bile pigment ($\mu\text{g./ml.}$)	1.961 $\pm$ 0.465	0.816 $\pm$ 0.230	1.2-2.9	0.5-1.4	13.852*
Total bile pigment ($\mu\text{g./ml.}$) Protein (mg./ml.)	0.319 $\pm$ 0.0504	0.219 $\pm$ 0.037	0.250-0.405	0.154-0.283	6.554*
$\Delta E(454-574 \text{ nm.})$	0.190 $\pm$ 0.045	0.0817 $\pm$ 0.0203	0.108-0.280	0.047-0.122	11.011*
$\Delta E(454-574 \text{ nm.}) \times 10$ Protein (mg./ml.)	0.321 $\pm$ 0.072	0.221 $\pm$ 0.037	0.240-0.463	0.150-0.334	6.136*
$\Delta E(490-520 \text{ nm.})$	0.078 $\pm$ 0.022	0.0308 $\pm$ 0.0098	0.042-0.139	0.017-0.052	9.819*
$\Delta E(490-520 \text{ nm.}) \times 10^*$ Protein (mg. ml.)	1.292 $\pm$ 0.254	0.821 $\pm$ 0.146	0.920-1.850	0.466-1.06	7.944*

Δ = Difference. *Probability $P < 0.001$.

TABLE II.—Relationship in Liquor Amnii from Rh-positive Normal Pregnancies between (a) Chemically Determined and Spectrophotometrically Determined Total Bile Pigment, (b) Bile Pigment and Protein Concentration, and (c) Gestation Time and Protein, Bile Pigment, or the Ratio Bile Pigment/Protein

Gestation Period	No. of Cases	Values Correlated	Correlation Coefficient	Probability
16-26 weeks	22	Bilirubin* and $\Delta E(490-520 \text{ nm.})$	+0.8503	0.000000286
30-34 "	25	Bilirubin* and $\Delta E(490-520 \text{ nm.})$	+0.8630	0.000000346
16-26 "	22	Bilirubin* and $\Delta E(454-574 \text{ nm.})$	+0.8925	0.000000166
30-34 "	25	Bilirubin* and $\Delta E(454-574 \text{ nm.})$	+0.8060	0.00000116
16-26 "	22	Bilirubin* and protein	+0.6321	0.00121
30-34 "	25	Bilirubin* and protein	+0.7547	0.0000131
16-26 "	22	Gestation time and protein	+0.2669	Not significant
30-34 "	25	Gestation time and protein	-0.4546	0.0224
16-34 "	47	Gestation time and protein	-0.6505	0.000000488
16-26 "	22	Gestation time and bilirubin*	-0.0441	Not significant
30-34 "	25	Gestation time and bilirubin*	-0.5642	0.0033
16-26 "	22	Gestation time and bilirubin*/protein	-0.3331	Not significant
30-34 "	25	Gestation time and bilirubin*/protein	-0.1978	Not significant

*Total bile pigment determined by diazo-coupling.

TABLE III.—Relationship between Cord Haemoglobin (Hb) and the Single Measurement of Liquor Bile Pigment compared with that between Cord Haemoglobin and the Ratio of Bile Pigment to Protein by including the Significance of the Difference between these Correlations. Data from the Same Specimens Taken at First Amniocentesis on Rh-negative Mothers Whose Babies Survived and were Not Transfused in Utero

Gestation Period (Weeks)	No. of Cases	Bile Pigment	Bile Pigment Cord Hb		Bile Pigment-Protein Ratio/Cord Hb		Significance of Difference between r_1 and r_2 using Fisher's z -Transformation	
			Correlation Coefficient (r_1)	Probability (P, 2-tail)	Correlation Coefficient (r_2)	Probability (P, 2-tail)	Normal Deviation (c)	Probability (P, 2-tail)
20-26	16	Bilirubin*	-0.5838	0.01758	-0.5717	0.0206	0.0412	Not significant
27-36	26	Bilirubin*	-0.4811	0.01284	-0.7442	0.0000131	1.4764	0.1 < P < 0.15
20-36	42	Bilirubin*	-0.3870	0.01136	-0.6547	0.00000258	1.6569	0.09 < P < 0.1
20-26	16	ΔE (490-520 nm.)	-0.7991	0.000204	-0.8189	0.0001048	0.1462	Not significant
27-36	26	ΔE (490-520 nm.)	-0.4888	0.01128	-0.7838	0.0000022	1.7656	0.07 < P < 0.08
20-36	42	ΔE (490-520 nm.)	-0.5199	0.000418	-0.7791	0.000000298	2.0616	0.03 < P < 0.04
20-26	16	ΔE (454-574 nm.)	-0.7567	0.00069	-0.7982	0.00021	0.26816	Not significant
27-36	26	ΔE (454-574 nm.)	-0.5564	0.00316	-0.8594	0.000000474	2.2498	0.02 < P < 0.03
20-36	42	ΔE (454-574 nm.)	-0.5119	0.00053	-0.8154	0.000000288	2.5507	0.01 < P < 0.02

*Total bile pigment determined by diazo-coupling.

the 20 to 26 weeks group, where correlations of pigment alone and ratio were about equal, the ratio of the bile pigment to protein, whether the latter was determined chemically or spectrophotometrically, was more highly correlated with cord haemoglobin than in this relationship with the corresponding single measurements. In the later group (27-36 weeks) the difference between the correlation coefficients of total pigment/haemoglobin and bile pigment to protein ratio/haemoglobin is significant. This is not so for the earlier group (Table III).

Infant survival was compared with the severity of haemolytic disease predicted from the liquor bile pigment to protein ratio by classifying as severe those cases in which the ratio was equal to or greater than 0.4 between 20 and 26 weeks and 0.3 from 27 to 36 weeks. Whereas about half of the 76 babies in the severe group died (Table IV) of the four deaths in the 39 cases of the non-severe group only one death was primarily due to haemolytic disease. In a second death, where haemolytic disease might have been contributory, the disease was probably only mild when the liquor was taken (22 weeks), since the indirect Coombs titre rose from 1 in 2 at this time to 1 in 256 at birth, when its haemoglobin was 16.4 g./100 ml. and necropsy showed minimal signs of haemolytic disease.

The superior accuracy of the liquor bile pigment to protein

FIG. 1.—Decrease in liquor bile pigment to protein ratio with increase in gestation time (16-34 weeks) in normal pregnancies (Rh-positive mothers).

TABLE IV.—Evidence of Accuracy of Liquor Total Bile Pigment to Protein Ratio in Prognosis and as a Guide to the Need for Intrauterine Transfusion

Treatment	Not Severe*		Severe*		Total
	Lived	Died	Lived	Died	
Intrauterine transfusion	1	1	2	22	26
Intrauterine transfusion + exchange transfusion	2	—	18	4	24
Exchange transfusion	11	1†	14	2	28
No transfusion	10	2‡	—	10	22
Rh-negative infant	10	—	2	—	12
Rh-negative infant transfused in error	1	—	1	—	2
Total	35	4	37	38	114§

*Prediction severe total bile pigment to protein ratio of ≥ 0.4 at 20-26 weeks or ≥ 0.3 at 27-36 weeks.

† Respiratory distress and pneumonia primary cause of death (indirect Coombs 1/2 at amniocentesis (22 weeks) had risen to 1/256 at birth. Hb at birth 16.4 g.).

‡ Haemolytic disease not cause of death.

§ 114 fetuses from 110 mothers.

ratio in the prediction of severity of haemolytic disease, as opposed to the bile pigment value alone, was demonstrated by comparing the predicted with the actual outcome of pregnancy in those cases where prediction by the two measurements differed (Table V). Of the 110 Rh-negative women tested there were differences in 19 cases. Only two of these were predicted less accurately by the ratio, and in one this inaccuracy could be accounted for by the presence of altered blood in the specimen. Even when the level of bile pigment taken as an index of a severe prognosis was reduced from just over two to under two standard deviations in excess of the normal mean, the ratio still gave a better prediction in 13 out of 15 cases (Table V).

Discussion

In an earlier paper (Morris *et al.*, 1967) we presented evidence showing that the severity of fetal haemolytic disease could be predicted more accurately from the ratio of liquor bile pigment to protein than from the bile pigment concentration alone. This conclusion has been questioned by Walker *et al.* (1969), who, using the ratio of the transmittance at 520 and 490 nm. (Knox *et al.*, 1965; Savage *et al.*, 1966) to measure bile pigment, found prediction less reliable when based on the bile pigment to protein ratio. Further studies in our laboratory involving tests on liquor from 110 Rh-immunized women do not endorse this finding but continue to favour the ratio as a better guide to prognosis. Evidence of this is provided by the results of linear regression analyses of cord blood haemoglobin levels against liquor bile pigment and the ratio of liquor bile pigment to protein.

Though significant correlations between cord haemoglobin and liquor bile pigment (Liley, 1961) and cord haemoglobin and liquor bile pigment to protein ratio (Cherry *et al.*, 1965) have been shown, the two regressions have not been compared previously on the same cases. In spite of the fact that variable erythropoietic activity detracts from the value of cord haemoglobin as a guide to the extent of erythroblastosis in the neonate (Watson *et al.*, 1965), the higher correlations between cord haemoglobin and the ratio suggest that com-

TABLE V.—Prediction and Outcome. Demonstrating the Superiority of the Total Bile Pigment to Protein Ratio over the Total Bile Pigment Estimation Alone in 19 Cases where These Two parameters gave Different Prognoses

Gestation (Weeks)	Prediction				Outcome		Comment on Outcome	Comment on Accuracy of Prediction
	From Bile Pigment Alone (μg./ml.)		Bile Pigment Ratio		Cord Hb (g./100 ml.)	No. of Exchanges		
20	2.6	Not severe	0.554	Severe	—	—	Abortion	Ratio better
20	2.9*	Not severe*	0.485	Severe	—	—	Stillbirth	Ratio better
20	2.2	Not severe	0.441	Severe	12.6	1	Moderately severe	Ratio marginally better
20	2.3	Not severe	0.415	Severe	14.9	0	Rh-negative baby	Ratio wrong†
20	2.8*	Not severe*	0.445	Severe	3.7	2	Severe	Ratio better
20	2.6	Not severe	0.554	Severe	—	—	Intrauterine death	Ratio better
21	2.6	Not severe	0.434	Severe	18.7‡	4	Very severe	Ratio better
21	2.6	Not severe	0.465	Severe	—	—	Intrauterine death	Ratio better
23	1.9	Not severe	0.475	Severe	8.6	—	Neonatal death	Ratio better
27	1.0	Severe	0.268	Not severe	13.6	2	Moderately severe	Ratio worse
30	1.0	Not severe	0.400	Severe	10.5	5	Very severe	Ratio better
31	1.4*	Not severe*	0.330	Severe	10.9	2	Severe	Ratio better
31	1.4*	Not severe*	0.369	Severe	8.0	2	Severe	Ratio better
31	0.9	Not severe	0.364	Severe	9.3	3	Severe	Ratio better
33	0.9	Not severe	0.333	Severe	10.0	1	Severe	Ratio better
33	1.0	Not severe	0.334	Severe	11.4	1	Moderately severe	Ratio better
34	0.9	Not severe	0.360	Severe	12.7	1	Moderately severe	Ratio marginally better
34	0.8	Not severe	0.364	Severe	12.0	1	Moderately severe	Ratio better
35	1.0	Not severe	0.423	Severe	11.7	4	Severe	Ratio better

Prediction severe { Total bile pigment alone ≥ 3.0 μg./ml. between 20 and 26 weeks or ≥ 1.5 μg./ml. between 27 and 36 weeks.

* Total bile pigment to protein ratio ≥ 0.4 between 20 and 26 weeks or ≥ 0.3 between 27 and 36 weeks.

† Bile pigment values of over two standard deviations greater than the normal mean for the respective gestational group.

‡ Prediction inaccurate owing to gross contamination of liquor with bilirubin derived from altered blood.

§ Cord haemoglobin raised by intrauterine transfusion.

pared with the single measurement the ratio is a better index of haemolytic disease.

A comparison of prediction with actual outcome of pregnancy in those cases where the prognoses given by the ratio and single measurement differed showed that the former was a superior index of the severity of haemolytic disease (Table V). As the criteria used to divide "not severe" from "severe" predictions were similar for both single measurements and ratios—namely, values of about two standard deviations in excess of their normal means—it is unlikely that the preponderance of not severe predictions given by the single measurements could arise from the designation of too high a value to the figure used to divide the two categories.

Close agreement between predicted and actual outcome of pregnancy was obtained when the 110 cases of Rh-isoimmunized women were classified as not severe or severe by using the bile pigment to protein ratio (Table IV). Of the 39 cases in the not severe group only one of the four deaths was primarily caused by haemolytic disease, whereas over half of the babies in the severe group died. All the surviving babies in the severe group were transfused, either in utero or by exchange transfusion or by both methods, except for three Rh-negative infants, two of whom were classified incorrectly because the ratio was increased artificially by the presence of altered blood in the liquor. Nearly one-third of the infants in the not severe category were Rh-negative, and of the remainder only just over one-half were transfused.

Though the bilirubin concentration in the liquor amnii is directly related to the severity of haemolytic disease, bile pigments in the liquor can be derived from breakdown of haemoglobin from both the fetus and the mother. While that from fetal cell destruction remains fairly constant in normal pregnancy, in Rh-isoimmunization the amount of pigment is related to the severity of haemolytic disease in the fetus. The pigment due to maternal haemoglobin occasionally shows vast increase in amount due to a leak of a relatively large quantity of blood into the liquor from placental damage. Such damage can of course be caused by an amniocentesis needle, or it can occur naturally without any clinical signs whatever. It is in these instances that large amounts of bilirubin and other breakdown products of haemoglobin are found. It seems that if the liquor is soiled by blood then in a few days the red colour disappears as haemoglobin is broken down and the liquor is tinged with yellow or brown according to the type and amount of pigment present. Halitsky *et al.* (1968), who also studied the effect of blood contamination in liquor, showed that this pigment is largely methaemalbumin. They pointed out that it is clinically important, particularly late in pregnancy (34–35 weeks), to differentiate between this pig-

ment and staining due to meconium. This may be done by inspecting the shape of the spectral curve before and after carrying out Schumm's test for methaemalbumin on the liquor.

It follows that no devices such as those of Liley (1963), Alvey (1964), Misenhimer (1964), Bonsnes (1965) Cherry *et al.* (1965), and Knox *et al.* (1965) can alter the fact that there may be present large amounts of pigment derived from altered blood which will absorb light at 450 nm. We would agree with Bartsch (1969) and Halitsky *et al.* (1968), therefore, that the liquor spectral curve should always be examined and that it is not enough simply to take readings at one, two, or three wavelengths. A complete spectral curve such as that labelled (a) in Fig. 2 readily shows that the liquor is contaminated by pigment derived from altered blood. This curve was obtained at 22 weeks in a Rh-isoimmunized woman who was carrying a Rh-negative baby, and had reliance been placed on simple bilirubin estimation, whether by pairs of wavelength observations or by chemical determination, the fetus would have been diagnosed as seriously affected. Curves b, c, and d showing the liquor spectral curves repeated at intervals from 22 to 32 weeks demonstrate a gradual return to normal (d). For comparison the curve (e) superimposed is taken from the liquor surrounding a fetus grossly affected with haemolytic disease.

Liley (1961) and others (Cherry *et al.*, 1965; Goldstein *et al.*, 1966; Bevis, 1967) noted in Rh-affected pregnancies that the liquor pigment concentration decreases with increasing gestation time during the third trimester. Liley (1968), Carlton and Sinha (1970), and Walker (1970) found a rise in pig-

FIG. 2.—Effect of altered blood on the spectral absorption curve of liquor and its decrease as pregnancy progressed from (a) 21½, (b) 23, (c) 25, to (d) 31 weeks in a case without haemolytic disease (Rh-negative fetus) having a placental bleed at or before 20 weeks. For comparison (e) shows the curve in a case of extremely severe haemolytic disease without altered blood.

ment concentration from 12 to 18 weeks with a plateau from 18 to 25 weeks and a fall from 26 to 36 weeks. Our results (Table II) shows the significant negative correlation between gestation time and bilirubin level from 30 to 34 weeks. The lack of correlation between pigment concentration and gestation time observed in our normal series in the second trimester (Table II) is consistent with a change from a rise to a fall in liquor pigment level around 20 to 22 weeks. From 16 to 26 weeks the liquor protein concentration was also unrelated to maturity (Table II) though by the third trimester the protein level, like the bilirubin concentration, was correlated with gestation time (Table II). Nevertheless, when these two measurements were expressed as a ratio the correlation with gestation time was no longer significant. These observations are in accord with those of Cherry *et al.* (1965) on Rh-isoimmunized women at 28 to 38 weeks who suggested a direct correlation between protein concentration and liquor volume. Dunstan's (1968) failure to find a significant correlation between amniotic fluid volume and protein concentration has cast doubt on the interdependence of these two quantities.

The highly significant correlation between the concentration of bile pigment and protein (Table II) in normal liquor agrees with the findings of others in Rh-affected pregnancies (Wild, 1961; Dunstan, 1968), and by inference with statistical evidence (Walker *et al.*, 1969), showing that both bilirubin and protein are indices of the severity in haemolytic disease. Though the manner in which bilirubin enters the liquor is still in doubt, it is thought (Wild, 1961), in view of the strong binding between bilirubin and serum albumin (Martin, 1949; Barac, 1953), that most unconjugated bilirubin is attached to liquor albumin, which forms about two-thirds of the total protein (Wild, 1961) and is far in excess of that required to transport the bilirubin (Watson *et al.*, 1965). Bilirubin diglucuronide may also be partly albumin-bound according to Brodersen *et al.* (1967). They claimed that this "direct reacting" pigment constitutes about 10 to 30% of the total bile pigment, though Watson *et al.* (1965) considered it to be an artefact formed in the chemical estimation of liquor bilirubin. The above evidence indicates that a complex set of conditions contributes to make the prognoses given by the ratio better than the single measurement. Bilirubin-protein binding no doubt plays a part, but at this stage it might be premature to dismiss completely any connexion with liquor volume.

Both chemical and spectrophotometric methods are suitable for the estimation of total bile pigment in liquor (Table II). Nevertheless, owing to the very low pigment concentration usually encountered, simple diazo-coupling techniques (Watson, 1962; Watson *et al.*, 1965) involve measuring small differences between low optical extinctions. For reliable results this requires considerable expertise and a colorimeter in first-class condition; while concentration of bilirubin by chloroform extraction and solvent evaporation can lead to losses (Brodersen *et al.*, 1967). For routine testing we therefore consider differential spectrophotometry (Alvey, 1964; Knox *et al.*, 1965) preferable to chemical methods, a conclusion also reached by Walker (1970). An accurate spectrophotometer, however, giving high wavelength resolution throughout the range 400 to 700 nm. is essential. A recent spectrophotometric method (Mallikarjuneswara *et al.*, 1970), using uncentrifuged liquor, based on chloroform extraction without evaporation and measurement at only one wavelength, possibly combines some of the advantages of chemical and spectrophotometric procedures; its authors, however, have yet to test it in a full clinical trial.

We have made the following guide to prognosis in Rh-isoimmunization. It is based on mean pigment to protein ratios found in normal liquor (Table I) and an assessment of

the ratio value in relation to outcome in Rh-negative pregnancies (Table IV). A bile pigment to protein ratio of under 0.4 between 20 and 26 weeks suggests that haemolytic disease is either mild or absent and that further analysis of liquor at about monthly intervals should be done. With ratios in excess of 0.43 the prognosis is so poor that intrauterine transfusion is indicated. Between 30 and 34 weeks ratios of under 0.3 are low enough to justify allowing the pregnancy to run to term provided there are no other indications for early delivery. We rarely undertake intrauterine transfusion for the first time after 30 weeks, but a ratio of 0.5 or more would suggest very severe haemolytic disease. These values may be converted for use with either of the spectrophotometric expressions

$$\frac{(\Delta E (454-574 \text{ nm.}) \times 10)}{\text{protein}} \quad \text{or} \quad \frac{(\Delta E (490-520 \text{ nm.}) \times 10^2)}{\text{protein}}$$

if multiplied by a factor obtained by dividing the appropriate mean ratio determined spectrophotometrically by that determined chemically; such ratios are given in Table I.

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REFERENCES

- Alvey, J. P. (1964). *American Journal of Obstetrics and Gynecology*, **90**, 769.
 Barac, G. (1953). *Archives Internationales de Physiologie*, **61**, 129.
 Bartsch, F. K. (1969). Communication given at the International Symposium on the Management of the Rh Problem, Milan.
 Bevis, D. C. A. (1956). *Journal of Obstetrics and Gynaecology of the British Empire*, **63**, 68.
 Bevis, D. C. A. (1967). *Hospital Medicine*, **1**, 408.
 Bonsnes, R. W. (1965). Quoted by J. T. Queenan in *Modern Management of the Rh Problem*. New York, Hoeber Medical Division, 1967.
 Bower, D., and Swale, J. (1966). *Lancet*, **1**, 1009.
 Brodersen, R., Jacobsen, J., Hertz, H., Rebbe, H., and Sørensen, B. (1967). *Scandinavian Journal of Clinical and Laboratory Investigation*, **20**, 41.
 Carlton, M. A. and Sinha, R. (1970). *Journal of Obstetrics and Gynaecology of the British Commonwealth*, **77**, 221.
 Cherry, S. H., Kochwa, S., and Rosenfield, R. E. (1965). *Obstetrics and Gynaecology*, **26**, 826.
 Dunstan, M. K. (1968). *Journal of Obstetrics and Gynaecology of the British Commonwealth*, **75**, 732.
 Fleming, A. F., and Woolf, A. J. (1965). *Clinica Chimica Acta*, **12**, 67.
 Freda, V. J. (1965). *American Journal of Obstetrics and Gynaecology*, **92**, 341.
 Goldstein, P. J., Rosenstein, D. L., and Bloomberg, G. (1966). *Obstetrics and Gynaecology*, **28**, 189.
 Halitsky, V., Krumholz, B. A., and Wiener, M. F. (1968). *Obstetrics and Gynaecology*, **32**, 339.
 Knox, E. G., Fairweather, D. V. I., and Walker, W. (1965). *Clinical Science*, **28**, 147.
 Liley, A. W. (1961). *American Journal of Obstetrics and Gynaecology*, **82**, 1359.
 Liley, A. W. (1963). *American Journal of Obstetrics and Gynaecology*, **86**, 485.
 Liley, A. W. (1968). *Bibliotheca Haematologica*, No. 29, part 1, p. 237.
 Mackay, E. V. (1961). *Australian and New Zealand Journal of Obstetric and Gynaecology*, **1**, 78.
 Mallikarjuneswara, V. R., Clemetson, C. A. B., and Carr, J. J. (1970). *Clinical Chemistry*, **16**, 180.
 Martin, N. H. (1949). *Journal of the American Chemical Society*, **71**, 1230.
 Misenhimer, H. R. (1964). *Obstetrics and Gynecology*, **23**, 485.
 Morris, E. D., Murray, J., and Ruthven, C. R. J. (1967). *British Medical Journal*, **2**, 352.
 Robertson, J. G. (1964). *British Medical Journal*, **2**, 147.
 Savage, R. D., Walker, W., Fairweather, D. V. I., and Knox, E. G. (1966). *Lancet*, **2**, 816.
 Walker, A. H. C. (1957). *British Medical Journal*, **2**, 376.
 Walker, A. H. C., and Jennison, R. F. (1962). *British Medical Journal*, **2**, 1152.
 Walker, W. (1970). *British Medical Journal*, **2**, 220.
 Walker, W., Fairweather, D. V. I., and Jones, P. (1964). *British Medical Journal*, **2**, 141.
 Walker, W., Landon, M. J., and Oxley, A. (1969). *British Medical Journal*, **1**, 605.
 Watson, D. (1962). *Proceedings of the Association of Clinical Biochemists*, **2**, 81.
 Watson, D., Mackay, E. V., and Trevella, W. (1965). *Clinica Chimica Acta*, **12**, 500.
 Wild, A. E. (1961). *Clinical Science*, **21**, 221.