J. Physiol. (1978), 276, *pp.* 395–402
With 3 text-figures
Printed in Great Britain

RENIN SUBSTRATE IN PLASMA OF UNANAESTHETIZED PREGNANT EWES AND THEIR FOETAL LAMBS

By J. G. CARVER and J. C. MOTT

From the Nuffield Institute for Medical Research, University of Oxford, Headley Way, Headington, Oxford OX3 9DS

(*Received 10 October 1977*)

SUMMARY

1. In unanaesthetized preparations 6–14 days after catheterization, the concentration of renin substrate (AI 212 ± 20 ng/ml. S.E. of mean) in the plasma of foetal lambs during the last third of gestation was significantly less than that of paired samples of maternal plasma (AI 350 ± 27 ng/ml.).

2. During the first 5 days following catheterization the concentration of renin substrate in maternal plasma was lower than subsequently but was raised if the uterus contained a nephrectomized foetus.

3. The concentration of renin substrate in the plasma of intact foetal lambs without nephrectomized twins varied little in the post-catheterization period and later; the concentration of substrate in nephrectomized foetal lambs averaged threefold that of intact lambs and a similar concentration of substrate was found in intact twins of such lambs in the immediate post-catheterization period.

4. It is suggested that the ratio of foetal/maternal plasma renin activity understates their relative enzyme activity since foetal substrate concentration is so considerably less than maternal.

INTRODUCTION

In immature foetal lambs of < 0·71 term, low plasma renin activity has been reported (Carver & Mott, 1975) compared with that found in older foetal lambs (Broughton Pipkin, Lumbers & Mott, 1974) where the rates of formation of angiotensin I were several-fold those in the ewe. Skinner, Dunn, Mazzetti, Campbell & Fidge (1975) have described the rate curve for angiotensin I formation by renin in sheep plasma *in vitro* and pointed out that at the concentrations of renin substrate found in this species *in vivo*, renin activity may be expected to depend on the concentration of endogenous substrate as well as on the amount of enzyme available. It was therefore of interest to investigate the concentration of renin substrate in foetal plasma compared with maternal.

This paper examines renin substrate concentration in the plasma of foetal lambs from 97 to 141 days gestation, its relation to that in the mother, and the associated plasma renin activities.

The effects of foetal nephrectomy on substrate concentration (which were not confined to the nephrectomized foetus) are also described.

METHODS

Arterial, venous and amniotic catheters were implanted at a sterile operation in thirty-five pregnant ewes and their foetal lambs under general anaesthesia as described by Boddy, Dawes, Fischer, Pinter & Robinson (1974). Both kidneys were removed from one foetal lamb at operation in twelve ewes. All the measurements of this paper were made on blood samples taken with the preparation in a resting condition, not less than 24 hr after any previous sampling.

Collection of blood. 4 ml. blood for plasma renin activity and renin substrate estimations were collected into cooled polypropylene tubes containing 0·1 ml. ethylene diamine tetra-acetate (EDTA) 0·3 M/ml. blood. Plasma was separated by centrifugation for 20 min at 4 °C and 2,500 rev/min and stored at −20 °C until assay.

Measurement of plasma renin activity and renin substrate concentration

Plasma renin activity. Angiotensin I was measured at appropriate time intervals during the incubation at 37 °C of 0·2 ml. aliquots of plasma containing inhibitors of angiotensinase activity as detailed below. The rate of generation of angiotensin I (plasma renin activity or endogenous velocity) by endogenous renin from endogenous substrate was calculated over the first 2 or 3 h and is expressed as ng/ml. hr ± S.E. of mean.

Renin substrate. Renin substrate was measured as the angiotensin I generated in plasma by exhaustive incubation with an excess of added sheep renin. A preliminary investigation showed that maximal liberation of angiotensin I from 0·2 ml. plasma (in the presence of inhibitors as detailed below) was achieved in 30 min following addition of 0·22 Goldblatt units of sheep renin in 0·05 ml. sodium chloride 0·9% (w/v).

Enzymic destruction of angiotensin I during incubations was prevented by the addition of either EDTA 0·3 M, 0·1 g/g plasma and 2,3-dimercaptopropanol (50 mg/ml., Boots Limited) 0·01 g/g plasma, or phenylmethyl sulphonyl fluoride in ethyl alcohol 5%, 5 μl./ml. plasma and neomycin sulphate 10%, 12·5 μl./ml. plasma (Sealey, Moon, Laragh & Atlas, 1977). Incubations were terminated at the appropriate times by addition of 0·2 ml. of cold acetone (Analar). The mixture was centrifuged for 15 min at 4 °C and 2,500 rev/min.

After dilution of the supernatants with bovine serum albumin (BSA) 2% in 0·0989 M-sodium phosphate buffer pH 7·5 (containing 0·001 M-EDTA, 0·0752 M-sodium chloride, 0·003 M-sodium azide, 0·0034 M-8-hydroxyquinoline sulphate, 0·004 M-2,3-dimercaptopropanol) the angiotensin I concentration was determined by radioimmunosssay.

Radioimmunoassay. The quantity of angiotensin I in aliquots of the diluted incubates was measured in triplicate. A standard curve was constructed in nephrectomized sheep plasma treated in a manner identical to the samples. [^{125}I]angiotensin I and angiotensin I antiserum were added and the mixture was incubated at 4 °C for 18–24 hr. Free [^{125}I]angiotensin I was separated from the bound fraction by adsorption on to 1·25 mg charcoal (Norit A, Sigma) coated with dextran T40 (Pharmacia Ltd) in 0·2 ml. sodium phosphate buffer 0·099 M, pH 7·5 containing sodium chloride 0·075 M (Herbert, Lau, Gottlieb & Bleicher, 1965).

[^{125}I]angiotensin I was obtained from commercial sources (Lepetit Ltd or New England Nuclear) or prepared by iodination of angiotensin I (Schwartz-Mann, Orangeburg, N.Y.) by the method of Hunter & Greenwood (1962). Purification was carried out on a Whatman CM32 cellulose column, or a CM-Sephadex C-25 column, from which free ^{125}I was eluted at pH 5·0 with sodium acetate 0·05 M. Labelled angiotensin was then eluted with sodium acetate 0·1 M or 0·2 M at pH 6·5. The active fractions of eluate were diluted with sodium phosphate buffer pH 7·5 containing enzyme inhibitors and BSA 2%; this labelled angiotensin was stored at −20 °C in convenient aliquots until use. The antiserum to angiotensin I was from the same batch as that used by Broughton Pipkin *et al.* (1974). *Renin units* were calculated from substrate concentration [S] and plasma renin activity [X] as described by Ryan, McKenzie & Lee (1968) from the relationship for a first-order reaction.

$$k[E]\,t = \log_e \frac{[S]}{[S-X]}.$$

This unit of renin is that activity which cleaves 1% of substrate/hr. Mean values are given ± S.E. of mean except where otherwise stated.

RESULTS

Renin substrate in plasma

Ewes

The average concentration of renin substrate in five non-pregnant ewes was 99 ± 6 ng/ml.

Pregnant ewes. 6–14 days after catheterization, the average concentration of renin substrate in plasma from pregnant ewes was 392 ± 26 ng/ml.; on preceding days lower concentrations (mean 178 ± 30 ng/ml.) were found (Fig. 1*A*, ○, ——). The latter progressively approached the range characteristic of established preparations ($r = 0{\cdot}66$, $P < 0{\cdot}01$).

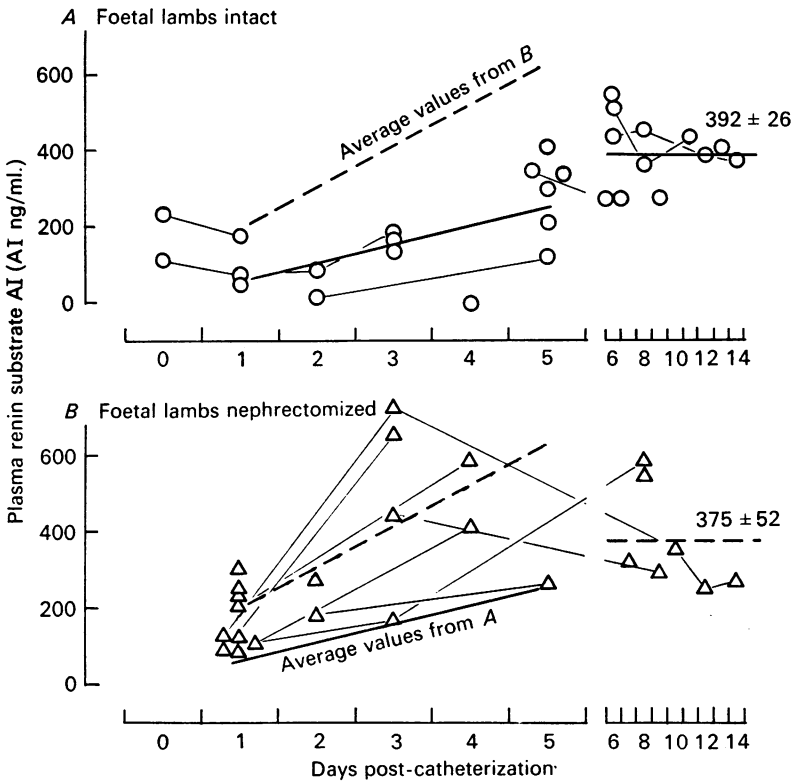

Fig. 1. Renin substrate (AI ng/ml., mean ± S.E. of mean) in plasma of pregnant ewes plotted against post-catheterization interval (days); values from individual animals joined by thin lines. *A*, ewes carrying only intact foetal lambs (○, ——). *B*, ewes carrying a nephrectomized foetus (△, – – –). Substrate concentration was significantly higher in *B* on days 1–5, but not on days 6–14. The regression lines for both groups from day 1 to day 4 are shown in both *A* and *B*.

Pregnant ewes carrying a nephrectomized lamb. The average concentration of renin substrate in the plasma of ewes carrying a nephrectomized lamb (Fig. 1*B*, △, – – –) was 293 ± 47 ng/ml. during the 5 days following catheterization and increased over this period at a rate ($r = 0{\cdot}58$, $P < 0{\cdot}02$) not different from ewes carrying only

normal foetal lambs; the difference (202 ± 52 S.D. ng/ml.) of the two mean concentrations at the midpoint of the regression lines (Fig. 1) was highly significant ($P < 0{\cdot}001$). However, the final level of substrate (375 ± 52 ng/ml.) in ewes with nephrectomized lambs 6–14 days post-catheterization was not different from that in normal pregnant ewes.

Foetal lambs

Unlike the ewes, systematic change of concentration of renin substrate following catheterization was not evident in any of the three categories of foetal lambs investigated.

Intact foetal lambs. The average concentration of renin substrate (223 ± 25 ng/ml.) found in intact foetal lambs during the first 5 days following catheterization did not differ from that on days 6–14 (171 ± 31 ng/ml., Fig. 2*A*, ●, ——.). These lambs were either singles or had intact twins.

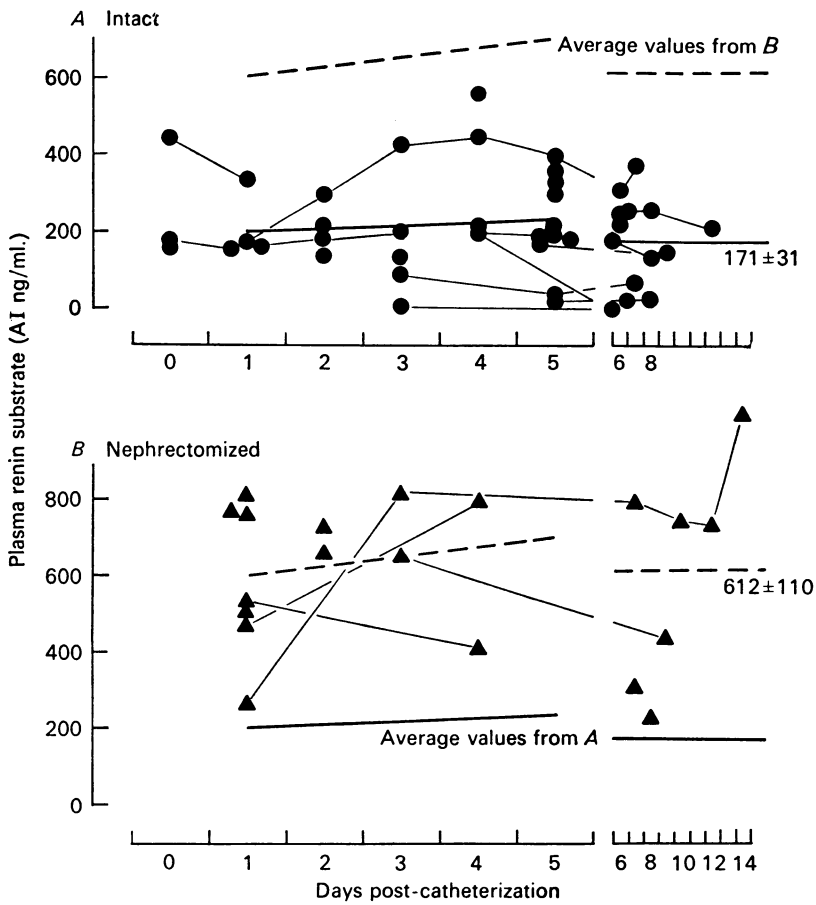

Fig. 2. Renin substrate (AI ng/ml. mean ± S.E. of mean) in plasma of foetal lambs plotted against post-catheterization interval (days); values from individual animals joined by thin lines. *A*, intact lambs without nephrectomized twins (see text ●, ——). *B*, nephrectomized lambs (▲, – – –). The average values for both groups are shown in both *A* and *B*.

Nephrectomized foetal lambs. Renin substrate concentrations were generally very much higher in nephrectomized (Fig. 2*B*, ▲, ---) than in otherwise similar intact lambs, even on the first day following catheterization. The average values were 630 ± 49 ng/ml. during the first 5 days and 612 ± 110 ng/ml. subsequently. The mean differences from intact lambs in both time periods ($P < 0.001$, < 0.05) were significant.

TABLE 1. Renin substrate concentration (ng AI/ml. plasma) in intact and nephrectomized lambs of twin pregnancies

Gestation age (days)	Time from Catheterization (days)	Renin substrate in	
		Intact twin	Nephrectomized twin
116	1	701	761
117	1	353	814
118	8	304	229
119	1	560	501
120	2	701	729
125	1	766	771
128	2	572	664

Intact lambs with nephrectomized twins. Substrate concentrations in the same range as those in nephrectomized foetal lambs were encountered in several intact lambs. Scrutiny of the data revealed that these lambs had nephrectomized twins with similar substrate concentrations (Table 1). Two additional lambs with recently dead nephrectomized twins also had substrate concentrations of 850 and 681 ng/ml. respectively. This contrasts with the average substrate concentration in six intact lambs in the absence of a nephrectomized twin on days 0–2 which was 221 ± 29 ng/ml. ($n = 11$).

Maternal and foetal plasma renin substrate in established preparations

The average concentration of renin substrate not less than 5 days post-catheterization in nineteen pregnant ewes was 352 ± 24 ng/ml. and in twenty-six foetal lambs 190 ± 23 ng/ml.; there was no correlation with gestation age in either group. Maternal concentration exceeded foetal in fourteen out of sixteen pairs of maternal and foetal samples for which the average values were, respectively, 350 ± 27 and 212 ± 20 ng/ml. (paired t test, $t = 4.77$, $P < 0.001$).

DISCUSSION

Renin substrate in pregnant ewes

The raised level of renin substrate (mean 392 ± 26 ng/ml. Fig. 1*A*) found in established preparations of pregnant sheep compared with non-pregnant sheep (mean 99 ± 6 ng/ml.) parallels the situation in pregnant women (Skinner, Lumbers & Symonds 1972). Skinner *et al.* (1975) have reported a value (using homologous renin) of 410 ± 30 ng/ml. for 'normal sheep' of unspecified reproductive state.

Substrate concentrations in ewes and lambs

The absence of correlation between maternal and foetal levels of renin substrate in the respective plasmas confirms the assumption that a molecule in this size range (mol. wt. 52,000, Skinner *et al.* 1975) must like renin, be assembled within the conceptus. In established preparations (6–14 days post-catheterization) in sixteen pairs of samples foetal renin substrate concentration averaged only 61 % of maternal (cf. Table 2).

The average concentration of renin substrate in the intact foetus varied little throughout the period of observation and reversals of the maternal/foetal substrate gradient were accounted for by low maternal levels in the days following catheterization. Maternal plasma renin activity is higher at this time than later (Broughton Pipkin *et al.* 1974) but the low substrate levels now reported may merely reflect a post-anaesthetization metabolic disturbance not similarly evident in the foetus.

Foetal nephrectomy. Bilateral nephrectomy is well known to allow renin substrate concentration in plasma to rise (Peach, 1977). The measurements in nephrectomized foetal lambs (Fig. 2*B*) are threefold those in intact lambs ($P < 0{\cdot}05$) and the difference evidently develops within 24 hr.

High concentrations of renin substrate were also found in ewes carrying nephrectomized lambs (Fig. 1*B*) and in twins of nephrectomized lambs in the early post catheterization period (Table 1). Possibly this reflects a temporary excess of precursors of renin substrate in pregnancies which include a nephrectomized foetus. While functional vascular anastomosis between twin lambs at this gestation age can occur (Valdes-Cruz, Taylor, Mott & Carver, 1977) its incidence appears to be low and is certainly precluded in five of six pairs of twins with similar substrate concentrations in Table 1 by differences of haematocrit greater than 3 %.

An observation (Carver & Mott, 1975) that in immature foetal lambs plasma renin activity was often low raised the question as to whether this was accounted for by low concentrations of renin substrate in the plasma of immature foetal lambs. Recent examination of the renin–renin substrate reaction in sheep (Skinner *et al.* 1975) has re-emphasized the important influence of substrate concentration in the endogenous range on plasma renin activity in this first-order reaction. As the data of this paper show, no systematic variation of foetal renin substrate concentration with gestational age was found between 97 and 141 days gestation. Thus low plasma renin activity in immature foetal lambs cannot be accounted for by a deficiency of renin substrate.

The fact that renin substrate concentration is substantially lower in foetal than maternal plasma means that the ratios reported for the foetal/maternal plasma renin activity ratio understate the foetal/maternal enzyme ratio. Fig 3 shows renin units (see Methods) calculated for plasmas from pregnancies of various gestation ages. The mean values for renin activity and substrate in those plasmas from pregnancies of 114 days gestation onwards are summarized in Table 2 together with renin units calculated from these means.

Fleischman, Oakes, Epstein, Catt & Chez (1975) measured plasma renin activity fifteen pregnancies (128–140 days gestation age) in which foetal activity was found to average 3·3 times maternal. The ratios reported in two smaller series were 2·9 (114–122 days, Smith, Lupu, Barajas, Bauer & Bashore, 1974) and 5·2 (114–144 days,

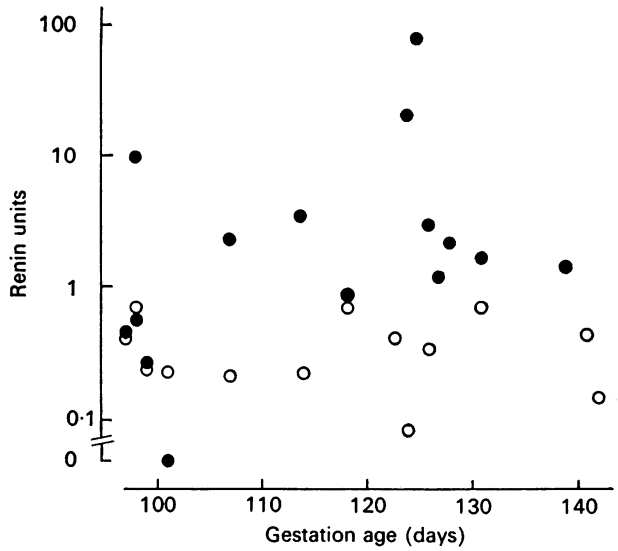

Fig. 3. Renin units calculated from renin activity and renin substrate concentration (see Methods) in foetal (●) and maternal (○) plasma of sheep pregnancies plotted against gestation age.

Broughton Pipkin, Lumbers & Mott, 1974). These observations are compatible with the ratio of 4·7 in Table 2. Most of the earlier measurements of plasma renin activity from this laboratory were made in the presence of additional sheep substrate (Broughton Pipkin *et al.* (1974) and yielded a foetal/maternal ratio of 7·1. Thus that procedure (which would have minimized any relative deficiency of renin substrate in the foetal plasma) yielded a foetal/maternal enzyme ratio close to that calculated from renin units derived in Table 2 from the average figures of this paper for renin activity and renin substrate in plasma.

TABLE 2. Renin activity (AI ng/ml. hr) and renin substrate (AI ng/ml.) in plasma of ewes and foetal lambs (114–142 days gestation). Mean values ± S.E. of mean. Renin units calculated from mean values for activity and substrate (see text)

Plasma	*n*	Renin activity	Renin substrate	Renin units
Non-pregnant ewes	5	1·3 ± 0·3	99 ± 6	1·32
Pregnant ewes	8	1·2 ± 0·2	337 ± 30	0·36
Foetal lambs	9	5·6 ± 1·0	225 ± 48	2·52
Foetal/Maternal ratio		4·7	0·67	7

Calculation of renin units from initial velocity and substrate concentration (Ryan *et al.* 1968) in effect standardizes the contribution of endogenous substrate to the renin activity of a plasma; thus the resultant figures permit comparison of the amount of *enzyme* in different plasmas. The intrinsic interest of the relative amounts foetal and maternal renin thus inferred from the measurements of this paper does not detract from the pragmatic merit of measurement of plasma renin activity in physiological or clinical circumstances. The production of angiotensin I by endogen-

ous renin from endogenous substrate provides the feedstock for the enzyme cascade which produces the more active angiotensins II and III (Peach, 1977). Nevertheless, the difference between maternal and foetal situations strikingly illustrates the conclusion of Skinner *et al.* (1975) that 'substrate concentration *in vivo* is therefore as important as enzyme concentration in determining production rate of angiotensin'.

We are grateful to Dr E. Haas for the gift of sheep renin which made this work possible and to Dr E. R. Lumbers for the angiotensin I antiserum. We also thank our colleagues and technical staff who participated in the preparation of the animals.

J.G.C. was supported by M.R.C. grant G972/841/C and by the Wellcome Foundation.

REFERENCES

BODDY, K., DAWES, G. S., FISCHER, R., PINTER, S. & ROBINSON, J. S. (1974). Foetal respiratory movements, electrocortical and cardiovascular responses to hypoxaemia and hypercapnia in sheep. *J. Physiol.* **243**, 599–618.

BROUGHTON PIPKIN, F., LUMBERS, E. R. & MOTT, J. C. (1974). Factors influencing plasma renin and angiotensin II in the conscious pregnant ewe and its foetus. *J. Physiol.* **243**, 619–636.

CARVER, J. G. & MOTT, J. C. (1975). Plasma renin, [Na^+] and [K^+] in immature foetal lambs with indwelling catheters. *J. Physiol.* **245**, 73–75*P*.

FLEISCHMAN, A. R., OAKES, G. K., EPSTEIN, M. F., CATT, K. J. & CHEZ, R. A. (1975). Plasma renin activity during ovine pregnancy. *Am. J. Physiol.* **228**, 901–904.

HERBERT, V., LAU, K. S., GOTTLIEB, C. W. & BLEICHER, S. J. (1965). Coated charcoal immunoassay of insulin. *J. clin. Endocr. Metab.* **25**, 1375–1384.

HUNTER, W. M. & GREENWOOD, F. C. (1962). Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature, Lond.* **194**, 495–496.

PEACH, M. J. (1977). Renin-Angiotensin System: Biochemistry and Mechanisms of Action. *Physiol. Rev.* **57**, 313–370.

RYAN, J. W., MCKENZIE, J. K. & LEE, M. R. (1968). A rapid simple method for the assay of renin in rabbit plasma. *Biochem. J.* **108**, 679–685.

SEALEY, J. E., MOON, C., LARAGH, J. H. & ATLAS, S. A. (1977). Plasma prorenin in normal, hypertensive and anephric subjects and its effect on renin measurements. *Circulation Res.* **40**, 41–45.

SKINNER, S. L., DUNN, J. R., MAZZETTI, J., CAMPBELL, D. J. & FIDGE, N. H. (1975). Purification, properties and kinetics of sheep and human renin substrates. *Aust. J. exp. Biol. med. Sci.* **53**, 77–88.

SKINNER, S. L., LUMBERS, E. R. & SYMONDS, E. M. (1972). Analysis of changes in the renin-angiotensin system during pregnancy. *Clin. Sci.* **42**, 479–488.

SMITH, F. G. JR., LUPU, A. N., BARAJAS, L., BAUER, R. & BASHORE, R. A. (1974). The renin-angiotensin system in the fetal lamb. *Pediatr. Res.* **8**, 611–621.

VALDES-CRUZ, L. M., TAYLOR, M. J., MOTT, J. C. & CARVER, J. G. (1977). Detection of conjoined placental circulation in twin foetal lambs. *J. Reprod. Fert.* **51**, 503–505.