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CHANGES IN THE PHYSICAL PROPERTIES AND IN THE COLLAGEN AND HEXOSAMINE CONTENTS OF THE FOETAL MEMBRANES DURING PREGNANCY IN THE RAT

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In the course of other work we observed that the foetal membranes (amnion and yolk sac) showed changes in physical properties at the end of pregnancy. From fairly tough, clean and well-defined structures earlier in pregnancy they became soft, friable, slimy and ill defined. These changes seemed likely to be of biological importance in facilitating the birth of the foetus, and we thought it of interest to examine them in more detail. We have now tested the strength of the membranes by measuring the pressure required to rupture a portion held across a standard, circular aperture. From the 15th to 18th day of pregnancy this pressure rises progressively. It then drops steeply and remains low until the end of pregnancy. During this fall change in the collagen content of the membrane per unit area was found to be relatively slight. The decrease in strength of the membranes was accompanied by a fall in the concentration of collagen per unit weight and an increase in the total hexosamine content of the membranes relative to collagen; these results suggest an increase in the polysaccharide component of the connective tissues. A preliminary account of this work has already been published (Harkness & Harkness, 1955*b*).

METHODS

The rats were albinos of the local strain (originally 'Glaxo'), weighing 180–220 g at the commencement of pregnancy, the date of which was determined by the same method as used previously (Harkness & Harkness, 1954). Each rat was killed by a blow on the head and breaking the neck. The contents of the uterine horns were removed intact, and the membranes were either dissected away and used for analysis, or tested mechanically.

Chemical methods. Collagen was extracted with hot trichloroacetic acid (Fitch, Harkness & Harkness, 1955) and estimated from the hydroxyproline content of the acid hydrolysate of the extract (Neuman & Logan, 1950*a*). Hexosamine was estimated after acid hydrolysis by the Elson–Morgan reaction (Elson & Morgan, 1933), using the procedure of Boas (1953) for removal of contaminating chromogens. In some cases nitrogen in the solid left after extraction of collagen

was estimated by the micro-Kjeldahl procedure, using the digestion technique of Chibnall, Rees & Williams (1943), and the ammonia estimated by distillation into boric acid with bromocresol green-methyl red as indicator (Conway, 1946). We have called this fraction 'non-collagenous protein nitrogen', though it will not represent all non-collagenous protein, since some material other than collagen is dissolved in hot trichloroacetic acid.

Method of examining the mechanical properties of the foetal membranes. The apparatus is shown in Fig. 1. The membranes were held across a circular aperture 3·5 mm in diameter, and the pressure required to burst them was measured. The aperture was surrounded by a concentric ring of holes through which gentle suction was applied in the preliminary stages of dissection to hold the tissue in place. The procedure was as follows. The bag of membranes containing the foetus and attached to the placenta was placed intact over the aperture, which was in the middle of a small bath of 0·9% (w/v) sodium chloride. The placenta was cut off, the membranes (yolk sac and amnion) opened and the foetus removed. A cap with a hole in the centre was placed over the membranes to hold them in position. This cap was subsequently held in place by finger pressure. Suction from a filter pump with the tap fully opened was then turned on, and the pressure at which the

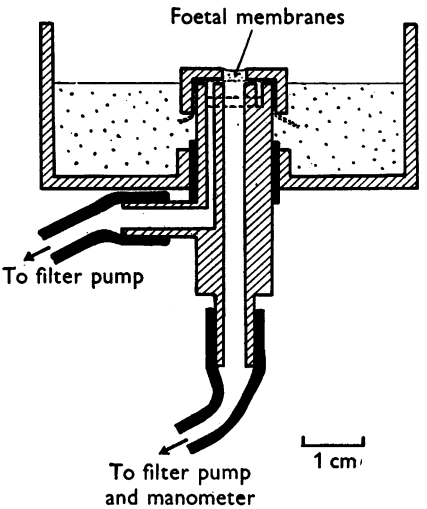

Fig. 1. Apparatus for measuring the strength of foetal membranes.

membranes burst was observed. The rate of change of pressure was in all cases the most rapid the filter pump could produce with the standard dead space of the apparatus and a trap. It took about a quarter of a minute for the pressure to fall to 50 cm Hg below atmospheric pressure. The observations reported here were all made at 20–22° C; a few made at 37° C gave similar results.

Measurement of the area of the membranes. From other rats we had figures for the weights at different times of pregnancy of individual conceptuses, which comprised the bag of membranes with its contents (foetus and amniotic fluid) plus the placenta. We estimated changes in the weight of the bag of membranes and contents by subtracting the average placental weight obtained from another series of rats. In order to obtain an estimate of changes in area of the bag of membranes we assumed the bag to be spheroidal. We estimated the length to breadth ratio for calculating surface area from photographs of horns of the uterus at various stages of pregnancy. The specific gravity of the bag and contents was assumed throughout to be unity. Clearly this is not strictly accurate, but the error is too small to affect the conclusions and we have therefore ignored it.

RESULTS

Physical properties and composition of membranes. The area of membranes examined was that covering the back and sides of the foetus. Observations on various parts of the membranes indicated that the bursting pressure was approximately uniform over this region, though rather higher in the small area where the membranes lie against the placenta.

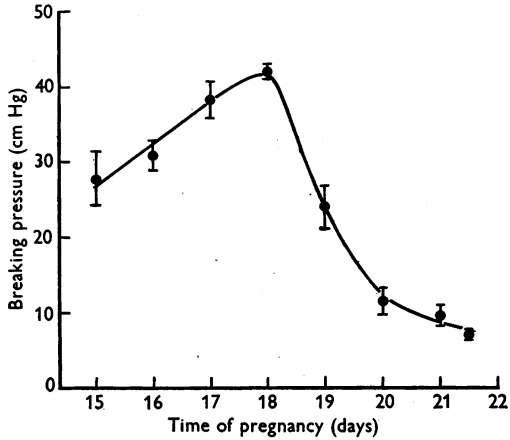

Fig. 2. Breaking pressure (cm Hg) of foetal membranes. The vertical lines extend from + to − the standard error of the mean.

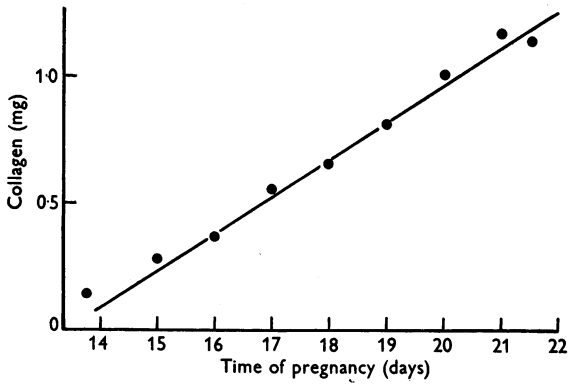

Fig. 3. Total collagen content of foetal membranes. The line is a calculated regression line representing the equation:

$$\text{Collagen (mg)} = -1{\cdot}96 + 0{\cdot}146\ (\pm 0{\cdot}075)\ t\ \text{(days)}$$

The pressure required to burst the membranes at various times of pregnancy is shown in Table 1 and Fig. 2. It will be seen that this pressure rises from the 15th to the 18th day. On the 19th day it drops sharply. This decrease is followed by a further but slower decline. While these changes are taking place

TABLE 1. Changes in the strength and composition of the foetal membranes during pregnancy

Time of pregnancy (days) ...	13–14	15	16	17	18	19	20	21	21½
Breaking pressure (cm Hg)	—	27·8±3·6 (6)	30·7±2·0 (5)	38·2±2·4 (5)	41·9±1·0 (5)	23·9±2·8 (5)	11·5±1·8 (5)	9·5±1·4 (4)	7·0±0·5 (4)
Wet weight of membranes per foetus (mg)	20±2 (4)	45±5 (5)	48±10 (3)	72±11 (3)	107±16 (2)	133±13 (4)	202±6 (5)	288±36 (4)	369±35 (6)
Total collagen of membranes per foetus (mg)	0·15±0·02 (4)	0·28±0·03 (5)	0·37±0·13 (3)	0·56±0·12 (3)	0·66±0·08 (2)	0·81±0·11 (4)	1·01±0·04 (5)	1·17±0·07 (4)	1·14±0·06 (6)
Collagen concentration (mg/g wet wt.)	3·38±0·68 (4)	4·86±0·28 (5)	6·95±0·95 (3)	7·49±0·57 (3)	6·21±0·43 (2)	6·28±0·72 (4)	5·00±0·37 (5)	4·50±1·07 (4)	3·20±0·27 (6)
Total hexosamine (μg)	—	24·3±4·5 (3)	36·5±3·6 (4)	43·0±1·3 (4)	69·7±9·7 (5)	109±14 (5)	156±17 (5)	201±26 (7)	203±32 (3)
Non-collagenous protein N (μg/g wet wt.)	←———	11·9±0·9 (2)	———→			10·7±0·5 (4)	10·5±0·3 (5)	7·7±0·4 (4)	7·2±0·6 (6)
Total non-collagenous N (mg)	←———	0·85±0·21 (2)	———→			1·41±0·17 (4)	2·12±0·08 (5)	2·18±0·28 (4)	2·59±0·26 (6)

The estimate of variation is the standard error of the mean. The number of rats in each group is given in parentheses.

the total amount of collagen in the membranes rises approximately linearly (Table 1 and Fig. 3). The total weight of the membranes follows a different curve, and there is a general correlation between the concentration of collagen

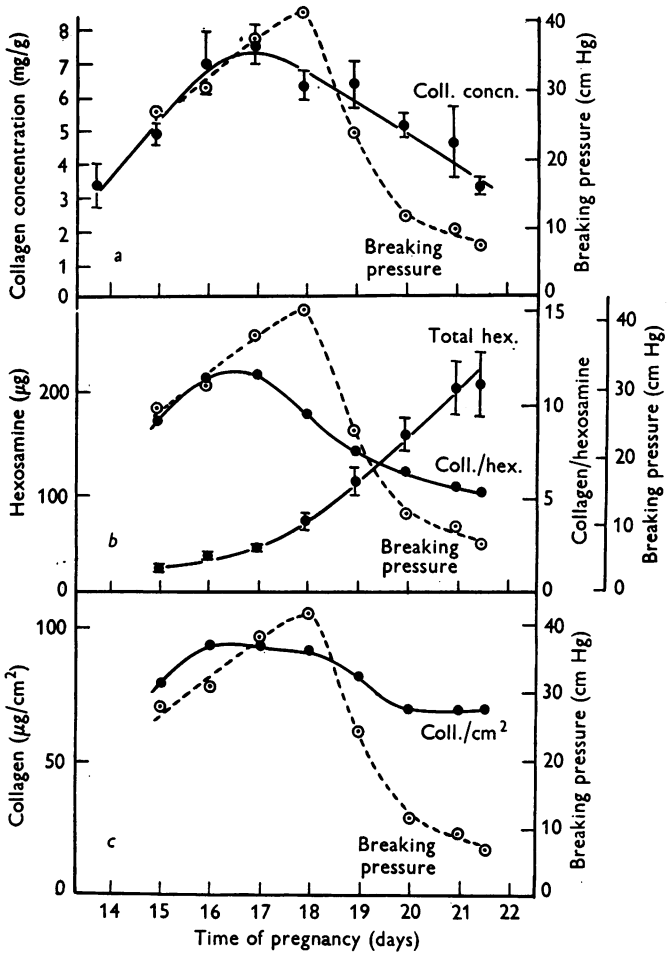

Fig. 4. Changes in chemical composition of foetal membranes with time of pregnancy. (*a*) Collagen concentration (μg/g wet weight). (*b*) Total hexosamine (μg) in membranes per foetus, and ratio collagen/hexosamine, calculated from the regression line for total collagen and curve for total hexosamine. (*c*) Quantity of collagen per unit area of membranes (μg/cm²). The change in breaking pressure with time of pregnancy is shown by a dotted line in each of the figures.

and breaking pressure (Table 1 and Fig. 4*a*). This is not exact, the time of highest concentration appearing to be earlier than the time of highest breaking pressure. These data on collagen concentration show that the previously

recorded relation between increase in weight and collagen (Harkness & Harkness, 1955*a*), based on a small sample, is an oversimplification.

The change in the total hexosamine content of the membranes is shown in Fig. 4*b*. The curve is convex to the time axis, hexosamine accumulating progressively more rapidly as pregnancy advances. The ratio of collagen to hexosamine calculated from the separate curves for total collagen and total hexosamine is approximately correlated with the breaking pressure.

TABLE 2. Weight and dimensions of uterine contents

Time of pregnancy (days) ...	11–13	14–15	16–17	18–19	20	21	21½
Weight of uterine contents per foetus (g)	0·26 ±0·04 (8, **12·6**)	0·52 ±0·08 (6, **14·3**)	1·55 ±0·20 (6, **16·8**)	2·94 ±0·30 (6, **18·7**)	4·69 ±0·25 (12)	5·62 ±0·24 (9)	6·06 ±0·17 (9)
Weight of placenta (g)	—	0·175 ±0·042 (9, **14·7**)	0·276 ±0·017 (10, **16·5**)	0·351 ±0·009 (14, **18·6**)	0·439 ±0·009 (7)	0·452 ±0·015 (9)	0·442 ±0·015 (10)
Ratio length/breadth of membranous sac	1·26 ±0·03 (6*, **12·3**)	1·44 ±0·06 (4*, **15·0**)	1·60 ±0·02 (4*, **17·0**)	1·73 ±0·18 (4*, **19·0**)	2·39 ±0·22 (8*)	2·20 ±0·15 (4*)	2·15 ±0·20 (4*)

The estimate of variation is the standard error of the mean. The figures in parentheses are, first, number of rats or horns (*); and second, in bold type, mean time of pregnancy.

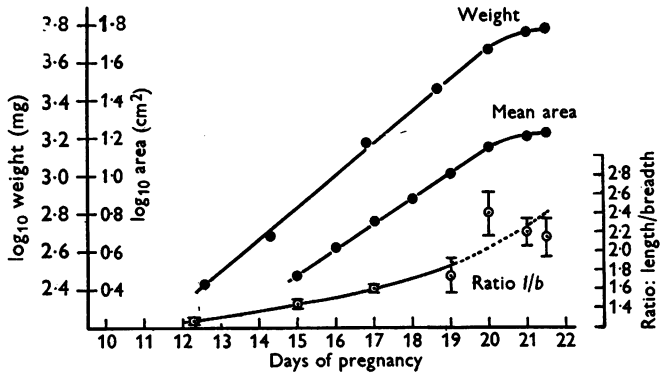

Fig. 5. Dimensions of sac of membranes.

The non-collagenous nitrogen was not as fully investigated as the other constituents; the total increased in the last 4 days of pregnancy, while the concentration decreased as in the case of collagen. It appears that the increase in weight of the membranes in this period is mainly due to an increase in material with a low protein content.

Changes in the area of membranes. Changes in the weight of uterine contents and of the placenta, and in the length to breadth ratio (*l*/*b*) of the bag of membranes are shown in Table 2 and Fig. 5. *l*/*b* shows large variation at the end of pregnancy, which is not unexpected if the restraint of the membranes is reduced, since the foetuses move about actively at this time. We have actually used the values obtained from a smooth line, drawn by eye through the points, for calculations of area, but the results would not be materially affected if the mean values for individual days were used. The changes in the

quantity of collagen present in unit area of membranes are shown in Fig. 4*c*, where they may be compared with changes in breaking pressure. The breaking pressures cover an approximately sixfold range, whereas changes in collagen per unit area of membranes are small, having a range of only about 30%.

DISCUSSION

The method we have used for measuring the mechanical properties of the foetal membranes is empirical, and the results could be affected by changes in properties other than tensile strength. When the membranes are subjected to pressure they bulge and stretch before they eventually break. They break by splitting somewhere in the centre of the bulge, not by tearing at the edge of the aperture across which they are stretched. The tension in the membrane is related to pressure difference on the two sides and to the radius of curvature of the bulge. We did not make any measurements of the changes in the radius of curvature at the breaking point at different times in pregnancy. It seems likely that, if they were allowed for, the changes in breaking point would be even greater than those recorded, since one would expect the radius of curvature to be greater the less stretchable the membranes; and the membranes seem to be much more easily stretchable in the last 2 or 3 days of pregnancy. This can be seen if one injects fluid into a whole sac of membranes. At the end of pregnancy they stretch extremely easily and at once at very low pressures. On the other hand, about the 18th to 19th day they may hold a pressure of as high as 70 cm of mercury without obviously stretching. It seems reasonable to conclude therefore that our measurements do represent a real weakening or relaxation of the structure of the foetal membranes. The mechanism of this weakening is unknown. Change in quantity of collagen present in unit area of membranes appears to be too small to account for it. It is of course possible, though perhaps unlikely, that the membranes are not held together by collagen at all, but by some other material. We have reason to think that elastin is very unlikely to be important in this respect; silver staining of the membranes shows a heavy reticulin network in the yolk sac membrane and a much lighter one in the amnion. No elastin network was demonstrable by Verhoeff's method. Extraction of the membranes with 0·1 N-NaOH at 100° C for 45 min and washing with water left a small amount of material which contained traces of hydroxyproline which, though too small for accurate estimation, would correspond to an amount of elastin equal to only about 5% of the total collagen on the basis of the hydroxyproline content of elastin given by Neuman & Logan (1950*b*). Elastin seems to have only about one-tenth the tensile strength of collagen (Wöhlisch, du Mesnil de Rochemont & Gerschler, 1927). It therefore seems unlikely that elastin plays any important part in determining the strength of the membranes. It is also unlikely that the physical changes that we have found are due to alterations

in some unknown structural element, though this possibility must be kept in mind. The structural function of other materials has been very little studied, and it is undoubtedly important for the understanding of the structural stability of tissues that more work should be done on this question. It is of interest to make a rough calculation to see whether the quantity of collagen present is enough to account for the strength of the membranes. One can get an approximate estimate of the minimum strength of the membranes if it is assumed that at the moment of breaking the bulge is in the form of a hemisphere. The pressure (p) will then be related to the tension in the membranes (T) by the formula $p=2T/r$, where r is the radius of curvature of the hemisphere. The breaking tension in the strongest (18-day) membranes works out at about 5kg/mm^2 of collagen. The breaking strength of tendon is of the order of 5–10 kg/mm^2 of fresh tissue (see Cronkite, 1936; Stücke, 1950), or 10–20 kg/mm^2 of collagen. In tendon the collagen is orientated in longitudinal bundles to give maximal strength in the direction of the stretching force. In the membranes with which we are concerned the fibres are not orientated all in one direction but are in the form of a two-dimensional network. We should thus expect about half the tensile strength, which is approximately what is found in unsoftened membranes. Though this is a rough calculation it does indicate that the collagen in the membranes could account for their physical strength.

None of the chemical changes that we have investigated exactly parallels the change in physical properties of the foetal membranes. Nevertheless, there is a suggestive general parallelism between these and the changes in percentage collagen, percentage total protein and in the ratio of collagen to hexosamine. The fact that the percentage total protein diminishes indicates that the collagen is not diluted by an increase in cellular material, but more probably, in extracellular material. The relative increase in hexosamine points in the same direction, and indicates that the increase is not a simple oedema but involves increase in extracellular polysaccharides. Though clearly much more information is required to define the relation between physical and chemical changes, the latter suggests infiltration with a watery lubricating material which allows the collagen fibres to slide over one another. Judging by properties of tendon, which breaks at about 10% extension (Wöhlisch *et al.* 1927; Stücke, 1950), the collagen fibres themselves are very inextensible. Paradoxically it appears from electron microscopical evidence that the finer protofibrils can be extended to double or more their resting length. Fibrils can be found with increased band widths, and these fibrils are presumed to have been stretched by contraction of neighbouring material in the preparatory procedure for electron microscopy (see Bear, 1952). Increased band widths have also been produced by deliberate stretching of specimens before electron microscopy (Mustacchii, 1951). Reduction in collagen concentration by dilution with some other material similarly takes place in the symphysis

pubis when it is relaxed by relaxin (Frieden & Hisaw, 1953). It is also found in association with mechanical weakness in wounds in scorbutic animals (Abercrombie, Flint & James, 1955). Another obvious instance of relaxation is that of the uterine cervix in pregnancy, but nothing seems to be known about its time course for comparison with chemical changes such as the reduction in collagen concentration in pregnancy (Harkness & Harkness, 1954). No measurements of hexosamine in any of these instances of tissue relaxation appear to be available for comparison with our own. The change in hexosamine we have found could be unrelated to the connective tissues and be caused by secretion of mucus or changes in other hexosamine-containing materials. The importance of a more detailed investigation of this point will be easier to judge when the results of comparable investigation of other cases of relaxation are available.

SUMMARY

1. Investigation of the physical strength of the foetal membranes of the rat showed a rise from the 15th day to a maximum about the 18th day, followed by a steep fall to low values at the end of pregnancy.

2. Over the same period of pregnancy there was an approximately linear increase in the total collagen content of the membranes.

3. Investigation of the surface area of the membranes showed relatively small changes in collagen per unit area. These were not large enough to account for changes in strength, and it was concluded that the weakening represents a relaxation of the structural material analogous to that which takes place in the cervix and symphysis pubis in pregnancy.

4. The weakening of the membranes was accompanied by an increase in material of low protein content, and a drop in collagen concentration per unit of wet weight.

5. There was a general but not exact parallelism between the strength of the membranes and the ratio of collagen to hexosamine.

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REFERENCES

Abercrombie, M., Flint, M. H. & James, D. W. (1955). Wound contraction in relation to collagen formation in scorbutic guinea-pigs. *J. Embryol. exp. Morph.* (in the Press).

Bear, R. S. (1952). The structure of collagen fibrils. *Advanc. Protein Chem.* **7**, 69–160.

Boas, N. F. (1953). Method for the determination of hexosamines in tissues. *J. biol. Chem.* **204**, 553–563.

Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). The total nitrogen content of egg albumin and other proteins. *Biochem. J.* **37**, 354–359.

Conway, E. J. (1946). *Microdiffusion Analysis and Volumetric Error.* London: Crosby Lockwood.

Cronkite, A. E. (1936). The tensile strength of human tendons. *Anat. Rec.* **64**, 173–186.

Elson, L. A. & Morgan, W. T. J. (1933). A colorimetric method for the determination of glucosamine and chondrosamine. *Biochem. J.* **27**, 1824–1828.

FITCH, S. M., HARKNESS, M. L. R. & HARKNESS, R. D. (1955). Extraction of collagen from tissues. *Nature, Lond.*, **176**, 163.

FRIEDEN, E. H. & HISAW, F. L. (1953). The biochemistry of relaxin. *Recent Progr. Hormone Res.* **8**, 333–378.

HARKNESS, M. L. R. & HARKNESS, R. D. (1954). The collagen content of the reproductive tract of the rat during pregnancy and lactation. *J. Physiol.* **123**, 492–500.

HARKNESS, M. L. R. & HARKNESS, R. D. (1955*a*). The growth of collagen in the foetus, placenta and foetal membranes of the rat. *J. Physiol.* **128**, 225–234.

HARKNESS, M. L. R. & HARKNESS, R. D. (1955*b*). Changes in the foetal membranes during pregnancy in the rat. *J. Physiol.* **129**, 78*P*.

MUSTACCHII, P. O. (1951). Extensibility of hydrated collagen. *J. Amer. Leather Chem. Ass.* **46**, 479–483.

NEUMAN, R. E. & LOGAN, M. A. (1950*a*). The determination of hydroxyproline. *J. biol. Chem.* **184**, 299–306.

NEUMAN, R. E. & LOGAN, M. A. (1950*b*). The determination of collagen and elastin in tissues. *J. biol. Chem.* **186**, 549–556.

STÜCKE, K. (1950). Über das elastische Verhalten der Achillessehne im Belastungsversuch. *Archiv. klin. Chir.* **265**, 579–599.

WÖHLISCH, E., DU MESNIL DE ROCHEMONT, R. & GERSCHLER, H. (1927). Untersuchungen über die elastischen Eigenschaften tierischer Gewebe. 1. Elastizitätmodul, Zereissfestigkeit, Arbeitsvermögen und elastische Volkommenheit. *Z. biol.* **85**, 325–341.