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SOME EFFECTS OF ENDOGENOUS HORMONES ON MUSCLES AND CONNECTIVE TISSUE, WITH SPECIAL REFERENCE TO THE EWE

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SUMMARY

This paper summarizes research over several years on muscle and connective tissue changes associated with oestrus, pregnancy and *post-partum* involution, chiefly with reference to the ewe, but embracing also eleven other mammalian species.

Macroscopical alterations, such as change in weight of certain muscles and relaxation of joints, effected by endogenous hormones, have been measured and are reported. The underlying microscopical changes in muscle and connective tissue, which are described and illustrated, consist of hypertrophy of muscle fibres, loosening of collagenous fibres, and great changes in morphology and behaviour of connective tissue cells. Fibroblasts enlarge, then eosinophils, plasma cells and lymphocytes appear. The possible function of eosinophils, plasma cells and lymphocytes in such modified connective tissue is discussed.

The findings may be related both to problems of veterinary obstetrics and meat production.

THE ENDOGENOUS HORMONES with which this paper is particularly concerned are those of reproduction, chiefly pregnancy. Its most obvious relationship to animal production is through problems of veterinary obstetrics such as vaginal prolapse (bearing trouble), undilated cervix at parturition (ring-womb at birth) and dystocia (difficult birth) with consequent neonatal mortality. All these conditions can have considerable economic consequences. (See Edgar (1952), Bassett (1956a) for vaginal prolapse; Hindson and Turner (1962) for undilated cervix; Wallace (1949), McFarlane (1955) for neonatal mortality in sheep; N.Z. Dairy Board records, 1948–49, for neonatal mortality in cattle; D. M. Smith (pers. comm.) for neonatal mortality in pigs.)

Less immediately obvious, but also relevant, is the need to know to what degree different components of the animal body vary during normal physiological processes.

If, for example, pregnant animals are used in meat production studies, another variable may be introduced which may deserve consideration in the interpretation of results. Again, in experimental treatment of animals with exogenous hormones, such as stilboestrol for growth stimulation in meat animals, it is important to know the effect of the endogenous hormones before one can assess the changes brought about by the exogenous hormones.

In all species which have been investigated, there are great changes in certain muscles and connective tissues during different phases of the reproductive cycle, and thus presumably related to different levels of endogenous sex hormones. The changes are usually much greater in pregnancy than in oestrus and are specific to structures involved in reproduction. There are species differences in the structures affected.

Most of the research of other workers in this field has been with small rodents, chiefly the guinea-pig, rat and mouse. (See Kochakian *et al.* (1956), Wainman and Shipounoff (1941) for striated muscle; Hisaw and Zarrow (1950), Frieden and Hisaw (1953), Harkness (1955–6) for connective tissue.) The above animals have also been used to produce experimental evidence that the alterations which occur are in fact due to the action of sex hormones. This evidence has been further substantiated experimentally on several other species during the work reported here.

In this work, the ewe has been taken as the chief example. Investigations on striated skeletal muscle have been confined to this species. The smooth muscle studies have been made on the ewe and on several other species. Observations on connective tissue have been extended to include ligaments, tendons and other connective tissues from the sheep, cattle, pig, rabbit, rat, mouse, guinea-pig, cat, ferret, hedgehog, phalanger (Australian possum), and human. This comparative approach has allowed the determination of a pattern of connective tissue behaviour, in response to endogenous hormones, in mammals generally. The conclusions are thus applicable to any species which is being considered, whether farm animal or human.

Several techniques have been employed (Bassett and Phillips, 1954a, b, 1955a, b; Bassett, 1956b, 1958, 1959, 1961, 1962) to study the changes brought about by endogenous hormones. The findings will be considered under three main headings:

(1) Gross anatomy of the pelvic region, describing particularly the structures which change.

(2) Gross quantitative changes, describing weight changes of muscles and organs, and other measurements made with mechanical instruments.

(3) Microscopical and electronmicroscopical changes.

GROSS ANATOMY OF PELVIC REGION

In considering the anatomy of the pelvic region, particularly in relation to reproduction, it is more logical to reconstruct it mediolaterally (*i.e.*, from the inside out) rather than lateromedially (from the outside in), which is the conventional way of dissecting it. A mediolateral reconstruction begins with the pelvic girdle, which immediately surrounds that part of the reproductive tract constituting the birth canal, and then the structures surrounding the pelvic girdle, in turn. The most important parts of the pelvic girdle, in the present context, are the pubic symphysis, joining the opposing bones of the "floor" of the pelvic canal, and the sacro-iliac joints, which join the pelvis to the spine. The pubic (or ischio-pubic in some species such as sheep, cattle and pig) symphysis does not loosen in most animals, including ewe, cow and sow, and indeed all the species listed above, except guinea-pig, mouse and hedgehog. The sacro-iliac joint, on the other hand, loosens in all the animals studied except hedgehog and phalanger. The sacro-iliac joints tend to be looser in ewes with vaginal prolapse than in normal ewes in the same sexual state; also, they can be made to loosen in spayed ewes by injection of stilboestrol. Loosening of either pubic symphysis or sacro-iliac joints, or both, allows alteration in size or shape of the pelvic canal during parturition. It will be appreciated that, if this loosening of joints at birth is to be effective, many related structures must also change, which they, in fact, do.

For instance, the muscles of the pelvic outlet change, both the muscles themselves, their intramuscular connective tissue, and tendon of origin (possessed only by ischiococcygeus).

In ewes with vaginal prolapse the ischiococcygeus tendon is even more elongated than in normal pregnant ewes at term. Another difference between prolapsed and normal ewes is in the deep perineal sphincter. Normally the two ends of this muscle, which is a band going right around rectum and vulva, meet each other at the ventral surface of the vulva; in prolapsed ewes they are often stretched as much as 10 mm apart. However, as with other differences in prolapsed ewes, it is difficult to decide between cause and effect.

The sacrosciatic ligament, which forms each lateral pelvic wall in sheep, cattle and pig, also changes greatly in pregnancy and, as further evidence for the hormone theory, it has been altered in steers treated with stilboestrol to a degree equal to that of a cow half-way through gestation.

Covering the sacrosciatic ligament is a layer of lateral pelvic muscles, including gluteus medius, and biceps femoris which extends right from the spine to the stifle joint. Part of biceps femoris is attached to the caudal margin of the sacrosciatic ligament, so that, as this ligament stretches at parturition, biceps femoris must also stretch, by divarification of its fibres.

The dermis of the vulval skin changes in some pregnant animals, such as the ewe, and this site in the oestrous ferret is the most remarkably modified of all the tissues which have been investigated.

Although there is a species variation as to the pelvic structures which change, modifications in both smooth muscle and connective tissue have been found in the reproductive tract in all species in which it has been examined.

The distended pregnant uterus is, of course, greatly altered, but changes are seen in the uterus in oestrus even when none of the other structures is affected in a particular animal. The broad uterine ligament, which attaches the reproductive tract to the abdominal and pelvic walls, becomes very much larger in area and thickness in pregnancy.

Samples of connective tissue from structures not implicated in reproduction, and in which no changes have been found in relation to the reproductive cycle, include the flexor tendon from the lower limb, intramuscular connective tissue of gastrocnemius and dermis of shoulder skin. Neither smooth muscle nor connective tissue of the rectum is affected.

GROSS QUANTITATIVE CHANGES

Striated (skeletal) Muscle Weight

Figure 1 shows typical muscle weight changes during pregnancy and *post-partum* involution. The example here is ischiococcygeus, but similar weight changes are found in the perineal sphincter and retractor clitoridis. Each point represents the mean weight of muscles from six ewes. There is a fall in weight in early pregnancy, with a subsequent rise after the tenth week, the muscle being heaviest immediately after lambing, after which it loses weight rapidly. Statistical analysis of the figures for these three muscles (which will be published in full elsewhere) showed highly significant differences between weights at each stage of the reproductive cycle.

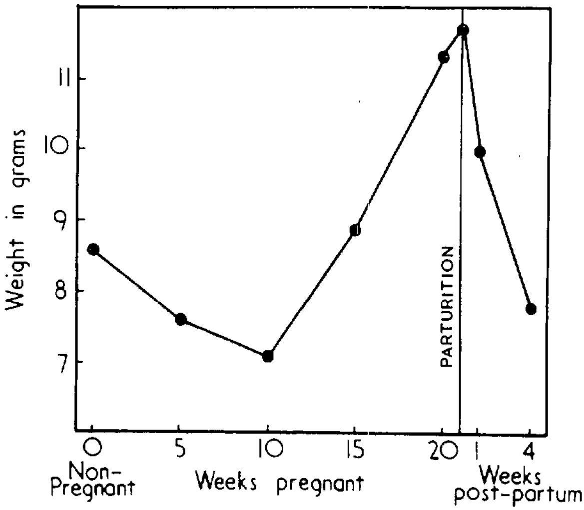

Fig. 1: Change in mean weight of ischiococcygeus muscle during pregnancy and post-partum *involution (6 ewes at each stage).*

The weight change of biceps femoris showed a similar trend, although there was a high variability between animals at each stage and the differences between stages were not significant. There were no significant weight changes in gluteus medius and gastrocnemius. Weighings made of triceps brachii, from the forelimb, indicated that pregnancy may be associated in some way with a low weight of this muscle. As there was no apparent effect of pregnancy on carcass weight of these ewes, there may be a redistribution of weight of different muscles in the pregnant animal. If some muscles become heavier, others must become lighter, for carcass weight to remain constant.

These results, showing that some muscles are affected by pregnancy and that others may be, suggest that pregnancy could be a complicating factor in meat production studies; also, it seems likely that hormone treatment may have a variable effect on different muscles. Further investigations are necessary, however, particularly on those muscles most valued as meat, before any firm conclusions may be drawn.

SMOOTH MUSCLE AND CONNECTIVE TISSUE WEIGHT

Figure 2 shows changes in weight of the empty uterus, composed chiefly of smooth muscle and connective tissue,

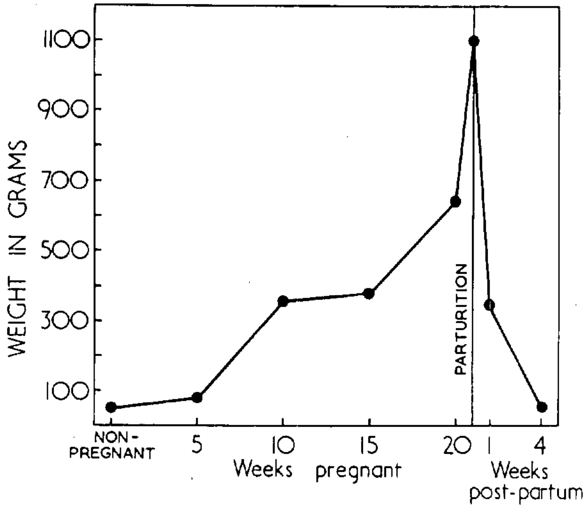

Fig. 2: Change in mean weight of empty uterus during pregnancy and post-partum *involution (same ewes as in Fig. 1).*

in the same ewes. The trend is similar to that for the skeletal muscles — a sharp rise in weight approaching parturition, and a sharp fall in weight during involution. There is, of course, no fall in weight in early pregnancy — the uterus becomes heavier as soon as it contains the products of conception.

GROSS CHANGES IN CONNECTIVE TISSUE

Figure 3 shows gross connective tissue change. The example here is movement of the sacro-iliac joint, measured with a mechanical instrument, at different stages of pregnancy and during involution. These were different ewes from the ones in the two previous figures and the means are of five animals at each stage. There is the same fall in early pregnancy and the same rise round about the 10th week as there is for the muscle weights.

It seems probable that this cyclic pattern has a physiological basis, as it has been recorded also for other characteristics such as potential displacement of the reproductive tract (Bassett and Phillips, 1955b) and excretion of oestrogens (Bassett *et al.*, 1955).

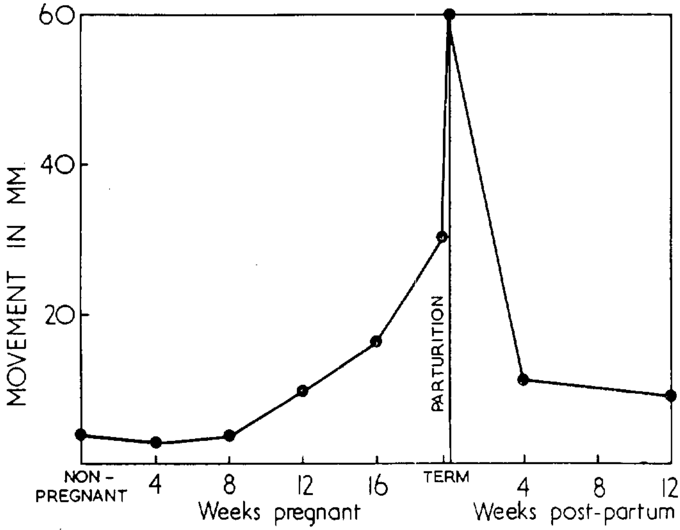

Fig. 3: Movability of sacrum relative to ilium during pregnancy and post-partum *involution (5 ewes at each stage, different ones from Figs. 1 and 2).*

Underlying this characteristic macroscopical pattern of progressive growth or proliferation and rapid regression when the climax is past (*i.e.*, after parturition or oestrus), is a corresponding microscopical pattern.

MICROSCOPICAL CHANGES

Muscle

For striated muscle, ischiococcygeus has again been taken as an example and it has been found that, for this muscle at least, the increased weight is associated with a greater fibre diameter (Bassett, 1956b). The same enlargement of muscle fibres has been observed in smooth muscle of the reproductive tract of several species.

Figures 4 and 5 contrast smooth muscle of the uterus of a non-pregnant rat and that of a pregnant rat at term, respectively. In the pregnant rat the muscle fibres are much larger and there are some marked histochemical changes (basophilic substance round the nucleus) as well.

Connective Tissue

Collagenous Fibres and Fibroblast Cells

The microscopical connective tissue modifications are even more remarkable than the muscle changes. Figure 6, which shows the sacrosciatic ligament of a non-pregnant dioestrous ewe, is typical of the dense connective tissue of most ligaments and tendons in a resting state. The collagenous fibres are straight and closely packed and the only cells present are fibroblasts (the ubiquitous cells of connective tissue) with small, narrow nuclei and no visible cytoplasm. Contrast this with Fig. 7 which shows the same ligament of a ewe killed just after parturition. The collagenous fibres are exceedingly loose and the fibroblasts are now quite different. The nuclei are larger and rounder and there is much visible cytoplasm which stains characteristically with basic dyes. Very similar modifications are seen in other dense pelvic connective tissue, such as ischiococcygeus tendon and the fibrous part of the sacro-iliac joint.

In loose connective tissue, such as that in a broad uterine ligament, there is, in pregnancy, a relatively much greater increase in the ground substance in which the collagenous fibres lie, than there is in the more dense kinds of connective tissue.

The two extremes — non-pregnant and pregnant at term — have been compared to emphasize the point, but, in fact, the proliferative process is a gradual one closely following the cycle of gross changes in connective tissue except that the microscopical differences occur first. The cell nuclei begin to enlarge and then more and cytoplasm appears. During involution the fibroblast nuclei become small, no cytoplasm is visible and the collagenous fibres are more densely packed, so that in the end the tissue returns to its non-pregnant form.

Eosinophils, Plasma Cells and Lymphocytes

Fibroblasts are the common cells of connective tissue. In greatly modified connective tissue, at the time when the fibroblasts are extremely enlarged, and also during the period of involution, other cells, which are not usual inhabitants of connective tissue, infiltrate into the tissue from the blood (Bassett, 1962).

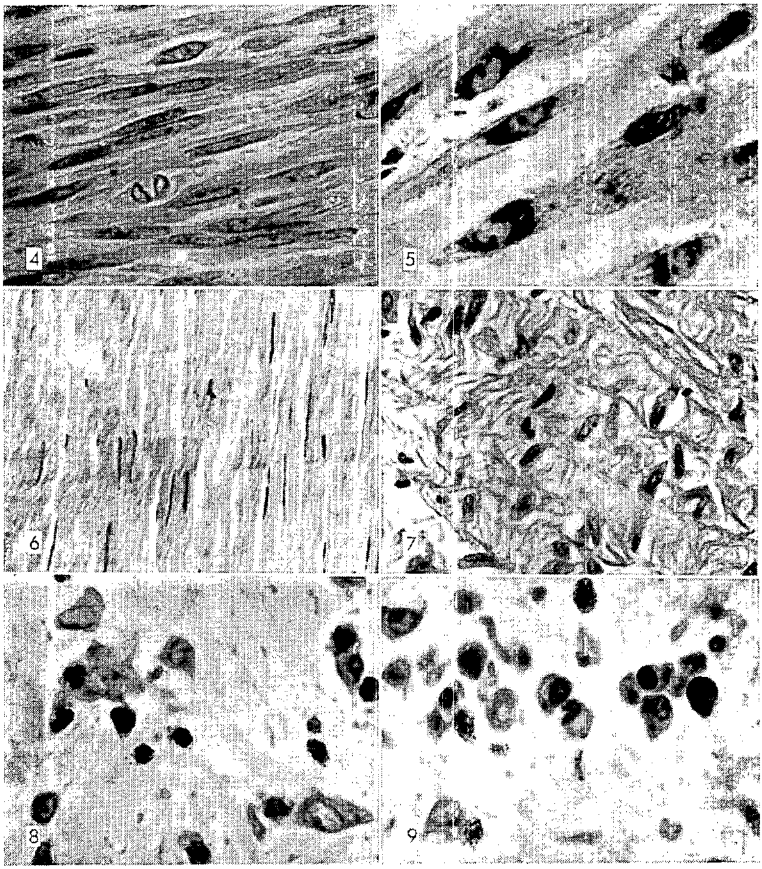

Fig. 4: Smooth muscle in uterus of dioestrous rat. Narrow nuclei and muscle fibres. (Methyl green-pyronin. ×600).

Fig. 5: Smooth muscle in uterus of pregnant rat, at term. Basophilic substance round enlarged nuclei. Wider muscle fibres. (Methyl-green pyronin. ×600).

Fig. 6: Sacrosciatic ligament of dioestrous ewe. Straight densely arranged collagenous fibres. Narrow fibroblast nuclei, no visible cytoplasm. (Iron haematoxylin-light green. ×250).

Fig. 7: Sacrosciatic ligament of ewe, immediately post-partum. *Loosened collagenous fibres. Fibroblast nuclei enlarged, much cytoplasm. (Iron haematoxylin-light green. ×250).*

Fig. 8: Eosinophils and enlarged fibroblasts, many in close contact, in vulval skin of oestrous ferret. (Haematoxylin-azur II-eosin. ×600).

Fig. 9: Plasma cells, lymphocytes, eosinophils and enlarged fibroblasts, in early involuting vulval skin of post-oestrous ferret. (Haematoxylin-azur II-eosin. ×600).

Figure 8 shows a section of the grossly swollen vulval skin of an oestrous ferret. There are dramatically enlarged fibroblasts, but also numerous eosinophil cells (one type of leucocyte), which in many cases are in close contact with the fibroblasts. This infiltration by eosinophils at the time of maximum connective tissue proliferation and during early involution is quite characteristic of the various tissues from the different species, although the appearance is not always quite so striking as it is in the oestrous ferret.

At the same time as the eosinophils infiltrate, or slightly later, plasma cells and lymphocytes appear. Figure 9 shows these cells and a few eosinophils in the involuting vulval skin of a ferret just going out of oestrus. The plasma cells and lymphocytes stay in the involuting tissue for longer than the eosinophils, which disappear fairly early in the process.

The next problem is to form some idea of the role of the different cells in this modified connective tissue.

Since the fibroblasts are the only cells present during the proliferative phase in all the affected structures in all

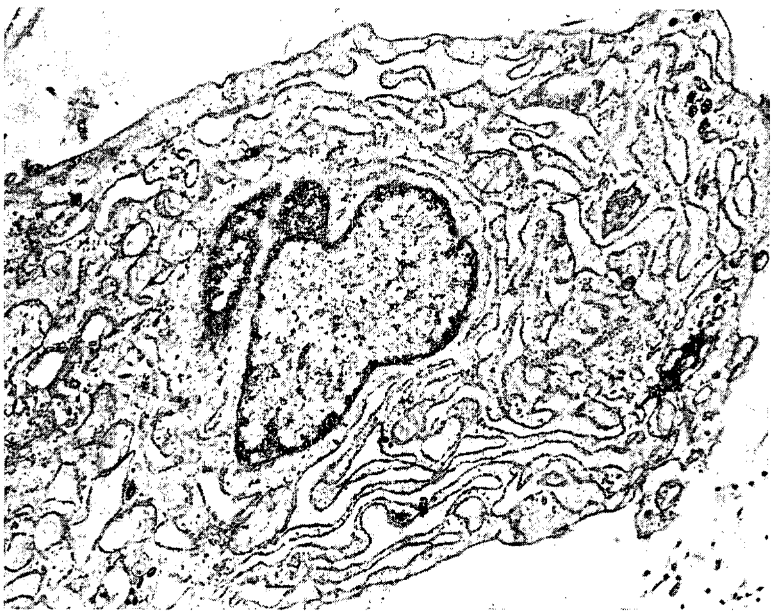

Fig. 10: Electronmicrograph of active fibroblast from symphyseal ligament of pregnant guinea-pig, at term. Much rough-surfaced folded membrane (endoplasmic reticulum) denotes ribonucleic acid, thus protein synthesis. (×10,250.)

the species, they must be responsible for the loosening of collagenous fibres and increase in ground substance. There is much evidence — histological, histochemical and electronmicroscopical — that the fibroblasts are actively synthesizing and secreting protein, which could be in the form of new fibres or precursors, ground substance, and perhaps also an enzyme which splits existing fibres.

Figure 10 illustrates the fine structure of such an active fibroblast from the symphyseal ligament of a pregnant guinea-pig. The rough-surfaced, much-folded membrane in this enlarged cell, known as endoplasmic reticulum, indicates the presence of ribonucleic acid, which is known to be a prerequisite for protein synthesis in a cell.

There is good evidence that eosinophils are attracted into the tissues by the fibroblast secretions to perform a necessary function by acting on or with the secretions so that a pathological condition cannot develop (Bassett, 1962). In extreme cases, they are attracted right to the source of secretion so that the cells are in contact with one another. In tissues such as the oestrous ferret vulval skin, which, during the breeding season, remains swollen for as long as the animal is unmated, the fibroblasts are constantly stimulated to synthesize and secrete, and some such regulatory mechanism is essential. The fact that eosinophils are also especially numerous during the involutionary process strengthens the idea that they are involved in the removal of substances not required by the organism. This suggested function of eosinophils is interesting because the purpose of these cells in the body is at present unknown. The accumulation of eosinophils in tissues in which they are not always present is usually regarded as pathological — they accumulate, for instance, at the site of an immune reaction, that is reaction against a foreign protein — hydatid infection, for example. Many authors have thus considered that protection against foreign protein is the chief reason for an abnormal accumulation of eosinophils. The ideas suggested by the present findings are thus an extension of this theory to include the regulation of extracellular protein produced under certain normal conditions by the cells of the animal itself.

The function of the plasma cells and lymphocytes is at present even less clear. However, they are also characteristic of an immune reaction and, again, usually regarded as signs of a pathological condition. It could be that the animal has a temporary immune reaction against certain of its own proteins — all the proliferated and enlarged tissues which must somehow be rapidly removed after parturition (or oestrus), when the need for them is past. Auto-immunity, a fashionable word in medical research today, is applied to certain diseases (Burnet, 1959) but the concept could perhaps be enlarged to include some temporary but normal conditions.

Although this very detailed connective tissue research may seem far removed from the practical problems described at the beginning of this paper, there are several connecting links which suggest further lines of investigation in both fundamental and applied fields. For instance, because fibroblasts are responsible for loosening of connective tissue, study of their ultrastructure and metabolism, and the effects of different hormone levels on them, are quite relevant to conditions in which there is a deviation from the normal amount of structural change, such as vaginal prolapse, when the pelvic tissues are unusually loose, ring-womb, when the connective tissue of the cervix may be too tight, and dystocia, when tissues of pelvis and reproductive tract may be too tight. The numbers of eosinophils in the tissues at the time of normal maximum change may be important — too few could result in excessive proliferation, and vice versa.

More immediately practical are trials of the effects of hormone treatment on different obstetrical abnormalities. Some preliminary trials in England using the hormone relaxin (which, with oestrogen, loosens the symphyseal ligament of guinea-pig and mouse) have given quite promising results in treatment of undilated cervix (Hindson and Turner, 1962), and the same hormone could well be of value in dystocia. Hormones, such as testosterone, which have been shown to prevent symphyseal relaxation in pregnant mice (Gardner and van Heuverswyn, 1940), might be useful for treating vaginal prolapse.

Problems in this applied approach are that in most conditions best results would be obtained if the dysfunction could be treated early, at its onset — not always possible under field conditions. Also, some conditions, such as vaginal prolapse, are probably due to a combination of causes, of which the tissue looseness is only one. For any of these conditions, it might be necessary to try many different dose levels on a large number of animals before the effectiveness of a hormone treatment could be assessed.

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DISCUSSION

Q: *Has Dr Bassett any information or ideas on the pathways by which specific oestrogens act on specific tissues? Is any such highly specific mechanism likely to be involved in the laying down of fat, discussed in Mr Barton's paper?*

DR E. G. BASSETT: Work by Villee and co-workers (*Rec. Progr. Hormone Res.*, *16*: 49) shows that an oestrogen-stimulable enzyme, a transhydrogenase, is present in target organs such as endometrium, placenta and mammary gland, but is absent in non-target organs such as liver and heart. These workers consider that the effect of oestrogen on specific tissues is mediated by the presence of this enzyme. It could well be that such an enzyme is present also in connective tissues which are specifically sensitive to oestrogen. I do not know how such a concept could be applied to the laying down of fat. Many different body functions depend on some specific mechanism, the tissues having developed their particular sensitivity in embryonic life.

Q: *Is it known if oestrogens influence the permeability of tissues to eosinophils?*

DR BASSETT: I cannot give a direct answer to that. It is known that sex hormones do act on blood vessels in specific tissues so as to increase their size. I think I have read that the permeability of certain blood vessel walls to erythrocytes is increased in pregnancy. It is known that in inflammation there is an increased permeability of blood vessel walls to leucocytes, including eosinophils. So is seems likely that the change in blood vessels effected by oestrogens could lead to an increase in permeability to eosinophils.

Q: *A regression phase also occurs in the mammary gland. Are there any parallel anatomical changes in mammary connective tissue and similar tissue in the reproductive tract?*

DR BASSETT: I have not made any microscopical examinations on the regressing mammary gland, but this could easily be done, as I still have uncut wax blocks of mammary gland tissue from the same ewes from which most of the other connective tissue samples were taken.