Sites of Synthesis of Plasma Proteins in the Foetal Rat

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1. The foetal rat of 16 or more days incorporates ^{14}C -labelled amino acids into all the demonstrable plasma protein fractions *in vivo*. 2. Slices of foetal rat liver incubated *in vitro* incorporate ^{14}C -labelled amino acids into the main plasma protein fractions, including the foetal-specific 'post-albumin'. 3. Slices of placenta are unable to incorporate ^{14}C -labelled amino acids into plasma proteins *in vitro*. 4. Liver slices from maternal rats incubated *in vitro* incorporate ^{14}C -labelled amino acids into plasma proteins. The presence of post-albumin cannot be demonstrated after incubation. 5. Liver slices from foetal rats, but not from adult rats, contain demonstrable amounts of haemoglobin into which ^{14}C -labelled amino acids are incorporated.

The synthesis of the albumin and globulin fractions of rat plasma was shown by Miller, Bly, Watson & Bale (1951) to take place in the liver. This study was carried out on the perfused intact organ. Albumin synthesis by rat-liver slices *in vitro* was demonstrated by Campbell (1955) and Campbell & Stone (1957). Although much work has been done on the passage of proteins across the placenta (Hemmings & Brambell, 1961) the possible role of the foetus in the supply of foetal plasma proteins has received little attention. Kelleher, Kenyon & Villet (1963) showed that the specific activity of the albumin and globulin plasma protein fractions was much higher in foetal than in maternal plasma after injection of labelled amino acids into the foetus *in utero*. This finding suggested that the foetus was capable of the new synthesis of plasma proteins but the tissue sites of synthesis were not identified. In the human foetus, Dancis, Braveman & Lind (1957) found that the liver can synthesize all plasma proteins except γ -globulins. The placenta was not able to synthesize plasma proteins.

In previous work (Wise, Ballard & Ezekiel, 1963) a specific foetal plasma protein (post-albumin) was identified by starch-gel electrophoresis. The protein occurs in concentrations similar to albumin in the foetus but is absent from the maternal and adult rat plasma. The protein has been partially purified and has a sedimentation coefficient similar to that of rat albumin (R. W. Wise & I. T. Oliver, unpublished work). In the present work, the tissue sites of synthesis of post-albumin, albumin and some other foetal plasma proteins were investigated.

MATERIALS AND METHODS

Animals. The rats used in this study were of the Wistar Albino strain, species *Rattus norvegicus*.

Radioactive labelling of plasma proteins *in vivo*. Pregnant rats were anaesthetized with ether and the uterus of each was exposed by an incision in the abdominal wall. Each of two foetuses was injected intraperitoneally with $8\mu\text{C}$ of ^{14}C -labelled algal-protein hydrolysate ($200\mu\text{C}/\text{mg}$; The Radiochemical Centre, Amersham, Bucks.). The maternal wound was sewn up and the rat allowed to regain consciousness. After 2 hr. the rat was killed by a blow on the head and the maternal blood sampled from the heart. The injected foetuses, as well as control animals that had not been injected, were removed and blood was obtained by severing the jugular vessels. Plasmas obtained were stored at -20° until required.

Plasma samples ($40\mu\text{l}$. of foetal and $10\mu\text{l}$. of maternal plasma) were separated by paper electrophoresis according to the method of Aronson & Gronwall (1957). The stained strips were scanned for radioactivity with a Nuclear-Chicago Actigraph 4π gas-flow detector. The strips were counted with the windows removed. A slit width of $\frac{1}{8}$ in. was employed. The strips were evaluated for protein with a Carl Zeiss Jena electrophoresis scanner.

Radioactive labelling of plasma proteins by tissue slices. Liver slices were prepared as described by Ballard & Oliver (1964). Slices of placenta were prepared in the same manner. Krebs-Ringer bicarbonate buffer (Krebs & Henseleit, 1932) with KH_2PO_4 omitted was used for the incubations. A 150 mg. portion of blotted slices was added to 1 ml. of the gassed buffer containing $5\mu\text{C}$ of ^{14}C -labelled protein hydrolysate. After shaking for 3 hr. at 37° (120 oscillations/min.) in a Dubnoff metabolic shaker, the slices were homogenized in a coaxial homogenizer, treated ultrasonically for 2 min. with a Dawe Sonifier ($\frac{1}{4}$ in. probe, strength 5) and cleared by centrifugation for 3 hr. at 20000g. The clear supernatant fluid was dialysed against water, freeze-dried and dissolved in a minimum volume of

0.9% NaCl (about 15 μ l.). A 1 μ l. portion of this sample was used for immunoelectrophoresis.

Preparation of rabbit immune sera. (i) Antisera to foetal rat plasma. Pooled foetal rat plasma was used for preparation of antisera. Rabbits were given four subcutaneous injections of 2 ml. each of equal parts of whole foetal plasma and Freund's complete adjuvant (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). Four weeks after the first injection, three injections were given at 1-week intervals. The rabbit was bled from the ear on alternate days for 1 week commencing 2 weeks after the last injection. After the blood had clotted the antiserum was separated and stored at -20° until required.

(ii) Antisera to purified plasma proteins. Rabbit antisera to rat plasma albumin was prepared in a similar manner, by injection of equal volumes of 1% protein solution in 0.9% NaCl and Freund's adjuvant. The albumin was prepared by the method of Korner & Debro (1956). Rat transferrin (Morgan, 1964) was kindly supplied by Dr E. Morgan (Department of Physiology, University of Western Australia) and antiserum obtained by intramuscular injection of transferrin solution with Freund's adjuvant.

Immunoelectrophoresis. Electrophoresis was carried out in 1.5% agar in the barbital buffer described by Laurell, Laurell & Skoog (1956). A current of 2.5 ma/slide was passed for 2.5 hr. at 4° . Antisera (diluted 1:4 with 0.9% NaCl) were placed in the diffusion wells and the slides stored at 4° for 48 hr. The slides were washed exhaustively with 0.9% NaCl and stained with 0.5% Ponceau S in 5% (v/v) acetic acid or in 0.5% Naphthalene Black in ethanol-water-acetic acid (5:5:1, by vol.).

Radioautography. (a) ^{14}C . The stained slides were placed in contact with the emulsion side of Kodak Royal Pan film and stored in a cassette for 7 days at room temperature.

(b) ^{59}Fe . The dried Ouchterlony plates were exposed to Kodak Kodirex film in an X-ray cassette for 3 days.

Identification of immunoelectrophoretic components of liver extracts. (a) Albumin. This was identified by comparing the precipitin lines obtained with the standard protein and the liver extracts after agar electrophoresis and diffusion against anti-(rat plasma albumin) and anti-(foetal rat plasma) (Fig. 2).

(b) Post-albumin. This was identified as that component with an electrophoretic mobility similar to albumin which was present in foetal liver extract but absent from adult liver extract. This was shown by (i) electrophoresis of adult and foetal liver extract and diffusion against anti-(foetal rat plasma) (Fig. 2), and (ii) incubation of anti-(foetal rat plasma) with adult plasma before diffusion against the electrophoretically separated extracts. In this case, only the precipitin lines assigned to post-albumin and haemoglobin were present in foetal liver extract. The absorbed antiserum gave no reaction with albumin or adult liver extract.

(c) Haemoglobin. The foetal plasma used to obtain the antiserum was partially haemolysed. Extracts from foetal liver slices gave a precipitin line in immunoelectrophoresis against anti-(foetal rat plasma) that disappeared after preincubation of the antiserum with haemoglobin. Haemoglobin was prepared by ultrasonic treatment of washed foetal erythrocytes.

(d) Transferrin. The immunodiffusion pattern obtained by allowing liver-slice extracts to react against anti-transferrin showed a precipitin reaction in a position

corresponding to that obtained with standard transferrin.

Reaction of foetal liver extracts against anti-transferrin occasionally gave more than one precipitin line. Foetal liver extracts were stripped of iron by dialysis against 0.1 M-sodium acetate buffer, pH 5.0, containing EDTA (0.01%). After further dialysis against distilled water and then against electrophoresis buffer, 0.1 μC of ^{59}Fe was added as FeCl_3 in acid solution (Abbott Laboratories, North Chicago, Ill., U.S.A.). The extract was concentrated and double diffusion against serial dilutions of anti-transferrin carried out under conditions similar to those used for immunoelectrophoresis. Radioautography of the dried unstained plates was carried out. The major precipitin line was heavily labelled with ^{59}Fe . Two components present in minor amounts were not labelled.

RESULTS

Fig. 1 shows the paper-electrophoretic pattern and radioactivity scan of the plasma proteins of the 20-day foetal rat. The main protein components (albumin, post-albumin, α -globulin and β -globulin) were labelled after the injection *in vivo* of ^{14}C -labelled amino acids into the foetus. Under the conditions described, no radioactivity was detectable in the plasma proteins of the maternal rat or of the control fetuses. Similar labelling of the

Fig. 1. Radioactive labelling of plasma proteins by the rat foetus *in vivo*. (a) Paper-electrophoretic separation of foetal plasma proteins from 20-day foetus, after injection of ^{14}C -labelled amino acids; (b) densitometric scan of (a); (c) scan of (a) for radioactivity due to incorporated ^{14}C -labelled amino acids. Numbered components are: 1, albumin; 2, post-albumin; 3, α_2 -globulin; 4, transferrin; O is the point of application of the sample.

plasma proteins was observed in test foetuses of ages between 16 days and term.

Fig. 2 shows the immunoelectrophoretic pattern and Fig. 3 the radioautography record of liver-slice extracts from the pregnant rat and its 16-day foetus. In the foetal sample it is possible to identify albumin, post-albumin, transferrin and haemoglobin (Fig. 2). All were labelled with ^{14}C (Fig. 3). In the maternal liver extracts, no post-albumin or haemoglobin was detected, although an α -globulin can be seen (Fig. 2). All components were labelled with ^{14}C (Fig. 3). Both maternal and foetal extracts contained a diffuse protein area, migrating to the cathode. It was also labelled.

The immunoelectrophoretic pattern of placental-slice extract from the 19-day pregnant rat shows that albumin, post-albumin and transferrin are present. However, no label was detected in these proteins by radioautography. Small amounts of

radioactivity were present in diffuse precipitin lines that were not identifiable as plasma proteins. The presence of unidentified labelled protein in placental extracts indicates that the slices were active in protein synthesis under the conditions used *in vitro*. Dancis *et al.* (1957), in studies on human placental slices *in vitro*, found incorporation of ^{14}C -labelled glycine into protein fractions that were electrophoretically similar to the plasma α - and β -globulins. The immunological properties of these fractions were related to plasma globulins only in the term placenta and with 3-month placenta were found to be unrelated. At neither stage of pregnancy did the placenta synthesize proteins electrophoretically identifiable as albumin or γ -globulin.

DISCUSSION

The occurrence of a foetal-specific plasma protein component and its absence from the maternal blood does not constitute definitive

Fig. 2. Tracing of immunoelectrophoretic patterns of labelled rat-liver extracts. (a) The sample on the left is rat serum albumin; the sample on the right is liver extract from a 16-day foetal rat. (b) The sample on the left is rat serum albumin; the sample on the right is a liver extract of maternal rat, 16 days pregnant. Anti-(foetal rat plasma) was placed in all wells for diffusion.

Fig. 3. Radioautography of immunoelectrophoretic patterns of labelled liver extracts. The radioautograph in this Figure is that obtained from the samples shown in Fig. 2.

evidence of its synthesis by foetal tissues. Some of the experiments reported here were designed to clarify this issue.

The results of experiments *in vivo* indicate that the rat foetus synthesizes foetal plasma proteins including the foetal-specific post-albumin. The absence of label from the plasma proteins of the control fetuses in the litter and the maternal plasma clearly demonstrates that radioactive amino acids were incorporated primarily by the injected foetus. Although Morgan (1964) has demonstrated that several intact maternal plasma proteins can be transferred to the foetus, the present results show that the foetal tissues also bring about synthesis. However, these experiments do not identify the tissue sites of synthesis, and experiments with isolated tissue slices *in vitro* were performed to allow such analysis.

Foetal liver slices bring about synthesis of albumin, haemoglobin, transferrin and post-albumin. The maternal liver slices were also capable of plasma protein synthesis *in vitro* but haemoglobin and post-albumin were not among the products. Since the perinatal liver contains large amounts of haematopoietic tissue until 5 days after birth (Oliver, Blumer & Witham, 1963), the identification of haemoglobin among the labelled foetal products is not surprising.

The rat placenta did not synthesize any of the plasma proteins described above under the conditions of the experiments, since the specific proteins extracted from the placenta were found to be unlabelled. This result also demonstrates that the labelling of liver-slice proteins was not due to unspecific binding of radioactive amino acids. In addition, when albumin is isolated as the antigen-antibody complex from liver slices incubated *in vitro*, its quantitative labelling with ^{14}C -labelled

amino acids is time-dependent (R. W. Wise & I. T. Oliver, unpublished work).

The experiments *in vivo* and *in vitro* demonstrate that the late-foetal rat liver brings about synthesis of foetal plasma proteins and the differentiation of liver function in this respect must occur early in embryogenesis. The transfer of maternal protein can probably account for only a minor fraction of the total plasma protein present in the foetal circulation.

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REFERENCES

- Aronsson, T. & Gronwall, A. (1957). *Scand. J. clin. Lab. Invest.* **9**, 338.
Ballard, F. J. & Oliver, I. T. (1964). *Biochem. J.* **90**, 261.
Campbell, P. N. (1955). *Biochem. J.* **61**, 496.
Campbell, P. N. & Stone, N. E. (1957). *Biochem. J.* **66**, 19.
Dancis, J., Braveman, N. & Lind, J. (1957). *J. clin. Invest.* **36**, 398.
Hemmings, W. H. & Brambell, R. F. W. (1961). *Brit. med. Bull.* **17**, 96.
Kelleher, P. C., Kenyon, C. D. & Villee, C. A. (1963). *Science*, **139**, 839.
Korner, A. & Debro, J. R. (1956). *Nature, Lond.*, **178**, 1067.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Laurell, C. B., Laurell, S. & Skoog, N. (1956). *Clin. Chem.*, **2**, 99.
Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951). *J. exp. Med.* **94**, 431.
Morgan, E. H. (1964). *J. Physiol.* **171**, 26.
Oliver, I. T., Blumer, W. F. C. & Witham, I. J. (1963). *Comp. Biochem. Physiol.* **10**, 33.
Wise, R. W., Ballard, F. J. & Ezekiel, E. (1963). *Comp. Biochem. Physiol.* **9**, 23.