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Protein Content of Liquor Amnii in Prediction of Severity of Haemolytic Disease of Newborn

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Summary: Two series of cases of Rh isoimmunization were subjected to liquor examination for bilirubin and protein level. Series 1 comprised 298 cases for the years 1962 and 1963. Series 2 comprised 179 consecutive cases for 1967 in which preliminary selection for liquor examination had been made on the basis of previous history and maternal antibody titre.

Bilirubin was measured as the liquor bilirubin ratio, and protein levels were estimated in series 1 by the Folin and Ciocalteau technique and in series 2 by a modified biuret method.

Correlations with severity of haemolytic disease in the foetus was made, taking into account the stage of gestation of liquor examination. Both bilirubin and protein levels correlate with severity, but bilirubin is superior to protein. Interrelation of these measurements as bilirubin/protein ratio was inferior to bilirubin level as a method of forecasting severity.

Introduction

The spectroscopic examination of liquor amnii during pregnancy for the presence of bilirubin is established in the management of Rh isoimmunization, though different methods of measuring the concentration of this pigment have been advocated (Scott, 1959; Liley, 1963; Walker *et al.*, 1964; Knox *et al.*, 1965). Wild (1961) suggested that liquor bilirubin levels may be influenced by the protein level as has been demonstrated for cerebrospinal fluid (Stempfel and Zetterström, 1955). Both bilirubin and protein levels normally fall as pregnancy advances, so allowances must be made for the stage of gestation when interpreting these values. Cherry *et al.* (1965) and Morris *et al.* (1967) recommended that both bilirubin and protein levels are taken into account by calculating the bilirubin/protein ratio. If, as they believe, the protein value gives an indication of liquor volume, this method should eliminate errors due to fluctuation of the liquor volume. We (Walker *et al.*, 1964) had previously estimated protein and bilirubin levels and in view of this new suggestion calculated the bilirubin/protein ratio for that material. Our findings were at variance with those of Cherry *et al.* and Morris *et al.*, but we had used a different method of estimating protein. We have therefore studied a further series of cases in which protein was estimated by the biuret method as they recommend.

This confirms the findings of the earlier study and the results of both series are presented.

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Materials and Methods

All liquor samples were obtained by amniocentesis and immediately placed in brown universal containers. Any contaminated with blood were discarded. Samples were Seitz filtered by means of a Hemming filter and the absorption spectrum was traced over the range 360–630 mμ with a 0·5-cm. light path. The level of bilirubin was expressed as the ratio transmission 520 mμ/transmission 490 mμ (Knox *et al.*, 1965).

The sample was then frozen until required for protein estimation.

Two series of patients with Rh isoimmunization were studied: (1) 298 samples from 1962 and 1963 where the protein was estimated by the Folin and Ciocalteau method (Papadopoulos *et al.*, 1959), and (2) 179 consecutive samples from 1967 where protein was estimated by the biuret method as recommended by Morris *et al.* (1967).

The levels of bilirubin and protein and the bilirubin/protein ratio were related to severity of haemolytic disease in the infant, initially for the six categories: (1) Rh-negative unaffected infant; (2) mild disease, Coombs positive, no treatment required; (3) moderate disease, transfusion required, cord Hb >11·5 g./100 ml.; (4) severe disease, transfusion or repeated exchange transfusion required, cord Hb usually <11·5 g./100 ml.; (5) very severe disease, overt severe clinical disease at birth, cord Hb usually <7·4 g./100 ml.; and (6) stillbirth due to haemolytic disease.

The essential purpose of liquor examination, however, is to select patients for premature induction and/or intrauterine transfusion. We have therefore found it satisfactory to forecast the results in two major classes—that is, those resulting in stillbirth or very severe disease in whom special treatment is justified compared with those having less severe disease or negative infants.

Similarly, in the tables only the protein or bilirubin value giving best discrimination has been used, so that the original $6 \times N$ tables are simplified to 2×2 tables and for these χ^2 and Q have been calculated.

Results

Reliability of Protein Estimation

Twenty specimens of liquor were divided into two aliquots each. One was assayed fresh—that is, after less than five days' storage at 4° C.—and the other after storage at −20° C. for a period of three months (Table I). There was no significant difference between the two sets of results.

TABLE I.—*Effect of Storage on Protein Values*

Assayed Fresh. Mean ± S.D.	Assayed after 3 months. Mean ± S.D.	Change	*t*
0·356 ± 0·14 g./100 ml.	0·350 ± 0·135 g./100 ml.	−1·7%	0·83

Effect of Haemoglobin on Protein Estimation

Although samples obviously contaminated with blood were discarded, haemoglobin was found to be present on the spectrum tracing in 30% of samples, but in more than trace amounts in less than 10%. One sample of liquor was treated with haemolysed washed cells to give a series of four haemoglobin levels, each far in excess of levels seen in test liquors. The protein value on the clear liquor was 0·14 g./100 ml. and in the treated specimens 0·14, 0·13, 0·13, and 0·13 g./100 ml. respectively.

We calculate that the presence of trace amounts of haemoglobin in specimens would not alter the protein value significantly and, furthermore, significance in relation to severity of disease was not altered by omitting all samples in which haemoglobin was detected.

Bilirubin and Protein Levels and Severity of Disease

The results of series 1 are summarized in Table II. All three methods allow a forecast of severity, but that based on bilirubin is most accurate, both in forecasting very severe disease and in excluding mild cases. Wrong predictions were made; 10% in the case of bilirubin but 27% on protein and 16% on bilirubin/protein ratio.

TABLE II.—*Series 1. Bilirubin and Protein in Relation to Severity of Haemolytic Disease of the Newborn in 298 Cases*

Liquor		Foetus: Stillborn or Very Severe	Foetus: Less Severe or Rh-negative	χ^2	Q
Bilirubin ratio ..	> 1·06	44	15	128	0·94
	≤ 1·06	18	221		
Protein (g./100 ml.)	≥ 0·2	40	59	35	0·69
	< 0·2	22	177		
Bilirubin/protein ratio	< 3·0	29	14	69	0·87
	≥ 3·0	33	222		

In 15 cases a wrong forecast of severe disease was made because of a high bilirubin level, but 12 of these were correctly predicted as less severe on the results of protein/bilirubin ratio. However, a correct prediction of severe disease on a high bilirubin value was made in 44 cases, and had the protein/bilirubin ratio been used 14 of these would have been wrongly forecast. Thus on balance knowledge of the protein level in liquor does not improve prediction based on liquor bilirubin alone.

The cases in series 2 were all collected in one year during which liquor examination was being carried out systematically, the selection of cases for and timing of amniocentesis being made on the basis of previous history and maternal antibody titre (Walker, 1968).

Because levels of bilirubin and protein are related to gestation their relation to severity of haemolytic disease was studied at two gestational stages and the results are summarized in Tables III and IV.

TABLE III.—*Series 2. Bilirubin and Protein at 33 Weeks' Gestation or Earlier in Relation to Severity of Haemolytic Disease of the Newborn in 85 Cases*

Liquor		Foetus: Stillbirth or Very Severe	Foetus: Less Severe or Rh-negative	χ^2	Q
Bilirubin ratio ..	≥ 1·12	43	5	56	0·96
	< 1·12	5	32		
Protein (g./100 ml.) ..	≥ 0·35	38	15	13	0·69
	< 0·35	10	22		
Bilirubin/protein ratio	≤ 3·0	17	21	3·6	0·39
	> 3·0	31	16		

Altogether in series 2 there were 59 cases of stillbirth or very severe disease and 120 of less severe disease or normal infants compared with 62 and 236 respectively in series 1. This is because some preliminary selection on history and titre had been carried out to try to exclude patients with very mild disease.

Thus the level of χ^2 in Tables III and IV is lower than in Table II. None the less bilirubin ratio gives the best prediction and it is not improved by relating it to protein level. Although the protein value is significantly related to severity it is inferior to bilirubin mainly because it fails to distinguish milder forms of disease.

TABLE IV.—*Series 2. Bilirubin and Protein at 34–36 Weeks' Gestation in Relation to Severity of Haemolytic Disease of the Newborn in 94 Cases*

Liquor		Foetus: Stillbirth or Very Severe	Foetus: Less Severe or Rh-negative	χ^2	Q
Bilirubin ratio ..	> 1·06	7	11	16·6	0·84
	≤ 1·06	4	72		
Protein (g./100 ml.)	≥ 0·25	7	22	6·3	0·65
	< 0·25	4	61		
Bilirubin/protein ratio	< 4·0	7	22	6·3	0·65
	≥ 4·0	4	61		

These conclusions apply for both stages of gestation considered. Higher correlations were achieved for cases tested before 34 weeks, but only patients thought to have a high risk of stillbirth were tested early in pregnancy and this probably accounts for the finding.

To consider further predictive value for the three assessments we have calculated the percentage of correct predictions achieved by each at three different stages of gestation, before 31 weeks, 31–33 weeks, and 34–36 weeks. The results are summarized in Table V.

TABLE V.—*Series 2. Prediction of Stillbirth or Very Severe Disease by Liquor Bilirubin and Protein Values at Different Stages of Gestation in 179 Cases*

Gestation	Method	Line of Best Discrimination	No. of Cases	% Correct Predictions*
< 31 weeks	Bilirubin ratio	1·12	55	87
	Protein (g./100 ml.) ..	0·35		71
	Bilirubin/protein ratio ..	2·0		58
31–33 weeks	Bilirubin ratio	1·1	30	93
	Protein (g./100 ml.) ..	0·35		72
	Bilirubin/protein ratio ..	4·0		65
34–36 weeks	Bilirubin ratio	1·06	94	84
	Protein (g./100 ml.) ..	0·25		72
	Bilirubin/protein ratio ..	4·0		72

* Regarded as correct if high level associated with stillbirth or very severe disease or Rh-negative infant.

At all stages of gestation the bilirubin value is confirmed as the best method and is not improved by relating the protein level. This also applies if a detailed study is made of these cases in which a wrong forecast was made on bilirubin value—for example, at 34–36 weeks' gestation three out of five very severe cases not predicted on bilirubin level would have been identified by the protein value but at the expense of also selecting 18 cases of less severe disease. Similarly, the forecast was not improved when the protein value was taken into account in patients in whom very severe disease had been wrongly forecast.

Discussion

Premature induction and intrauterine transfusion are used in the management of Rh isoimmunization with the object of preventing stillbirth or early intrauterine death. Both procedures carry serious risks to mother and foetus, so that stringent criteria should be used in selection of patients. The final decision is usually based on liquor bilirubin value, but wrong forecasts are made in at least 10% of cases, while amniocentesis is not without danger. Because the liquor bilirubin

level falls with advancing pregnancy allowance must be made for the stage of gestation at which the test is carried out, and a bilirubin value at 34 weeks that might indicate the need for active intervention would not necessarily have the same import at 24 weeks. Technical errors—for example, owing to exposure of liquor to daylight, to contamination with maternal or foetal blood, to interference by haemoglobin absorption, etc.—can be minimized by careful technique, but each laboratory needs to standardize and evaluate the method it uses.

Little is known of how bilirubin enters liquor or of its rate of turnover, but the water content changes rapidly, and if this were associated with variation in the total liquor volume, changes in the bilirubin value might be secondary to this.

Bilirubin is insoluble in water, and hence in liquor, as in plasma, it is attached to protein. Protein turnover in liquor amnii is slow in comparison with the water component, so theoretically could give an indirect measurement of liquor volume. Thus by relating bilirubin to protein as the bilirubin/protein ratio errors secondary to changes in liquor volume would be eliminated. Dunstan (1968), however, did not find any relation between protein content and liquor volume.

The present study of two series of cases of Rh isoimmunization comprising 477 cases in all and using two different methods of protein estimation confirms that the bilirubin and protein values do correlate with severity of haemolytic disease, but that bilirubin is superior to protein and is not improved, indeed is impaired, by relating to protein values.

TABLE VI.—*Protein Levels in Liquor Amnii—Summary of Results in Present and Two Relevant Published Series*

	Method	No. of Cases	Gestation	Protein Level (g./100 ml.)	
				Range	Mean
Present series 1 ..	F & C	298	All	0·1 –1·39	0·213
Present series 2 ..	Biuret	179	All	0·05 –1·1	0·318
Morris *et al.* (1967)	Biuret	25	30–32 weeks	0·236–0·587	0·374
Cherry *et al.* (1965)	Biuret	39	All	0·155–1·520	0·463

F & C = Folin and Ciocalteau technique.

Because these findings are in direct conflict with those of the other two groups of workers cited, we have considered whether technical differences in protein estimation between the three laboratories could be the explanation. A direct comparison of the protein values in these series is summarized in Table VI.

For the three laboratories the mean values are not different, though the ranges differ. This is probably because the published figures are based on extremely small series, but the method of selecting patients for amniocentesis may also have played a part.

We therefore conclude that the estimation of protein in liquor amnii does not give as good a forecast of severity of haemolytic disease as is possible by bilirubin estimation and that the value of bilirubin is not improved by relating it to the protein value.

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Sickle-cell Anaemia, Sickle-cell Thalassaemia, Sickle-cell Haemoglobin C Disease, and Asymptomatic Haemoglobin C Thalassaemia in one Ghanaian Family

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Summary : A Ghanaian family is described in which a sickle-cell haemoglobin C man married to a sickle-cell thalassaemia woman produced 12 children (eight alive). Four children have sickle-cell anaemia, two sickle-cell haemoglobin C disease, one has sickle-cell thalassaemia, and one is asymptomatic haemoglobin C thalassaemia.

It is emphasized that the contribution that adult sickle-cell disease patients make, through procreation, to the persistence of the S gene may be greater than is normally supposed, and that this contribution may soon outstrip that made by balanced polymorphism through falciparum malaria. Widespread haemoglobin genotyping in schools leading to genetic counselling is advocated to decrease the incidence of sickle-cell disease.

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Introduction

Ghana abounds in abnormal haemoglobins. One person in four or five has either the sickle-cell trait (Hb AS) or the haemoglobin C trait (Hb AC). For West Africa as a whole Lehmann (1954) described the incidence of the sickle-cell trait as between 20 and 30%. Edington and Laing (1957) made the incidence of the sickle-cell trait in Southern and Northern Ghana to be 19% and 7% respectively. Edington and Lehmann (1954a, 1954b) first described Hb C outside the U.S.A. The main focus for Hb C is in West Africa (Allison, 1956a, 1956b ; Neel *et al.*, 1956), with the highest frequency in Northern Ghana of 20–21% (Edington and Lehmann, 1956 ; Edington and Laing, 1957 ; Neel, 1957).

The most recent surveys in Ghana involved young adults from the north and south of the country. In the south frequency of the sickle-cell trait (AS) is 20% and the Hb C trait (AC) is 9%, while in the north (among 143 persons) Hb AS is 7%, Hb AC is 18%, β-thalassaemia is 4–5%, with one case of α-thalassaemia (Ringelhann *et al.*, 1968). Other