A COMMENT ON THE FRACTIONAL INDIRECT DIAZO REACTION

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Laurence and Abbott (1956) published a description of a micro-method for the estimation of serum bilirubin by the indirect diazo reaction. They indicated that two patterns of colour development are obtainable with foetal and adult sera from cases of severe haemolytic disease due to Rh incompatibility and from obstructive jaundice due to blockage of the common bile duct from carcinoma of the head of the pancreas (personal communication). Adult sera were described as exhibiting a rapid change in colour with maximum colour development at approximately 10 minutes of starting the reaction. Foetal sera exhibited a slower change, maximum colour developing at approximately 30 minutes from starting the reaction. Commercial "pure" bilirubin was found to give a pattern similar to that of foetal sera. These differences in behaviour were interpreted by the authors as indicating the existence of different "adult" and "foetal" substances which gave the indirect diazo reaction in sera.

The implications of such a finding are far-reaching with regard to both theoretical and clinical considerations. This study was undertaken to investigate whether different patterns of colour development were obtainable with the indirect diazo reaction using both normal and pathological, adult and foetal sera.

Materials and Method

Samples of adult blood were obtained from healthy normal individuals, from patients with obstructive jaundice, and from patients with haemolytic anaemia. Samples of foetal blood were obtained from umbilical cords at normal deliveries. Samples of pericardial fluids were obtained at necropsy from cases of kernicterus (not attributable to ABO Rh incompatibility) and from cases of congenital jaundice.

The method and reagents were those of Laurence and Abbott (1956). Serial readings were made in an EEL photoelectric colorimeter using a green filter ("O.G.R.1") at minute intervals for 30 minutes.

Haemolysis was looked for with the aid of a pocket spectroscope and by naked-eye inspection. No measurements of the actual amount of haemolysis were made.

Results are summarized in Table I.

TABLE I
SUMMARY OF RESULTS

Material	No. of Specimens	Haemolysis		Time of Maximum Colour Development
		Spectroscopic	Macroscopic	
Adult serum:				
Normal ..	4	Absent	Absent	10 minutes
Pathological ..	9	"	"	10 "
Umbilical cord serum ..	10	"	"	10 "
	4	Present	"	10 "
	5	"	Present	30 "
Pericardial fluid ..	1	Absent	Absent	10 "
	1	Present	"	10 "
	2	"	Present	30 "

Results

Unhaemolysed sera obtained from adult normal and pathological cases gave similar patterns in which maximum colour developed at approximately 10 minutes from starting the reaction.

Sera obtained from umbilical cord bloods exhibited two patterns depending on the degree of haemolysis. When haemolysis was present on naked-eye inspection, maximum colour developed at approximately 30 minutes. When haemolysis was absent on naked-eye inspection, and when it was only detectable spectroscopically, maximum colour developed at approximately 10 minutes.

Pericardial fluids behaved like umbilical cord sera, in that the 10-minute or the 30-minute pattern was obtained depending on the degree of haemolysis.

Serial dilutions of haemoglobin were made by adding a solution of haemoglobin obtained by freezing and thawing adult erythrocytes to unhaemolysed adult serum. Concentrations of haemo-

globin not exceeding approximately 3.5 g. per 100 ml. serum produced no change in the pattern of colour development. Higher concentrations changed the pattern from the 10-minute to the 30-minute type. Serial dilutions of the same haemoglobin solution in water without serum gave results similar to those for the dilutions of the haemoglobin solution in serum.

Discussion

The results here presented demonstrate that, with the indirect diazo reaction, maximum colour develops at approximately 10 minutes in the case of both adult and umbilical cord sera, when haemolysis is absent or slight. Naked-eye haemolysis changes the pattern of colour development so that maximum colour occurs at approximately 30 minutes. The pattern due to the bile pigments in adult and foetal sera is similar, and there is no evidence that the pigments of adult and foetal bloods are not identical.

Najjar and Childs (1953) obtained crystalline indirect serum bilirubin from icteric sera from cases of haemolytic anaemia, sickle cell anaemia, haematoma fluid, jaundice of the newborn, kernicterus, and congenital familial non-haemolytic jaundice with kernicterus. All these sera yielded, by the same technique, crystals which had identical physical and chemical properties.

Using the method of "reverse phase" chromatography, Cole and Lathe (1953) separated and extracted the direct-reacting material and the indirect-reacting pigment of serum. The direct-reacting material was obtained from sera of patients with obstructive jaundice and with hepatitis. The indirect-reacting pigment, probably bilirubin, was obtained from sera of cases of haemolytic disease of the newborn attributed to Rh incompatibility and from adult haemolytic anaemia. With both pigments maximum colour developed at approximately 10 minutes of starting the reaction. No differences were described between the adult and foetal pigments.

More recently Cole, Lathe, and Billing (1954) produced additional evidence that the indirect-reacting pigment is bilirubin and separated the direct-reacting material into two pigments with spectral curves similar to, but not identical with, that of bilirubin, and suggest that these pigments probably have a tetrapyrrole structure like that of bilirubin. Lathe (1954) has stated categorically that the predominant pigment of neonatal jaundice is bilirubin and that in nearly all cases examined the sole pigment present during the period of

rising concentration was bilirubin, the direct-reacting pigments appearing during the period of falling concentration.

Bilirubin is a product of the metabolism of the haem nucleus of haemoglobin. The haemoglobins of the species studied have been shown to contain an identical haem nucleus, and differences between haemoglobins, normal and abnormal, human and animal, seem to be attributable to differences in the globin moieties. Pauling, Itano, Wells, Schroeder, Kay, Singer, and Corey (1950) showed that the haems of haemoglobins A and S are identical. No evidence is available that the bilirubins arising from haemoglobins A and F are not similar.

It is interesting to note that the level at which added haemoglobin showed a definite effect on the quantitative micro-estimation of Laurence and Abbott (1956) coincides with that at which haemoglobin added to serum or water changed the pattern of colour development in this study.

No attempt was made to study the details of the reaction between haemoglobin and the diazo reagent, though the almost immediate change in colour to a dark brown is probably explained by the conversion of haemoglobin to acid haematin.

Since haemoglobin not only affects the quantitative estimation of serum bilirubin but also interferes with the normal pattern of colour development, inferences made from serial estimation of serum bilirubin in the presence of haemolysis may be misleading.

Conclusions

(1) Unhaemolysed adult and umbilical cord sera exhibit similar patterns of colour development with the indirect diazo reaction.

(2) Adult and umbilical cord sera showing haemolysis on naked-eye inspection exhibit similar abnormal patterns of colour development with the diazo reaction.

(3) No evidence was obtained to indicate the existence of different indirect-reacting pigments in adult and foetal unhaemolysed sera, in normal or pathological specimens.

(4) No evidence is available in the literature that the bilirubin of adult sera is not identical with that of foetal sera.

REFERENCES

- Cole, P. G., and Lathe, G. H. (1953). *J. clin. Path.*, **6**, 99.
- and Billing, B. H. (1954). *Biochem. J.*, **57**, 514.
- Lathe, G. H. (1954). In *The Chemical Pathology of Animal Pigments* (Biochemical Society Symposia No. 12), p. 34. University Press, Cambridge.
- Laurence, K. M., and Abbott, A. L. (1956). *J. clin. Path.*, **9**, 270.
- Najjar, V. A., and Childs, B. (1953). *J. biol. Chem.*, **204**, 359.
- Pauling, L., Itano, H. A., Wells, I. C., Schroeder, W. A., Kay, L. M., Singer, S. J., and Corey, R. A. (1950). *Science*, **111**, 459.