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The Mechanism of Fructose Formation in Goat Placenta with Special Reference to the Possible Involvement of Sorbitol or of Phosphoric Acid Esters

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The presence of fructose in the foetal blood of ungulates and its virtual absence from the maternal blood have been frequently observed (e.g. Goodwin, 1956). Huggett, Warren & Warren (1951) provided evidence, by means of both infusion and perfusion experiments, that the placenta was the site of fructose formation in the sheep. Walker (1960) used the infusion technique with the separation of one of twin foetuses to demonstrate that the placenta is a site of fructose formation from glucose in the goat also. Perfusion experiments are reported in the present paper which confirm this finding for the goat.

The mechanisms of fructose formation from glucose remains obscure. Two hypotheses to explain fructose formation are considered in this paper. One, put forward by Winterton (1949), suggested that fructose was formed in sheep placenta by the action of a phosphatase on a fructose phosphoric acid ester. The second hypothesis, that fructose might be formed from glucose via sorbitol, arose from the observation by Williams-Ashman & Banks (1954) that sorbitol dehydrogenase (ketose reductase) was present in the seminal vesicles of the rat and the suggestion that this could be part of a mechanism for the formation of fructose by this tissue. That fructose is indeed formed from glucose via sorbitol in sheep seminal vesicles has recently been convincingly demonstrated by Hers (1960*a, b*).

The results reported in this paper supply some evidence alongside which these hypotheses may be considered. A preliminary account of some of this work was given by Neil, Walker & Warren (1957).

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EXPERIMENTAL

Details concerning goats, surgical procedure, infusion of sugars (and, in this paper, sorbitol), collection of blood samples, chromatography of blood extracts and the determination of sugars have been given by Walker (1960).

Perfusion of the placenta. A Dale-Schuster pump unit was employed. In the initial experiments heparinized maternal-goat plasma was used as perfusion fluid, but more satisfactory results were obtained later with a dextran-saline solution having the following composition: dextran, 5.1% (w/v); NaCl, 0.76%; KHCO_3 , 0.24%; equilibrated with $\text{O}_2 + \text{CO}_2$ (95:5) at 37°. The umbilical-cord vessels were dissected and one vein and one artery cannulated. Perfusion into the artery was started, and the remaining pair of uncannulated vessels immediately ligatured. The foetus was then removed by cutting the cord. The perfusion fluid was sampled at intervals for sugar analyses.

Detection of sorbitol-dehydrogenase activity. Homogenates of placental tissue (10% in 0.25M-sucrose) or extracts of acetone-dried powders of placental tissue were assayed by the method of Blakley (1951) as described by Wolff (1955). The advisability of using whole homogenates where possible for detection of the enzyme was indicated by Blakley (1951). He found, using the Warburg technique, that extracts of rat kidney, for example, failed to oxidize sorbitol, whereas a whole homogenate of this tissue did so. In this work the Thunberg-tube technique was chosen as a rapid and simple method for detection of the enzyme with whole homogenates.

The tubes contained the following mixture: potassium phosphate buffer, 1.0 ml., 0.1M, pH 8.0; sorbitol, 0.2 ml., 0.1M; diphosphopyridine nucleotide (DPN), 0.1 ml., 5 mM; sodium malonate, 0.3 ml., 1.0M; nicotinamide, 0.3 ml., 1.0M; methylene blue, 0.1 or 0.3 ml., 1 mM; placental homogenate, 0.5 or 0.25 ml., 10% in water in most experiments; water to 3.0 ml. Control tubes contained water in place of sorbitol solution. The tubes were evacuated and incubated at 37°. Enzyme activity was expressed as the

ratio of the decolorization time of the control (C) to that of the assay solution (T).

Human placentas were obtained at parturition and analysed immediately. Pig placentas were obtained through the co-operation of T. Wall and Sons Ltd., were placed on ice immediately after removal from the animals, and assayed 2-3 hr. later. One sample of pig placenta was placed on ice immediately after being shed at parturition, and analysed 2-3 hr. later. Cow placenta was obtained similarly and analysed 24 hr. after being placed on ice. Horse placentas, obtained by the kind co-operation of Professor W. C. Miller, Equine Research Station, Newmarket, were analysed immediately after being shed at parturition. Samples of placentas from other species were obtained in the laboratory by laparotomy, and analysed immediately.

Analysis of phosphoric acid esters

Preparation of extracts of placenta. Cotyledons were removed from the placenta by tying off and cutting on the cotyledon side of the ligature, so as to avoid haemorrhage. The samples were rapidly freed from connective tissue, blotted, and then plunged into liquid N_2 . When frozen the material was crushed to a powder with a stainless-steel mortar and pestle. The powder was ground up with an equal weight of ice-cold $HClO_4$ (15%, w/v) and allowed to thaw. The mixture was centrifuged at 0° and the supernatant decanted. The residue was re-extracted twice with the same volume of ice-cold $HClO_4$ (7.5%, w/v) with centrifuging. All the supernatants were pooled and adjusted to pH 5.0 with carbonate-free NaOH. Fractions of the phosphorus-containing compounds in the placental extracts were prepared by slight modifications of the conventional barium acetate-ethanol precipitation procedures described by LePage (1951), with precautions to exclude CO_2 . The precipitates were removed by centrifuging, washed first with ice-cold 80% ethanol, followed by absolute ethanol, and finally with ether. The resulting powders were dried *in vacuo* over $CaCl_2$. Samples were then extracted with ice-cold HCl (0.1N) three times. The residue after extraction was practically phosphate free. The combined extracts were ultrafiltered at 0° through shells (Membranfiltergesellschaft, Göttingen) to remove mucoid material. The ultrafiltrate was neutralized and stored at -10° before column analysis.

Paper ionophoresis. This was carried out on extracts of placenta during preliminary experiments. The conditions used were: Whatman no. 4 paper, washed as recommended by Walker (1953); barbitone buffer, pH 8.6, I 0.05; 400 v, corresponding to approximately 0.4 mA/cm. width of paper; 3-4 hr. This method achieves a separation of phosphoric acid and its esters into groups (Neil & Walker, 1954).

Detection of phosphoric acid and its esters on paper. The methods available for detection of phosphoric acid and its esters on paper (e.g. Hanes & Isherwood, 1949; Wade & Morgan, 1953) have not proved entirely satisfactory in this Laboratory, especially when used for detection on buffered paper. The following method was therefore devised and has proved satisfactory for a large range of buffered papers.

After ionophoresis or chromatography the paper is dried and dipped in an acetone solution of $FeCl_3$ [0.025% $FeCl_3 \cdot 5H_2O$ in acetone containing 0.25% (v/v) of 10N-HCl]. The acetone is allowed to evaporate, and the paper then dipped in an acetone solution of $\alpha\alpha'$ -dipyridyl (0.1%) and dried at room temperature. The paper is now exposed to a

damp atmosphere containing SO_2 . Generation of SO_2 in a tank containing a beaker of hot water has been found to be satisfactory. The phosphoric acid and its esters appear as white spots on a rose-pink background after about 10-30 sec. exposure in the tank. The paper is immediately removed, and the spots remain unchanged for several days. The resulting paper is suitable for photography and further chemical analysis of the phosphorus-containing areas. The sensitivity of the method is affected by the buffer present in the paper, but on unbuffered paper the sensitivity limit is about 0.5 $\mu g.$ of P/cm.² with inorganic orthophosphate as test substance.

Analysis of extracts by gradient elution from an ion-exchange column. Placental extracts, prepared as above, were placed on a De-Acidite FF (borate form) column and developed by gradient elution according to the method described by Parr (1955). About 350 fractions (10 ml. each) were collected. Each fraction was assayed for total phosphorus by the method of Allen (1940). The phosphorus-containing peaks thus revealed were further analysed for acid-labile phosphoric acid derivatives ($n-H_2SO_4$, 100° , 10 min.); hexose esters were estimated as total hexose by the anthrone method (Trevelyan & Harrison, 1952); fructose esters as fructose by the method of Cole (as described by Bacon & Bell, 1948); pentose esters by the method of Brown (1946); purine and pyrimidine nucleotides by measurement of absorption at 260 $m\mu$.

RESULTS

Effects of injecting sorbitol into the maternal and foetal circulations

Sorbitol was injected into both maternal and foetal-goat circulations with a view to determining whether this hexitol can act in the same way as glucose in promoting fructose formation by the placenta. The sorbitol was injected in all cases as an aqueous 20% (w/v) solution previously warmed to 37° . All the figures quoted refer to the sugar concentrations in samples of whole blood.

Injection of sorbitol into the maternal circulation. Sorbitol (1.5 g./kg. maternal body wt.) was injected into the maternal circulation. Two experiments of this type were performed. Blood samples were taken before and at intervals over a period of several hours after the injection of sorbitol. The result of such an experiment is shown in Fig. 1. The maternal-blood fructose and glucose values started to rise immediately after sorbitol injection. The increases in concentration above the initial values were similar in both cases, but the peak in the fructose concentration was reached before that of glucose. Both peaks were reached within about 80 min. of sorbitol injection and both values returned slowly to normal after a further 2-2½ hr. That fructose and not sorbose was present in the maternal blood was confirmed by paper chromatography. The foetal-blood total reducing sugar rose slowly after the sorbitol injection. This rise was almost entirely due to a rise in the blood-fructose

value, and was greater than that which would have been expected had it been caused solely by the increase in maternal-blood glucose noted above. Since the maternal-blood fructose could not have entered the foetal blood (Walker, 1960), this result suggests that the sorbitol injected into the maternal circulation acted as a precursor of the fructose that appeared in the foetal blood. The result of this experiment when viewed in the light of the perfusion experiments described below suggests that the goat placenta is permeable to sorbitol and that the site of the fructose formation resulting in the increase in foetal-blood fructose observed in the present experiment was the foetus and not the placenta.

Injection of sorbitol into the foetal circulation. Sorbitol (approx. 1 g./kg. estimated foetal body wt.) was injected via the umbilical cord into the foetal circulation. Two experiments of this type were performed. Samples of maternal and foetal blood were analysed for total reducing sugar and fructose. The maternal-blood total reducing sugar rose slowly over a period of about 3 hr. after the sorbitol injection to a value about 50 % above the initial concentration (Fig. 2). The foetal-blood total reducing sugar rose steadily, after the injection, to a value about 100 % above the starting concentration. This rise was almost entirely due to an increase

Fig. 1. Pregnant goat no. 40. Foetal age 138 days; singleton. Sorbitol (1.5 g./kg. maternal body wt.) was injected intravenously into the maternal circulation between 36 and 44 min. ●, Maternal glucose; ■, maternal fructose; △, foetal total reducing sugar; □, foetal fructose. The arrow indicates mean time of injection.

in the fructose concentration. Since this fructose cannot pass into the maternal circulation, the rise in the maternal total reducing sugar suggests that sorbitol passed across the placenta from foetus to mother. No increase in foetal-blood glucose occurred, a result in contrast with that observed in maternal blood when sorbitol was injected into the maternal circulation. It was found by Walker (1960) that the injection of fructose into the foetal circulation also failed to evoke a rise in foetal-blood glucose.

Perfusion of the placenta

The volume of the perfusion fluid was about 120 ml. and the perfusion of the placenta was continued for 4 hr. Sorbitol (1 g./kg. estimated foetal body wt.) was injected into the perfusion circulation after an initial control period of 2 hr. Samples of perfusion fluid were taken at intervals for total reducing sugar and fructose determinations. The results are shown in Fig. 3.

Fig. 2. Pregnant goat no. 42. Foetal age 134 days; singleton. Sorbitol (1 g./kg. foetal body wt.) was injected into the umbilical vein between 28 and 32 min. ▲, Maternal total reducing sugar; △, foetal total reducing sugar; □, foetal fructose. The arrow indicates mean time of injection.

Fig. 3. Pregnant goat no. 48. Foetal age 135 days; singleton. Sorbitol (4 g. in 15 ml. of water) was injected into the perfusion fluid between 120 and 124 min. △, Perfusion-fluid total reducing sugar; □, perfusion-fluid fructose. The arrow indicates mean time of injection.

During the control period (i.e. up to 120 min.) the fructose concentration rose slowly to a value of about 75 mg./100 ml. of perfusion fluid. On injection of the strongly hyperosmotic sorbitol solution (15 ml.) at the 120th min., the volume of perfusion fluid increased slowly by a further 20 ml., owing to the passage of water from the maternal blood into the pump system. During this period of 1 hr. (i.e. up to the 180th min.) no increase in fructose concentration in the perfusion fluid was noted. Thereafter, over a period of about 1 hr., the fructose concentration slowly rose again at approximately the same rate as during the control period. There was no evidence during the period over which determinations were made of any marked increase in the rate of fructose formation due to the presence of sorbitol. The glucose concentration in the perfusion fluid remained fairly steady throughout the experiment. In all, three perfusions were carried out, with injection of sorbitol, and in no case was evidence obtained indicating that sorbitol influenced the rate of fructose formation by the placenta.

The rise in fructose concentration in the perfusion fluid during the control period supports the findings of Walker (1960) that, in the goat, as in the sheep, the placenta is a site of formation of foetal-blood fructose.

*Detection of sorbitol dehydrogenase
in placentas*

Sorbitol-dehydrogenase activity was detected by the methods described above and the results are shown in Table 1. Some confirmatory experiments on sheep placenta were carried out on acetone-dried

powders kindly supplied by Dr E. C. Long. The conditions for detecting the enzymic activity were varied considerably during the experiments on pig and horse placentas but every test was completely negative.

It is quite clear from Table 1 that the presence of the dehydrogenase cannot be correlated either with the fructose-forming potential of the placenta or with gross placental morphology.

*Phosphoric acid and its esters in goat-placental tissue
before and after injection of glucose into the maternal
circulation*

Preliminary analyses on perchloric acid or trichloroacetic acid extracts of placental tissue for inorganic orthophosphate and acid-labile and acid-stable phosphoric acid derivatives were carried out by the methods outlined above. The variability of the results (Table 2) suggested that such analyses would not be helpful in obtaining the information required. Further preliminary analyses were carried out by ionophoresis of solutions of fractions of the phosphorus-containing compounds which had been obtained from the extracts by barium acetate-ethanol precipitation. This procedure separated the phosphorus compounds present into four distinct groups. Quantitative analysis of these groups indicated that the amount of fructose 1:6-diphosphate in the placental extracts rose by a factor of two to three after the injection of glucose into the maternal circulation. Examination of an acid hydrolysate of the barium fraction by ionophoresis indicated the absence of phosphoric acid esters of sorbitol.

Table 1. *Placental type, principal foetal-blood sugar and placental tissue
sorbitol-dehydrogenase activity in various species*

The enzyme was detected as described in the Experimental section, and activities are expressed in terms of the ratio (C/T) of the time taken for decolorization of the control solution to that for decolorization of the assay solution. + + +, indicates C/T > 15; +, C/T 4-9.9; -, indicates no activity detected.

	Gross placental morphology (all chorioallantoic)	Sorbitol-dehydrogenase activity in placenta
Principal foetal-blood sugar: fructose		
Cow	Polycotyledonary, villous	+
Goat	Polycotyledonary, villous	+ + +
Sheep	Polycotyledonary, villous	+
Pig	Diffuse, villous	-
Horse	Diffuse, villous	-
Principal foetal-blood sugar: glucose		
Man	Discoid, villous	-
Dog	Annular or zonary, labyrinthine	-
Cat	Annular, labyrinthine	-
Guinea pig	Subspheroid, labyrinthine	+ + +
Rabbit	Discoid, labyrinthine	+ + +
Hamster	Discoid, labyrinthine	+ + +
Mouse	Discoid, labyrinthine	-
Multi-mammate rat	Discoid, labyrinthine	-
Jird (<i>Meriones libycus</i>)	Discoid, labyrinthine	-
Albino rat	Discoid, labyrinthine	-

Table 2. *Analyses of acid extracts of goat placentas for inorganic orthophosphate and acid-labile* and acid-stable phosphoric acid derivatives*

Values given (mean $\pm$ s.d.) are phosphorus (P) as a percentage of the total acid-soluble phosphorus extracted. Cotyledons were removed for analysis immediately before sugar infusion into the maternal circulation (zero time) and at subsequent intervals as indicated.

Sample time (min.)	No. of goats	Inorganic phosphate	Acid-labile* P derivatives	Acid-stable P derivatives
0	6	24.6 $\pm$ 4.6	10.2 $\pm$ 3.3	65.2 $\pm$ 4.6
30	6	26.4 $\pm$ 6.2	8.3 $\pm$ 2.1	65.3 $\pm$ 5.4
60	5	26.9 $\pm$ 7.9	8.7 $\pm$ 2.4	64.4 $\pm$ 5.7
120	3	29.7 $\pm$ 5.4	6.4 $\pm$ 1.6	63.9 $\pm$ 4.7

* Acid-labile P derivatives are represented by the increase in inorganic P after 10 min. at 100° in $\text{N-H}_2\text{SO}_4$.

During the development of methods for separating the phosphorus-containing compounds from the perchloric acid extracts from cotyledons, it was observed that the amount of these compounds precipitated by the barium acetate-ethanol procedure was much increased if the sodium hydroxide used to neutralize the acid extracts contained appreciable amounts of carbonate. The extra phosphorus-containing material which was precipitated in these conditions, but which appeared only in very small amounts in precipitates obtained from carbonate-free solutions, comprised 50–70 % of the acid-soluble phosphorus compounds in the acid extracts; further, it consisted of an acid-stable phosphoric acid mono-ester fraction which contained practically no esterified hexose, pentose, glycerol or glyceric acid. Qualitative tests indicated the presence of esterified choline in the fraction and a more detailed analysis of the compounds present is presented in the addendum to this paper.

Extracts were prepared, as described in the Experimental section, of placental tissue taken before, $\frac{1}{2}$ hr. after and 2 hr. after the injection of glucose into the maternal circulation. Quantitative analyses of the three ultrafiltrates prepared from these extracts were carried out with the gradient elution method, and eleven peaks containing phosphoric acid and its esters were observed in each of the three analyses. Each of the phosphate-containing fractions was present in each sample analysis and there were no additional peaks.

Most of the fractions were identified by using the special colour reactions indicated in the Experimental section and by paper chromatography. Over 98 % of the second elution peak was inorganic orthophosphate and 75–80 % of the ninth peak proved to be fructose 1:6-diphosphate. Quantitative comparison of the elution patterns obtained from the cotyledons taken at the three different times showed that the only significant changes in the distribution of phosphorus between the elution fractions occurred in the inorganic orthophosphate and the fructose 1:6-diphosphate peaks. Half an

hour after the injection of glucose into the maternal circulation the percentage of phosphorus present as fructose 1:6-diphosphate in the placental tissue extract had risen from 2.4 to 7.6, while that present as inorganic orthophosphate had fallen from 66.8 to 56.3. This pattern of altered P distribution was still apparent 2 hr. after glucose injection but the percentages observed (6.2 and 59.6 respectively) indicated a slow return towards the control values.

DISCUSSION

Walker (1960) demonstrated that the placenta of the goat was freely permeable in both directions to glucose, galactose, mannose and xylose, but not to fructose or sorbose or to the disaccharides sucrose, maltose and lactose. The effects of sorbitol injected into the maternal and foetal circulations of the goat on the foetal- and maternal-blood sugar concentrations respectively, as reported in this paper, suggest that the placenta is also permeable to sorbitol in both directions. The present experiments do not give any direct measure of the rate of sorbitol transport across the placental barrier, although it is probably much less rapid than that of glucose. The rise in foetal fructose caused by an injection of sorbitol into the maternal circulation is only a fraction of that caused by a similar injection of glucose. This could be explained by the much slower transport of sorbitol across the placenta than is the case with glucose. The rise in foetal fructose on injection of sorbitol into the foetal circulation is greater and more prolonged than that produced by glucose injection. Sorbitol in the foetal circulation is readily converted into fructose, presumably by the action of sorbitol dehydrogenase present in the foetal liver (Hers, 1960b). These observations, together with the absence of enhanced fructose formation by the placenta perfused with dextran-saline containing sorbitol, suggest that fructose formation from sorbitol does not occur in goat placenta.

Injection of sorbitol into the maternal circulation leads to the appearance of fructose in the maternal

blood and to an increase in the glucose concentration. This additional glucose is probably formed from fructose since the latter reaches its maximum concentration first. However, neither sorbitol nor fructose injected into the foetal circulation gives rise to an increase in foetal-blood glucose concentration [this paper and Walker, (1960)]. This constitutes an interesting difference between the metabolism of these compounds in adult and foetal tissues.

Perfusion of the goat placenta [and also of sheep placenta; (Long, 1957)] did not enhance the rate of increase of fructose concentration in the perfusion fluid. These findings are perhaps surprising in view of the sorbitol-dehydrogenase activities demonstrated in these tissues *in vitro* (Table 1) but may conceivably be attributable to failure of the sorbitol, in the perfusion fluid, to penetrate to the site of the placental enzyme. Wick & Drury (1951) reported such a permeability difference between glucose and sorbitol in rabbit muscle. Hers (1960*b*), using an assay system in which DPN was not added, found that sorbitol was rapidly converted into fructose by foetal-sheep liver but only slowly, if at all, by sheep placenta. The latter observation is in agreement with the results of the perfusion experiments described above. Hers (1960*b*) also demonstrated the presence of an active aldose reductase in both sheep and cow placentas, and suggested that reduction of glucose to sorbitol occurs in the placenta and that the sorbitol is converted into fructose in the foetal liver. Nevertheless, the perfusion experiments described in the present paper demonstrate that fructose is formed by the goat placenta [as it is in the sheep (Huggett *et al.* (1951))] in the absence of the foetus. This suggests that foetal-blood fructose in these species may arise by more than one mechanism.

Although sorbitol dehydrogenase was shown to be present in placental tissue from goat, cow and sheep, attempts to demonstrate its presence in the placental tissues from pig and horse (which also have fructose as the main foetal-blood sugar) failed. The presence of sorbitol dehydrogenase in the placentas from non-ungulates and the absence of appreciable amounts of fructose in the foetal blood of such species strengthens the conclusion that the occurrence of this enzyme in mammalian placentas is not associated with the formation of foetal-blood fructose.

If phosphoric acid esters, rather than sorbitol, are involved in the placental conversion of glucose into fructose it seemed reasonable to suppose that the administration of large amounts of glucose might evoke changes in the concentrations of phosphoric acid esters indicative of the mechanism of conversion. The experiments reported here show that, in goat placenta, only fructose 1:6-diphosphate

accumulates to any marked extent when glucose is injected into the maternal circulation. The accumulation of fructose 1:6-diphosphate in particular may merely reflect enhanced glucose metabolism under the experimental conditions. Dempsey & Wislocki (1947), using histochemical techniques, showed that fructose 1:6-diphosphatase activity was present in placental tissue from the guinea pig, cat, sow and human. However, their results did not suggest that notably high activity, or marked specificity, of the enzyme responsible could be associated particularly with ungulate placentas.

Therefore, although the accumulation of fructose 1:6-diphosphate in the experiments described here may well be a reflexion of the mechanism for the formation of foetal-blood fructose by goat placenta, more specific evidence for this view is at present lacking.

SUMMARY

1. Experiments designed to examine current hypotheses concerning the mechanism of fructose formation from glucose in ungulate placentas have been carried out.

2. The goat placenta is permeable to sorbitol. Injection of sorbitol into the maternal circulation increases the concentration of both fructose and glucose in the maternal blood, but injection into the foetal circulation increases only the foetal-blood fructose.

3. In the absence of the foetus, perfusion *in situ* of the foetal side of the goat placenta with sorbitol does not increase the rate of fructose formation.

4. The presence or absence of sorbitol dehydrogenase (ketose reductase) in the placentas of 15 species of animals cannot be correlated with the nature of the principal foetal-blood sugar.

5. Injection of glucose into the maternal blood leads to a marked increase in fructose 1:6-diphosphate in the placenta and to a decrease in placental inorganic orthophosphate.

6. Current hypotheses of fructose formation from glucose in ungulate placenta are reviewed in the light of these findings.

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ADDENDUM

The Occurrence of Phosphorylcholine and Phosphorylethanolamine in Goat Placenta

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In the main part of this paper it was mentioned that of the phosphorus-containing compounds obtained from goat cotyledons by acid extraction followed by precipitation as barium salts in the presence of ethanol (fraction A; preparation described below) between 50 and 70% consisted of an acid-stable phosphoric acid monoester fraction that contained practically no esterified hexose, pentose, glycerol or glyceric acid. Further characterization of this fraction is presented as an addendum for two reasons: (1) The analyses reported here were carried out on a single specimen of material obtained by pooling fractions A obtained from cotyledons from two goats. (2) The specimen analysed represents material isolated in the first instance for hexose phosphate analyses, and the method of isolation is not necessarily optimum for quantitative estimation of phosphorylcholine and phosphorylethanolamine in goat-placental tissue.

Preliminary electrophoresis of fraction A revealed that a group of the phosphoric acid derivatives present, representing 59.1% of the total phosphorus in the material, could be separated from the hexose phosphoric acid derivatives and inorganic phosphate. This group was slow-moving relative to inorganic orthophosphate and bore a net negative charge at pH 8.6 in barbitone buffer; it contained nitrogen, and preliminary qualitative analysis indicated the presence of esterified choline. Material having similar properties was prepared by fractionation of fraction A on De-Acidite FF ion-exchange resin (see below); the material so treated is referred to as fraction B.

As considerable amounts of phosphorylcholine and phosphorylethanolamine occur in rabbit uterus, and perhaps also in human, cow and pig uteri (Cawkwell, 1958), it seemed of interest to investigate further the goat-placental fraction B. The results presented here indicate that phosphorylcholine and phosphorylethanolamine together account for about four-fifths of the esterified phosphorus in the fraction. A preliminary account of this work was given by Neil (1960).

MATERIALS AND METHODS

Preparation of fraction A. The HClO_4 extract of cotyledons was adjusted to pH 4 with bench 10N-NaOH (which was about 0.1N with respect to Na_2CO_3), 4 vol. of ethanol (95%, v/v, pH 4) were added and the solution was kept at 2° for 18 hr. The precipitate was removed by centrifuging and NaOH added to the supernatant solution until it was just pink to phenolphthalein. An excess of barium acetate (25%, w/v) was added and the ethanol content adjusted to about 80% (v/v), after which the solution was kept at 2° for 18 hr. in a vessel open to the atmosphere. The material that was precipitated during this period was removed by centrifuging at 2° and washed twice with ice-cold ethanol [80% (v/v) adjusted with NaOH to be just pink to phenolphthalein], twice with absolute ethanol, twice with dry ether and placed in a vacuum desiccator over CaCl_2 .

Preparation of fraction B. The dry fraction A prepared as above was suspended in water and dissolved by addition of a minimal quantity of HCl; it was then freed from barium by passage through a column of Zeo-Karb 225 (H^+ form). The effluent was passed through a De-Acidite FF (Cl^- form) column and washed through with water. The eluate comprised fraction B, together with Cl^- , and contained 71.4%

of the total phosphorus present in fraction A. The eluate was freed from Cl^- by successive passage through two columns of Zeo-Karb 225, the first being in the Ag^+ form and the second in the Na^+ form. The Cl^- -free eluate was concentrated by distillation under reduced pressure at a temperature not exceeding 40° . The final concentrate of fraction B contained $27.6 \mu\text{g. atoms of P/ml}$.

Hydrolysis of fraction B. Phosphomonoesterase hydrolyses of fraction B were carried out at 37° in carbonate-bicarbonate buffer, pH 10, until there was no further increase in the inorganic phosphate content of the solution even upon further addition of enzyme. Controls were carried out with boiled enzyme.

Chromatography. Fraction B was fractionated on chromatograms run for 24 hr. on Whatman no. 1 paper, the mobile phase being 80% (w/v) phenol in water, in an atmosphere of NH_3 . The phosphoric acid esters were detected by the $\alpha\alpha'$ -dipyridyl method described in the main part of this paper. Parallel chromatograms were dipped in a solution of 0.2% ninhydrin in 95% (v/v) acetone. Chromatography of dinitrophenyl derivatives was carried out with the butanol-water system of Mellon, Korn & Hoover (1953).

Quantitative analysis. Phosphorus as inorganic orthophosphate was estimated either by the method of Allen (1940) or by that of Fiske & Subbarow (1925). Choline was assayed by the periodide method of Appleton, LaDu, Levy, Steele & Brodie (1953). Ethanolamine was estimated by the fluorodinitrobenzene method of Axelrod, Reichenthal & Brodie (1953).

Differential hydrolyses. Estimations of glycerylphosphorylethanolamine and phosphorylethanolamine and of glycerylphosphorylcholine and phosphorylcholine in admixtures were carried out by differential hydrolyses as described by Cawkwell (1958), followed by assays of the free bases liberated.

Materials. Phosphorylcholine was prepared by the method of Riley as described by Schmidt (1957). Dinitrophenylethanolamine was a gift from Dr J. S. Fawcett. Dinitrophenylserine was prepared by an adaptation of the method of estimation of serine (Axelrod *et al.* 1953). Phosphomonoesterase was obtained from the Sigma Chemical Co., St Louis 18, Mo., U.S.A., and was virtually free from diesterase activity. A second sample of phosphomonoesterase having negligible diesterase activity was kindly supplied by Dr P. Hele.

RESULTS

Paper chromatography of fraction B in phenol-ammonia revealed four principal phosphorus-containing areas which had R_F values of 0 (6%), 0.16 (10%), 0.35 (8%) and 0.86 (68%). Two of these areas (R_F 0.16 and 0.35) also gave positive reactions with ninhydrin. The phosphorus contents of these areas (indicated in parentheses above as a percentage of the total phosphorus in the extract applied to the paper) and also of the intervening areas were determined by a conventional ashing technique. The phosphorus recovered in these analyses ranged from 92 to 97% of that applied to the start-line. The R_F values of 0.35 and 0.86 corresponded to those of phosphorylethanolamine and phosphorylcholine respectively. Glycerylphosphorylethanolamine has an R_F of 0.63 in this system and was

absent from the extract. Glycerylphosphorylcholine is not clearly separated from phosphorylcholine in this system but its absence from the extract was indicated by the differential hydrolysis experiments described below. The phosphorus-containing material that remained on the start-line represents relatively non-specific adsorption since unwashed paper was used (Walker, 1953). The nature of the material of R_F 0.16 was not determined.

Choline analyses. The absence of glycerylphosphorylcholine and presence of phosphorylcholine in fraction B is indicated by the results of differential-hydrolysis experiments. Hydrolysis by N -hydrochloric acid for 30 min. at 100° (which liberates free choline from glycerylphosphorylcholine but not from phosphorylcholine) liberated no free choline from fraction B. Hydrolysis by $2N$ -hydrochloric acid for 24 hr. at 123° (which liberates choline from both the mono- and the diesters) liberated choline equivalent to 69.9% of the total phosphorus present in fraction B. The agreement between the results obtained by chromatography and by hydrolysis is reasonably satisfactory. Qualitative evidence confirming the presence of free choline after long hydrolysis of fraction B was provided by preparation of choline reineckate from the hydrolysate and by pyrolytic cleavage (Feigl, 1956). Further quantitative evidence, obtained by electrophoresis, confirming the analytical data is given below.

Ethanolamine analyses. The presence of phosphorylethanolamine in fraction B was suggested by preliminary electrophoresis experiments and by chromatography as described above. Estimation of free ethanolamine and serine by the fluorodinitrobenzene method (Axelrod *et al.* 1953), after acid hydrolysis of fraction B, did not prove satisfactory because the solvent extraction procedure is not very specific and because fraction B as prepared above is contaminated by small amounts of impurities that, after acid hydrolysis, yield dinitrophenyl derivatives that are extracted into both the chloroform and the isobutyl methyl ketone solvents. Hydrolysis by pure phosphomonoesterase in carbonate-bicarbonate buffer avoided these artifacts for ethanolamine determination. The results of ethanolamine estimations before and after hydrolysis by phosphomonoesterase revealed that this process liberated ethanolamine equivalent to 10.4% of the total phosphorus present in fraction B. The agreement between this result and that obtained by chromatography is not entirely satisfactory. The extent of recovery and the non-specific adsorption of material on the start-line mentioned above would be reflected by a much larger percentage error in the analyses of the relatively small amount of phosphorylethanolamine than in those for the large amount of phosphorylcholine. The figures obtained from

the chromatograms, therefore, are likely to be minimal.

Evidence that ethanolamine was liberated by the action of phosphomonoesterase was obtained by paper chromatography of the dinitrophenyl derivatives prepared from the hydrolysate. Only one derivative appeared in the hydrolysate but not in the control and it behaved just like pure dinitrophenylethanolamine in the butanol-water system of Mellon *et al.* (1953). Dinitrophenylserine was absent from both preparations.

Electrophoresis. Electrophoresis (barbitone buffer, pH 8.6, I 0.05, 0.4 mA/cm. width, Whatman no. 4 paper) of fraction A prepared as above from goat placenta revealed the presence of a phosphoric acid ester fraction that moved towards the anode at a rate of about 0.46 relative to inorganic orthophosphate (1.0). Most of the phosphorus-containing material in fraction B moved at the same rate as did a mixture of phosphorylethanolamine and phosphorylcholine under the same conditions. Determination of the total phosphorus on the paper after electrophoresis showed that 59.1% of the total phosphorus present in fraction A from goat placenta was present in the region of relative rate 0.46. Since fraction B accounts for 71.4% of the total phosphorus in fraction A, and phosphorylcholine and phosphorylethanolamine account for 80.3% of the total phosphorus in fraction B, the two esters account for 57.4% of the total phosphorus in fraction A. This figure is compatible with that obtained by quantitative electrophoresis described above.

DISCUSSION

It is reasonable to conclude from these results that phosphorylcholine and phosphorylethanolamine are quantitatively important constituents of

the phosphoric acid derivatives extractable from goat placenta by perchloric acid. From the nature of the extract analysed, as outlined in the introduction to this addendum, it is clear that the absence of the glyceryl derivatives of phosphorylcholine and phosphorylethanolamine does not exclude their presence in goat-placental tissue. All four of these derivatives have been shown to occur in human placenta (Porcellati, Curti & Luciani, 1959*a, b*).

SUMMARY

1. Phosphorylcholine and phosphorylethanolamine are shown to be quantitatively important constituents of the phosphoric acid derivatives present in goat placenta.

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The Electrophoresis of Histones and Histone Fractions on Starch Gel

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Histones can be divided into a number of fractions by the procedures of extraction, electrophoresis, sedimentation or chromatography (for review, see Butler, 1959). Chromatography on carboxymethylcellulose, as developed by Johns, Phillips, Simson & Butler (1960), gave three well-characterized fractions, a lysine-rich fraction (F1),

a moderately lysine-rich fraction (F2) and an arginine-rich fraction (F3). Two of these, F2 and F3, are reasonably homogeneous with respect to their *N*-terminal groups.

These fractions have now been studied by the method of electrophoresis in starch gel introduced by Smithies (1955). Neelin & Connell (1959) re-