The Proteins and Protein-Bound Carbohydrates of the Serum of the Developing Pig

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1. The concentrations of total nitrogen, hexosamine and protein-bound hexose and the amounts of these constituents precipitated by trichloroacetic acid were determined in the serum of pigs at various ages from 57 days after copulation to 42 days after birth. The concentrations of the same constituents were also determined in the serum of mature pigs. 2. A rise in the concentrations of total nitrogen, hexosamine and protein-bound hexose between 90 days' gestation and term was entirely due to material soluble in trichloroacetic acid. This material disappeared from the serum by 7 days after birth. 3. Electrophoresis of the serum proteins showed that the concentration of α_1 -globulin fell steadily during gestation, being lower at term than at 57 days' gestation. No α_1 -globulin was detected at 7 days of age. 4. It was concluded that high values for non-protein nitrogen in the serum of newborn piglets, determined after precipitation of the proteins with trichloroacetic acid, is largely due to the presence of one or more mucoproteins and not to α_1 -globulin. 5. The protein migrating in the α_1 -globulin position is possibly a foetal protein of the fetuin type.

The newborn piglet is a useful experimental animal for the study of neonatal physiology. The analysis of the serum of the unsuckled animal is, however, made difficult by the failure of its proteins to precipitate with the normal protein precipitants such as trichloroacetic acid and tungstic acid. The proteins are completely precipitated by these reagents if adult pig serum is added to the mixture (E. M. Widdowson, personal communication). The study reported here was undertaken in an endeavour to find an explanation for this phenomenon.

Moore, Shen & Alexander (1945) found that the proportion of the serum protein of pig foetuses accounted for by α_1 -globulin fell from 50 to 30% during gestation. Other workers (Foster, Friedell, Catron & Dieckmann, 1951; Rutquist, 1958; Miller *et al.* 1961) have reported that at term α_1 -globulin accounts for 46–68% of the serum proteins. Using moving-boundary electrophoresis, Rook, Moustgaard & Jakobsen (1951) found that newborn pig serum contained a large amount of a protein, distinguished as 'component X', which migrated between albumin and α_2 -globulin. This protein disappeared from the serum in the first 5 days after birth.

In newborn piglet serum these workers found that abnormally large amounts of nitrogen were soluble

in trichloroacetic acid and they presumed that this was due to the solubility of their protein 'component X'. Since, however, mucoproteins, as defined by Roseman (1959) are rarely precipitated by trichloroacetic acid (Kent & Whitehouse, 1955), it seemed possible that one or more of these substances might also be contributing to the nitrogen soluble in trichloroacetic acid.

The change with age from 56 days' gestation to maturity in the amounts of hexosamine and protein bound hexose in the serum has been studied. A concurrent study has been made of the serum protein pattern to find if changes in the concentration of the carbohydrates could be correlated with those of α_1 -globulin.

METHODS

Materials. Pigs of the Large White breed only were used. Blood was collected from foetuses at nine ages between 54 and 90 days' gestation by allowing it to drip from the severed end of the umbilical cord. The blood from two to six foetuses was pooled, and two pooled samples were obtained at each age. Blood was also obtained by heart puncture from nine newborn piglets before suckling, four at 1 day, two at 2 days, four at 4, 7 and 14 days respectively. Samples were also obtained from six adult pigs at slaughter. In each case, the blood was allowed to clot at room temperature, the serum separated by centrifugation and the separated serum stored at -15° until required.

Chemical methods. Total serum hexosamine and protein-bound hexosamine precipitated from serum by 10% (w/v)

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trichloroacetic acid (2 ml./0.1 ml. of serum) were determined by the Elson-Morgan reaction as described by Belcher, Nutten & Sambrook (1954). Protein-bound hexose was determined after precipitation of the proteins (a) with 95% ethanol, and (b) with trichloroacetic acid, by the orcinol method as described by Winzler (1955). Total N and N precipitated by trichloroacetic acid were determined by the micro-Kjeldahl method according to Chibnall, Rees & Williams (1943). The supernatant fluids obtained after precipitation of serum proteins with trichloroacetic acid were dialysed against distilled water at 4° and N, hexosamine and hexose determined in them by the methods described above.

To identify the hexoses bound to the serum proteins the proteins precipitated by trichloroacetic acid from samples of foetal, newborn and early post-natal sera were hydrolysed in 5% (v/v) H_2SO_4 for 2 hr. The hydrolysates were neutralized with solid BaCO_3 , the mixture was filtered, the filtrate evaporated to dryness *in vacuo* over P_2O_5 , dissolved in water and chromatographed on Whatman no. 1 paper with ethyl acetate-acetic acid-water (3:1:3, by vol.) as solvent.

Serum proteins were separated by electrophoresis on cellulose acetate paper (Oxoid, Oxo Ltd.) in barbiturate buffer (pH 8.6, 10.06). After fixation in industrial methylated spirit, proteins were stained with Ponceau S and protein-bound carbohydrate with a periodic acid-Schiff stain as described by Smith (1960). The strips stained with Ponceau S were cleared in liquid paraffin and the density of the protein bands was determined with an EEL (Evans Electroselenium Ltd.) scanner according to the makers' instructions.

Fetuin, prepared from calf serum according to Deutsch (1954), and γ -globulin (Armour Laboratories, U.S.A.) were used to make antisera as described by Pierce & Johnson (1960). Immuno-electrophoresis was carried out as described by Smith (1960).

RESULTS

Figs. 1, 2 and 3 show the amounts per litre of total N, hexosamine and protein-bound hexose respectively, and the amounts of these constituents precipitated by trichloroacetic acid in the serum of pigs from 54 days' gestation to a postnatal age of 42 days. Values found in adult pigs are also shown.

Fig. 1. Effect of development on the concentration of total N (●), and of N precipitated by trichloroacetic acid (x), in pig serum.

The concentrations of total N, hexosamine and protein-bound hexose did not change between 54 and 90 days' gestation. Between 90 days' gestation and term the small, but significant ($P < 0.01$), rise in the concentration of total N, and the greater rises in the concentrations of hexosamine and protein-bound hexose, were entirely due to the presence of soluble glycoproteins. On the first day after birth, when the concentrations of serum proteins had increased, largely because of the absorption of γ -globulin from the colostrum (Foster *et al.* 1951; Widdowson & McCance, 1956), the percentage of the total N, hexosamine and hexose precipitated by trichloroacetic acid rose to 98%, 80% and 70% respectively. Between the fourth and seventh days the concentrations of hexosamine and protein-bound hexose fell by about 80 mg./100 ml. of serum.

Fig. 4 shows representative electrophoretograms of the serum proteins of piglets at various ages between 56 days' gestation and a postnatal age of 28 days. In the present study most interest centred around the protein migrating in the α_1 -globulin position since this is probably identical with 'component X' described by Rook *et al.* (1951). The

Fig. 2. Effect of development on the concentration of hexosamine (●), and of hexosamine precipitated by trichloroacetic acid (x), in pig serum.

Fig. 3. Effect of development of the concentration of hexose precipitated by ethanol (●), and of hexose precipitated by trichloroacetic acid (x), in pig serum.

Fig. 4. Electrophoretic pattern of the proteins in pig serum at various ages. Electrophoresis was carried out on cellulose acetate strips in barbiturate buffer (pH 8.6, 10.06) at 0.5 mA/2.5 cm. strip for 6 hr.

amount of this protein was considerable in the serum of foetuses up to 90 days' gestation, smaller at term and progressively smaller up to 7 days of age, after which age it could seldom be detected. A small amount of γ -globulin, identified immunoelectrophoretically, was found in the serum of the newborn piglet before suckling. Immunoelectrophoresis of newborn pig serum against bovine fetuin antiserum did not produce a reaction with the α_1 -globulin.

The serum proteins from foetuses at 61, 65 and 79 days' gestation and from three different newborn piglets were subjected to electrophoresis on duplicated strips. One strip was stained for protein and the other for carbohydrate. In each of the foetal sera bands staining intensely for carbohydrate were found in the positions of the α_1 - and α_2 -globulin bands. In two of the three newborn sera examined only one band staining for carbohydrate, in the α_2 -globulin position, was found. In the other newborn serum a faint band was also found in the position of the α_1 -globulin.

Trichloroacetic acid-soluble material. The hexose, hexosamine and the bulk of the N soluble in trichloroacetic acid was non-diffusible. The concentration of these constituents thus increased to a maximum value in the serum of the newborn piglet and

Table 1. *Composition of serum components soluble in trichloroacetic acid*

Values are expressed as mg. of carbohydrate/mg. of non-hexosamine N. The number of samples is shown in parenthesis after description of the animals.

	Hexose	Hexosamine	Total carbohydrate
Foetus 57 days (1)	1.48	1.39	2.88
61 days (1)	1.33	1.00	2.32
65 days (1)	1.28	1.56	2.84
79 days (1)	1.37	1.73	3.00
Mean (4)	1.37	1.42	2.76
Newborn (3)	1.29	1.18	2.47
	(1.07-1.44)	(1.05-1.26)	(2.12-2.68)
1 day (2)	1.03	1.06	2.09
	(1.03, 1.03)	(0.93, 1.19)	(1.96, 2.22)
2 days (2)	1.10	1.18	2.29
	(0.80, 1.40)	(1.15, 1.22)	(1.95, 2.62)

decreased to a much lower value 1 day after birth (Figs. 1, 2 and 3). Table 1 shows that in spite of these changes in concentration the composition of the material, as expressed by the ratio of hexose, hexosamine and total carbohydrate to non-hexosamine N, did not change consistently between 57 days' gestation and a postnatal age of 2 days. The mean values at all ages were 1.22, 1.23 and 2.47 respectively. If the non-hexosamine N is assumed to be present as a protein containing 16% of N, the ratios indicate that the trichloroacetic acid-soluble material consists of one or more mucosubstances with a mean carbohydrate content of 19.5% hexose, 19.7% hexosamine and 39.5% total carbohydrate.

Mannose and galactose in approximately equal proportions by visual inspection were found in serum samples taken from pigs at all ages. After similar treatment of the trichloroacetic acid-soluble material galactose only was detected.

Electrophoresis of whole serum protein and of the proteins precipitated by trichloroacetic acid showed that in the foetuses all the proteins were represented in the precipitate and in amounts similar to those in the whole serum. At term the protein migrating in the α_1 position was not precipitated. Occasionally, too, some of the albumin had not been precipitated. In the ultracentrifuge most of the protein in the serum of the newborn piglet behaved as a single component and there was only a trace of material of high molecular weight present. The material soluble in trichloroacetic acid sedimented at a speed similar to that of the bulk of the serum proteins.

DISCUSSION

The protein described as ' α_1 -globulin' in this paper is probably identical with Rook *et al.* (1951)

'component X'. Its proportion in the serum was at its highest in the youngest foetus and fell steadily until it had practically disappeared by the seventh day. Between 90 days and term there was a rise in the concentration of nitrogen and of carbohydrates soluble in trichloroacetic acid. The fact that the amount of protein-bound hexose and hexosamine fell at the same time as α_1 -globulin disappeared from the serum appears to be fortuitous for the prenatal changes in concentration are quite different. It therefore appears that during about the last 25 days of gestation one or more compounds containing hexosamine and galactose enter the circulation of the piglet in greatly increased amounts, and disappear from it during the first 7 days of postnatal life.

The material soluble in trichloroacetic acid appeared to have a similar composition with regard to its hexosamine, hexose and nitrogen content at all ages. No attempt was made, however, to purify it and electrophoresis showed that it contained serum proteins in addition to mucoproteins. The true nitrogen content of the mucoprotein is probably therefore lower, and the true carbohydrate content higher, than that recorded. The recorded values for the carbohydrates, and for hexosamine in particular, are higher than those of α_1 -glycoproteins (Stacey & Barker, 1962) and lower than those of the blood-group substances (Pusztai & Morgan, 1961). The material was similar to the blood-group substances, however, in that the hexose present appeared to be galactose. An isoagglutination-inhibition test for blood-group A activity (Morgan & King, 1943) was negative.

The source of the mucosubstances is not known, but there are at least two ways in which they may enter the circulation. They may do so direct from the tissues, and might perhaps be related to the substances responsible for the high concentrations of non-protein nitrogen, as determined after precipitation of the proteins with trichloroacetic acid, in the skeletal muscle of the 90 days-old pig foetus and newborn piglet (Dickerson & Widdowson, 1960). Another possibility is that they are secreted into the intestine and absorbed. It is known that the intestine of the newborn piglet is permeable to large molecules. This permeability is non-specific (Leece, Matrone & Morgan, 1961; Asplund, Grummer & Phillips, 1962; Payne & Marsh, 1962) and is not present in fed piglets after about 24 hr. of age (Asplund *et al.* 1962; Payne & Marsh, 1962) though it persists for up to 106 hr. in piglets given water only (Payne & Marsh, 1962). The fourfold rise in the concentration of hexosamine in meconium between 87 days' gestation and term (J. W. T. Dickerson & D. A. T. Southgate, unpublished work) would seem to be in agreement with this latter hypothesis. It could be objected however, that as

the permeability is 'shut off' by the absorption of protein, the absorption of mucosubstances would have a similar effect. There seems to be no evidence at present that this is the case.

The substances discussed above seem clearly to be distinguished from the α_1 -globulin that is found in high concentrations in foetal pig serum. This protein was positive to periodic acid-Schiff stain and may contain a foetal glycoprotein similar to fetuin (Pedersen, 1944). It was, however, immunoelectrophoretically different from bovine fetuin.

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