

## SERUM PROTEINS AND THYROXINE-PROTEIN INTERACTION IN EARLY HUMAN FETUSES

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The concentration and electrophoretic pattern of serum proteins of the fetus differ from those of the adult in animals (1-3) as well as in man (4-7). In early fetuses of some species, serum proteins have been described which are either absent or modified at later stages of development (8, 9). Pedersen (10) first recognized in the newborn calf a large amount of an unusual  $\alpha$ -globulin for which he proposed the term *fetuin*. This material, which is absent in adults but present in fetuses of other species (11, 12), is an acidic glycoprotein containing glucosamine, mannose (13), galactose, galactosamine, and sialic acid (14).

Relatively few such studies have been made on humans, and most of the investigations have been concerned with newborn infants. It has been observed that total serum protein is very low in the first stage of fetal life and rises continuously during development (15). A fetal serum protein migrating between albumin and  $\alpha_1$ -globulin has been recently demonstrated very early in development and found to disappear by the fourth month of gestation (16). At the present time the nature and physiological significance of this human fetal protein is not understood.

The fetal thyroxine-binding proteins have been studied only in the rabbit and newborn humans. In fetal rabbits Osorio and Myant (17) found that the specific thyroxine-binding protein of the adult is not present until the later stages of development, but the fetal serum contains a different binding protein with an electrophoretic mobility intermediate between  $\alpha_2$ - and  $\beta$ -globulins. No qualitative difference between human newborns and adults was found (18, 19).

In the present study, serum proteins and thyroxine-protein interaction were examined in human fetuses after 8 to 20 weeks of gestation.

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### MATERIAL AND METHODS

Sera from 13 fetuses were collected at the Gentofte Hospital (Copenhagen) during the stay of one of us (J.R.) in Denmark.<sup>1</sup> The fetuses were obtained after legal abortion carried out by curettage.

The mothers, aged 25 to 40 years, were in good physical health, and the fetuses showed no gross abnormalities. The crown-rump length of the fetuses ranged from 5.8 to 14.3 cm; the gestation period, calculated from the last menstruation to the abortion less 14 days, corresponded to 8 to 20 weeks.

The fetal blood was collected immediately after delivery. Blood contained in the placenta was gently milked back into the fetus. The umbilical cord was clamped and cut, after which the fetus was cleaned to remove maternal blood and amniotic fluid. The blood was then allowed to drain from the umbilical cord by means of gravity and the fetal heart beat. The blood was permitted to clot at room temperature for approximately 1 hour and then centrifuged. The quantity of serum obtained ranged from about 0.1 ml in the smallest fetus to 4.0 ml in the largest. The sera were stored frozen for up to 12 months and were kept frozen during shipment. A normal adult serum, as a control, was stored and shipped under the same conditions.

The total protein content of the serum was determined by the biuret method of Dittelbrandt (20). Paper electrophoresis of the serum, at pH 8.6, was carried out both in barbital buffer, ( $\Gamma/2$ , 0.1) and in ammonium carbonate (0.1 M). Whatman 3MM filter paper was used in most experiments with serum aliquots of 0.03 ml. In some instances Whatman no. 1 filter paper or cellulose acetate strips (Oxo, Ltd.) were also used, in which case the quantity of serum was reduced to 0.01 ml. The electrophoresis was run for 18 to 24 hours at 2 ma per strip and 100 to 110 v by the conventional method, and the strips were stained either with bromphenol blue or amido black. Strips with fetal and adult sera were run simultaneously in the same apparatus. The concentration of the protein

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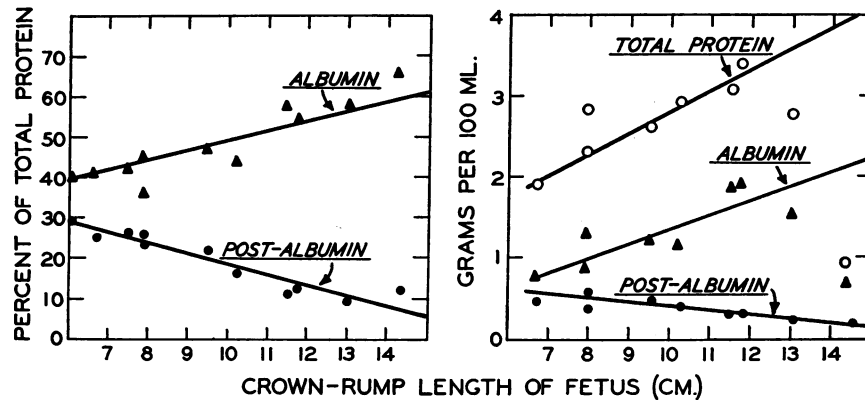


FIG. 1. RELATIVE (LEFT) AND ABSOLUTE (RIGHT) VALUES OF ALBUMIN AND POST-ALBUMIN PLOTTED AGAINST THE FETAL LENGTH. In this figure, as well as in those following, the straight lines are used only to indicate the general trend of the values and are not meant to imply that the progressions are linear.

fractions stained with amido black was measured by planimetry of the curve obtained with a scanning densitometer (Spinco Analytrol, model R). Glycoproteins were identified by staining the strips by the periodic acid-Schiff (PAS) reaction; lipoproteins were revealed by

staining with Sudan black. In these experiments 0.05 ml of serum was employed for each strip.

In order to identify the thyroxine-binding proteins,  $I^{131}$ -labeled L-thyroxine (Abbott Laboratories, Oak Ridge, Tenn.) was added *in vitro* to the serum. The preparations used were shown by paper chromatographic analysis to contain 90 to 95 per cent of the  $I^{131}$  as thyroxine. Approximately 0.01 and 0.5  $\mu$ g thyroxine dried under vacuum was added per ml serum. The mixtures were allowed to stand overnight at 4°C before electrophoresis was begun. Conventional and reverse-flow electrophoresis, at pH 8.6, was performed in barbital and in ammonium carbonate buffers (cf above), according to the methods previously described (21, 22).

Starch gel electrophoresis in 0.03 M borate buffer was carried out only at the level of 0.5  $\mu$ g of added thyroxine per ml of serum (22).<sup>2</sup>

The radioactive zones after electrophoresis were detected both by radioautography and by scanning the dry strips with a Geiger-Mueller tube and a continuously recording counting-rate meter. The areas under the radioactive peaks were quantitated with an electronic integrator.

The serum protein-bound iodine (PBI) was measured in only three of the oldest fetuses, because of the small amounts of serum collected. The analyses were performed at the Boston Medical Laboratory by the method of Zak, Willard, Myers and Boyle (23).

## RESULTS

The total serum protein concentration was lower in the fetuses than in adults, the values ranging from 1.9 g per 100 ml in the fetus 6.7 cm long, to 3.4 g per 100 ml in the fetus 11.7 cm long (Figure 1).

<sup>2</sup> We thank Dr. B. S. Blumberg and Dr. L. Farer, who kindly performed the starch gel electrophoresis.

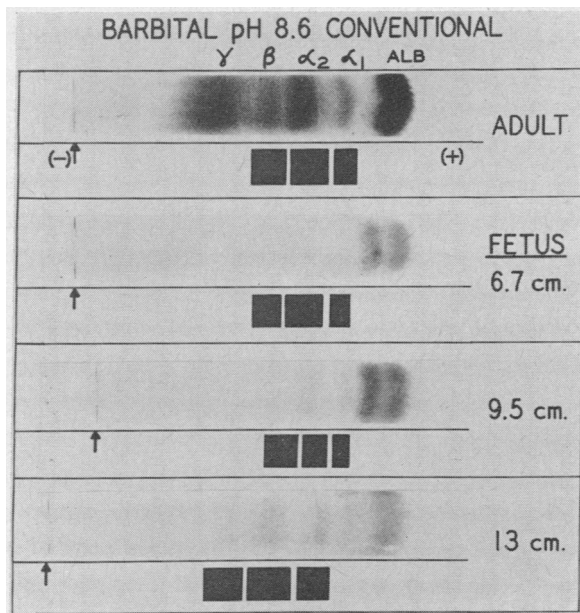


FIG. 2. ELECTROPHORETIC PATTERNS OF SERUM PROTEINS STAINED WITH AMIDO BLACK. The arrow indicates the point of application of the sample. The squares underneath each protein pattern indicate the positions of PAS-positive zones. The globulin zones in the fetuses were faint and were difficult to photograph;  $\alpha_1$ -,  $\alpha_2$ -, and  $\beta$ -globulin were seen in all, and increased with age;  $\gamma$ -globulin could be clearly identified only in the older fetuses. The fetal "postalbumin" protein is clearly ahead of the PAS-positive,  $\alpha_1$ -globulin.

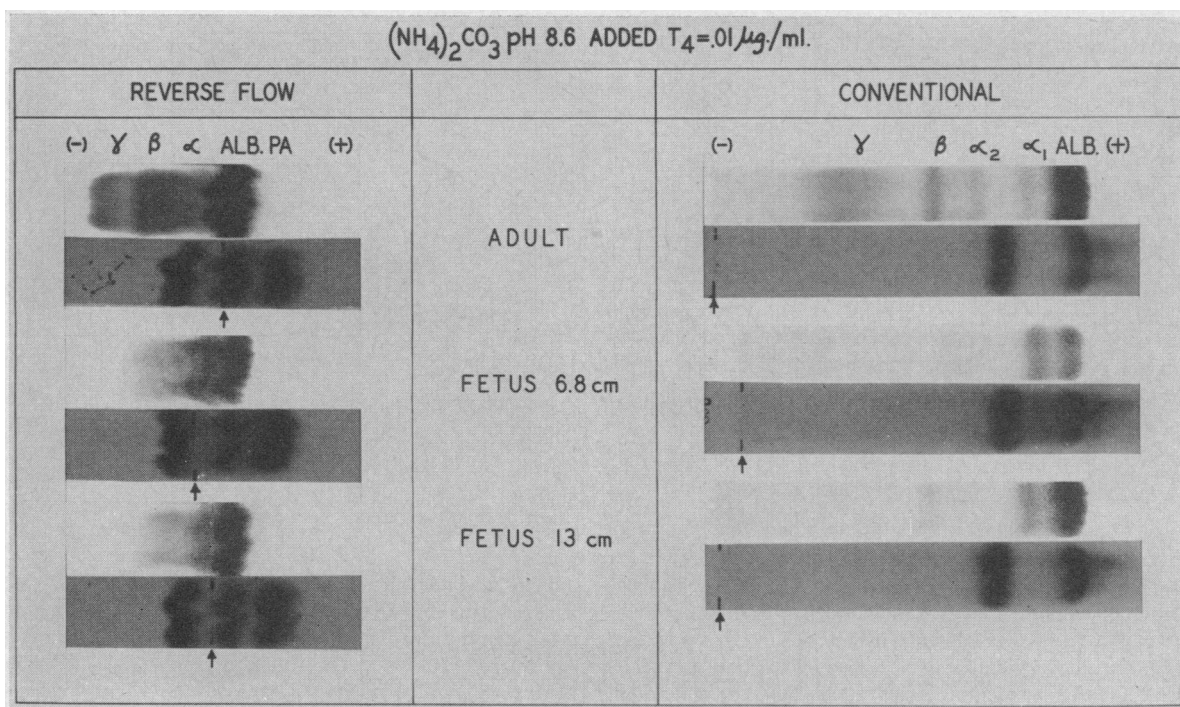


FIG. 3. ELECTROPHORESIS OF RADIOTHYROXINE-SERUM MIXTURES. Underneath each paper strip, stained with bromphenol blue, is the corresponding radioautograph (cf legend to Fig. 2). In ammonium carbonate buffer the fetal postalbumin fraction coincides with  $\alpha_1$ -globulin. A faint radioactive band corresponding to this zone is seen in the 6.8-cm fetus (PA = prealbumin).

The electrophoretic pattern of fetal serum was remarkably different from the adult. In barbital buffer a protein fraction, not present in the adult, migrated between albumin and  $\alpha_1$ -globulin (Figure 2). This "postalbumin" fraction comprised 29 per cent<sup>3</sup> of total protein in the 5.8-cm fetus and decreased to 10 per cent in the 13-cm fetus (Figure 1). In addition, the absolute amount of postalbumin showed a significant decrease from 0.46 g per 100 ml in the 6.7-cm fetus to 0.27 g in the 13-cm fetus (Figure 1). In ammonium carbonate the fetal postalbumin fraction coincided with  $\alpha_1$ -globulin (Figure 3).

The fetal albumin progressively increased with fetal size, both relatively and absolutely, from 39 per cent (0.75 g per 100 ml) in the 6.7-cm fetus to 57 per cent (1.6 g per 100 ml) in the 13-cm fetus (Figure 1). It is interesting to note that the inverse variations of albumin and postalbumin were such that the sum of these fractions, ex-

<sup>3</sup> These values are uncorrected for "tailing" along the paper strip.

pressed as per cent of total protein, was relatively constant at approximately 67 per cent. In absolute terms, the sum increased from 1.2 to 2.4 g per 100 ml.

Several attempts were made to characterize the postalbumin fraction by staining reactions. The PAS reaction failed to reveal glycoprotein in postalbumin even when 0.05 ml of serum was used per strip and despite the fact that other zones containing smaller amounts of protein gave positive reactions (Figure 2). Staining for lipoprotein resulted in a faint positive reaction in postalbumin and a positive reaction in albumin, and also in a smear extending from the origin to the  $\gamma$ -globulin area. These findings, however, were not consistently obtained and are unreliable owing to the fact that the sera were not fresh and had been frozen and thawed several times before use.

When bromphenol blue was added to serum before electrophoresis, the dye was found only in association with albumin and not with postalbumin. A similar study was performed on the serum of a subject with the genetic abnormality known

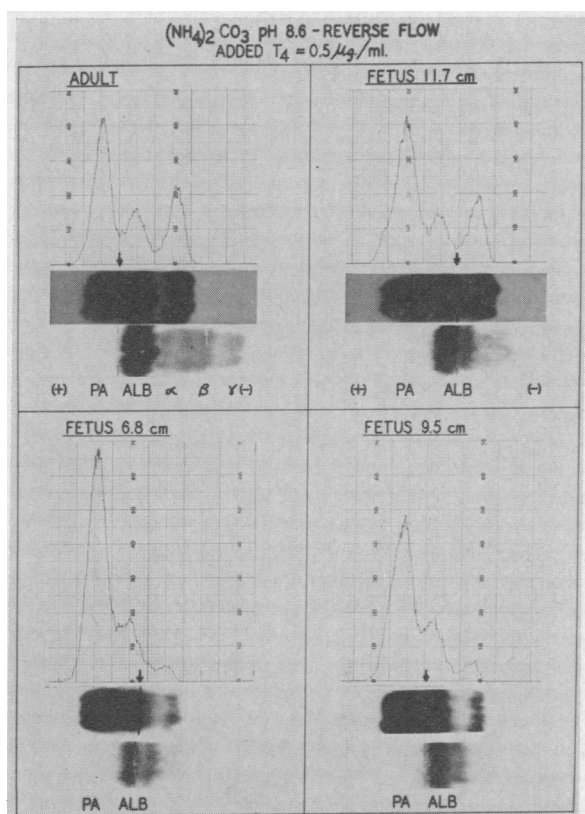


FIG. 4. ELECTROPHORESIS OF THYROXINE-SERUM MIXTURES. Above each paper strip, stained with bromphenol blue, is the corresponding radioautograph as well as the recorded distribution of radioactivity.

as bisalbuminemia (24).<sup>4</sup> Bromphenol blue migrated only with the more rapidly moving albumin A which is considered to be the normal type of albumin. Preliminary attempts to identify postalbumin by immunoelectrophoresis have been unsuccessful, and further study is in progress. Quantitation of the serum proteins other than albumin and prealbumin was not possible because of the small quantity of these fractions relative to the trailing of the faster proteins.

Conventional and reverse-flow paper electrophoresis of thyroxine-serum mixtures in the fetus revealed the same thyroxine-binding proteins as in the adult: inter- $\alpha$ -globulin (TBG), albumin, and prealbumin (TBPA) (Figures 3 and 4). All were detected at low ( $0.1 \mu\text{g}$  per ml) and high ( $0.5 \mu\text{g}$  per ml) levels of added thyroxine, but

<sup>4</sup> This serum was kindly made available in the form of a lyophilized powder by Dr. David Earle.

TBPA was not seen in barbital buffer. At the high level of thyroxine in barbital there was streaking of radioactivity in the area between the point of application of the serum and the  $\alpha$ -globulin zone in conventional electrophoresis; this trailing was more intense in the fetus than in the adult. Although not consistently detectable, a small amount of thyroxine was found in association with the postalbumin protein. This was more clearly seen in electrophoresis carried out in cellulose acetate membrane (Figure 5). It was also shown that both albumin A and B in bisalbuminemia form complexes with thyroxine (Figure 5).

Starch gel electrophoresis in the fetus revealed the normal TBPA and TBG bands, a band in albumin and a more slowly moving broad band between the origin and TBG (Figure 6). The latter was absent in the adult and corresponded to the migration of thyroxine in the absence of serum (25). The TBG and TBPA bands were less intense in the fetus than in the adult.

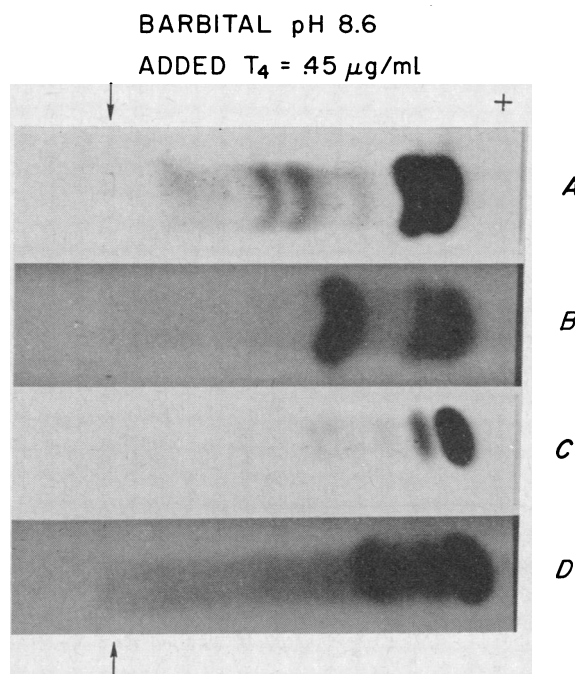


FIG. 5. ELECTROPHORESIS ON CELLULOSE ACETATE OF THYROXINE-SERUM MIXTURES FROM A PATIENT WITH BISALBUMINEMIA (A,B) AND FROM A FETUS 9.5 CM LONG (C,D). A and C are photographs of the strips stained with amido black. The corresponding radioautographs (B and D) show that both abnormal albumin B of bisalbuminemia and fetal postalbumin bind thyroxine.

## STARCH GEL BORATE pH 8.6 ADDED $T_4 = 0.5 \mu\text{g/ml}$ .

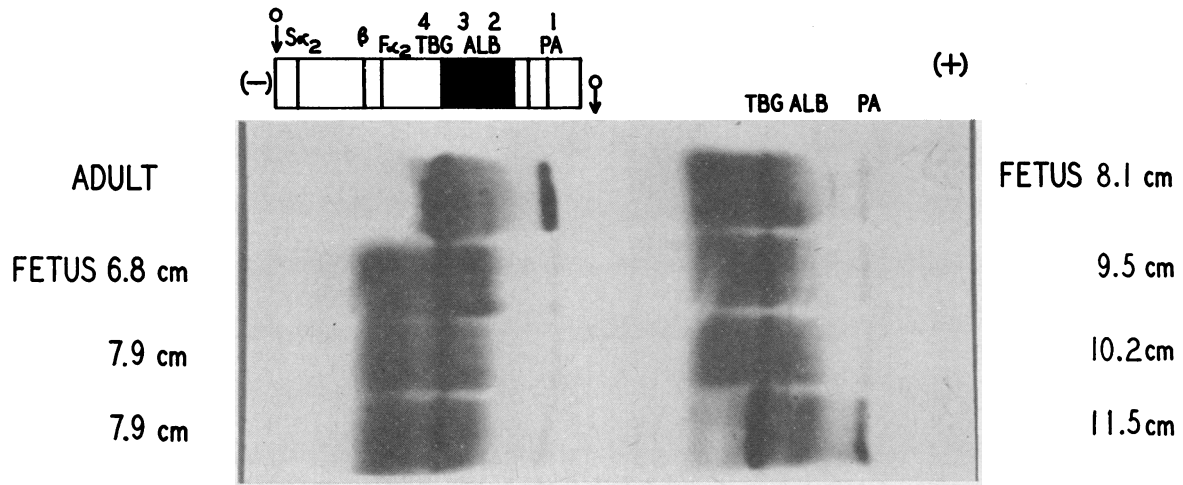


FIG. 6. STARCH GEL ELECTROPHORESIS OF THYROXINE-SERUM MIXTURES. Only the radioautograph is shown. At the upper left is a diagram indicating the location of protein zones in the adult.  $S\alpha_2$  and  $F\alpha_2$  are the slow and fast  $\alpha_2$ -globulins, respectively. The numbers 1, 2, 3, 4 indicate the location of the thyroxine zones in the adult (22); 0 indicates the point of application.

Quantitative studies of thyroxine-binding ability of the fetal sera were based on the reverse-flow technique. Both in barbital and ammonium carbonate buffers, the fraction of thyroxine bound to fetal TBG was lower than in the adult, tending to increase with embryonic development. With the addition of  $0.01 \mu\text{g}$  of thyroxine per ml of serum and electrophoresis in barbital, the amount of labeled thyroxine bound to TBG progressively increased from a minimum of 55 per cent of the total in the fetus 7.9 cm long to a maximum of 68 per cent in the fetus 13 cm. These may be compared with a value of 77 per cent in the adult (Figure 7). When the endogenous serum thyroxine (PBI,  $5.4 \mu\text{g}$  per 100 ml) is taken into account, the total quantity of thyroxine bound to TBG of the adult was  $0.046 \mu\text{g}$  per ml. In the fetus the endogenous thyroxine would be much smaller, since the PBI in the oldest fetuses was found to be 1.4 to  $1.6 \mu\text{g}$  per 100 ml, corresponding to a thyroxine concentration of 0.021 to  $0.024 \mu\text{g}$  per ml. Since the PBI could not be measured in most fetuses this calculation is not possible. In ammonium carbonate the amount of TBG-bound thyroxine rose from 20 per cent in the 7.5-cm fetus to 41 per cent in the 13-cm fetus, compared

with 40 per cent ( $0.025 \mu\text{g}$  per ml) in the adult (Figure 7).

With the addition of  $0.5 \mu\text{g}$  of thyroxine per ml, the relative and absolute amounts of thyroxine bound to TBG progressively increased, in barbital, from 4 per cent ( $0.02 \mu\text{g}$  per ml) in the 7.9-cm

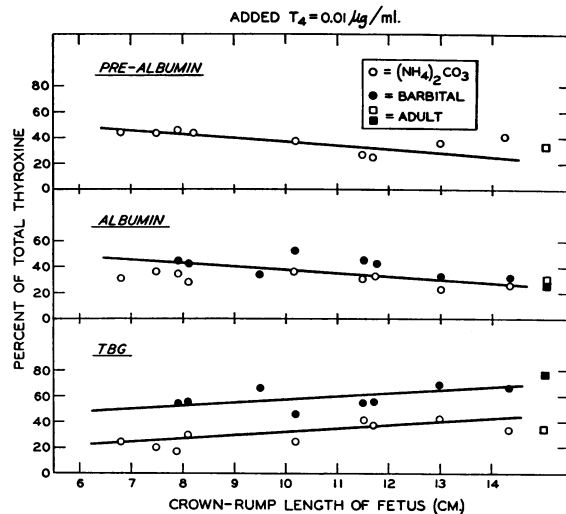


FIG. 7. THE DISTRIBUTION OF THYROXINE AMONG THE BINDING PROTEINS AT A LOW LEVEL OF ADDED RADIOTHYROXINE (SEE LEGEND TO FIG. 1).

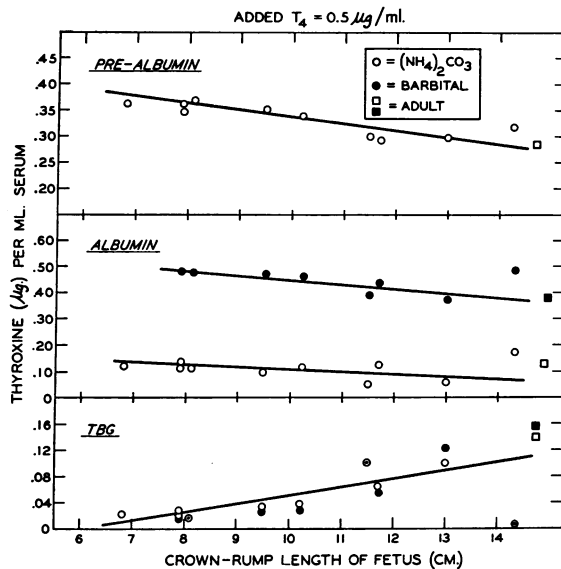


FIG. 8. THE DISTRIBUTION OF THYROXINE AMONG THE BINDING PROTEINS AT A HIGH LEVEL OF ADDED RADIOTHYROXINE. The values, given in absolute terms, are not corrected for endogenous thyroxine (cf text; see legend to Fig. 1).

fetus to 24 per cent ( $0.12 \mu\text{g}$  per ml) in the 13-cm fetus, as compared with 30 per cent ( $0.15 \mu\text{g}$  per ml) in the adult (Figure 8). In ammonium carbonate the values increased from 5 per cent ( $0.025 \mu\text{g}$  per ml) in the 6.8-cm fetus to 21 per cent ( $0.10 \mu\text{g}$  per ml) in the 13-cm fetus, in comparison with 25 per cent ( $0.13 \mu\text{g}$  per ml) in the adult (Figure 8). At this concentration of added thyroxine, correction for endogenous thyroxine has little effect. Thus, the corrected values for the adult are  $0.16$  and  $0.14 \mu\text{g}$  per ml in barbital and ammonium carbonate, respectively. In the fetuses, the correction would be even smaller.

Although no attempt was made to define the maximal binding capacity by adding increasing amounts of thyroxine, it is likely that TBG was saturated at the level of  $0.5 \mu\text{g}$  of added thyroxine per ml, at least in the young fetuses. In the adult the thyroxine-binding capacity was probably somewhat higher than the value found at the  $0.5 \mu\text{g}$  level.

The amount of thyroxine found in the fetal prealbumin zone was inversely related to the TBG level and progressively decreased with the age. At a concentration of  $0.01 \mu\text{g}$  of thyroxine added per ml of serum, 43 per cent was found in TBPA in the 6.8-cm fetus, and 27 per cent in the 11.7-cm

fetus, as compared with 30 per cent ( $0.02 \mu\text{g}$  per ml) in the adult. At a concentration of  $0.5 \mu\text{g}$  per ml there was 73 per cent ( $0.36 \mu\text{g}$  per ml) in TBPA in the 6.8-cm fetus, and 60 per cent ( $0.30 \mu\text{g}$  per ml) in the 13-cm fetus, as compared with 51 per cent ( $0.26 \mu\text{g}$  per ml, corrected value  $0.28 \mu\text{g}$  per ml) in the adult. It is likely, however, that a portion of the thyroxine in the prealbumin area was not bound to protein and did not represent a TBPA-thyroxine complex (Ref. 25 and Discussion).

In the fetus 14.3 cm long, the finding of a low total protein, a low TBG capacity, and a high proportion of thyroxine in the prealbumin region was comparable with the results obtained with the youngest fetuses. The reason for the unusual findings in this individual is not apparent.

#### DISCUSSION

The fact that the total concentration and the electrophoretic pattern of the serum protein in the human fetus differ from those of the mother and of the adult has been known for some time. However, the values obtained by different workers are not in quantitative agreement. The main reasons for these discrepancies are both the differences between the sources of the blood and the various methods employed.

Our data confirm the findings that the total serum protein concentration in the fetus is very low during the first stage of development and rises continually with age (6, 15, 16). We also found that during the period of our observation the relative and absolute amount of albumin, and the absolute value of  $\gamma$ -globulin, rises (4, 5, 7, 26). The fraction migrating between albumin and  $\alpha_1$ -globulin observed by Bergstrand and Czar (16), which we call postalbumin, constituted in our observations a considerable part of the total protein. In the youngest fetus it comprised 29 per cent of the total and decreased progressively to 10 per cent in the fetus 13 cm long. When compared on the basis of fetal size, the data of Bergstrand and Czar are in rough agreement with ours. These authors studied only fetuses greater than 10 cm in length, but they do not specify that these lengths are crown to rump. The correlation between size and age reported by these authors differs from our observations. Halbrecht, Klibansky, Brzoza and

Lahav (27) also found that postalbumin decreased in quantity between weeks 10 and 18 of fetal life.

Our finding that the postalbumin fraction does not contain PAS-positive glycoproteins confirms the results of Halbrecht and co-workers (27). Thus this material differs from fetuin of other species, which has been shown to be an acidic glycoprotein (cf opening paragraph). On the other hand, it has been suggested that postalbumin may be a fetuin in an initial carbohydrate-free stage (28).

The appearance of postalbumin, migrating just behind albumin, bears a superficial resemblance to the genetically determined "double albumin" recently described by Earle, Hutt, Schmid and Gitlin (24). In support of this possibility are the findings that neither postalbumin nor albumin B binds bromphenol blue when the dye is added before electrophoresis, and that both postalbumin and albumin B have the ability to bind thyroxine (Ref. 29 and Results). It has not been possible thus far to obtain more specific evidence in support of this speculation, so that the nature and significance of fetal postalbumin is at present unknown. Although maternal blood was not examined in the present study, there is no evidence that postalbumin exists in the mother (16), and hence we may presume it to be of fetal or placental origin.

Thyroxine binding by serum proteins of the human fetus has not been studied except at term. However, Osorio and Myant (17) found in rabbit fetal serum a special thyroxine-binding protein different from that in adults. These authors, from their studies on fetal and pregnant rabbits, suggest that the fetal thyroxine-binding substance reaches the mother's blood from the fetus and that some of the adult type thyroxine-binding protein in the fetal circulation is derived from the mother (30).

Our results demonstrate that human fetal serum contains the same thyroxine-binding proteins as the adult: prealbumin, albumin, and TBG. The fetal postalbumin, however, shows a slight affinity for thyroxine in the very young fetus. Quantitative studies were consistent with a low concentration of TBG in fetal serum, as compared with the adult, and a progressive increase with fetal size. The possibility of a difference between fetal and adult TBG in intensity of binding was not ex-

cluded, however. The amount of thyroxine in the albumin and prealbumin zones showed an inverse relationship to the amount of thyroxine bound to TBG. As indicated above, however, part of this thyroxine was probably not bound to protein, presumably as a result of dissociation during electrophoresis. At a high concentration of  $I^{131}$ -labeled thyroxine in the fetus, a zone of radioactivity between the origin and the TBG zone was also observed in starch gel electrophoresis. This band is absent in the adult and corresponds to the migration of free thyroxine in the absence of serum. Similar observations have been made in sera from patients with hypoalbuminemia and in animal sera containing little thyroxine-binding protein (25).

From the data available it is impossible to state whether the fetal TBG is derived from the maternal circulation or whether it is synthesized by the fetus itself or by the placenta.

#### SUMMARY

Serum has been examined in 13 human fetuses ranging in size (crown to rump) from 5.8 to 14.3 cm and in age from 8 to 20 weeks. Fetal serum contains a protein fraction moving between albumin and  $\alpha_1$ -globulin in barbital buffer and with  $\alpha_1$ -globulin in ammonium carbonate. This postalbumin component comprised 29 per cent of the total protein in the youngest fetus and decreased with age. It did not contain PAS-positive glycoproteins, did not bind bromphenol blue prior to denaturation, and exhibited a slight affinity for thyroxine.

During fetal development total protein, albumin, and  $\gamma$ -globulin increased.

Protein-bound iodine determined in the oldest fetuses was lower than in the adult, ranging from 1.4 to 1.6  $\mu\text{g}$  per 100 ml.

Zone electrophoresis in barbital, ammonium carbonate, or borate buffer, pH 8.6, revealed in the fetus the same thyroxine-binding proteins present in the adult: prealbumin, albumin and inter- $\alpha$ -globulin (TBG). In starch gel electrophoresis there was a more slowly moving band of thyroxine which was absent in the adult and corresponded to the large amount of thyroxine found by reverse-flow electrophoresis in albumin (barbital) or in prealbumin (ammonium carbonate). This phenomenon was inversely related to the amount of



TBG-bound thyroxine and appears to represent thyroxine dissociated from protein during electrophoresis.

Quantitative studies indicated that thyroxine binding by TBG in fetal serum was low, compared with the adult, and increased with fetal size.

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