Some Observations on Vitally Stained Rabbit Ova with Special Reference to their Albuminous Coat[1]

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WITH ONE PLATE

INTRODUCTION

THE gradual deposition of a homogeneous, jelly-like covering on the rabbit ovum during its passage down the uterine tube was first observed by Cruikshank in 1797. This observation has been confirmed repeatedly by many later workers (Barry, 1839; Bischoff, 1842; Thomson, 1859; Assheton, 1894; Gregory, 1930; and Pincus, 1936). Barry referred to this covering investing the zona pellucida as 'chorion', some called it the 'pro-chorion', while others (Lenhossek, 1911; Grosser, 1927) simply spoke of a deposit of mucus due to the glandular activity of the tubal epithelium. In the more recent literature (Gregory, 1930; Pincus, 1936) this layer is generally referred to as an 'albuminous' coat, probably because of its slight similarity to the covering of a hen's egg.

Lenhossek (1911) described a scanty but an identical coat on the ovum of the horse and dog. Hamilton & Day (1945) found a thin coat of material on the tubal ovum of the horse similar to that on the rabbit ovum. In monotremes (Hill, 1933; Flynn & Hill, 1939) and in many marsupials (Hartman, 1916; Hill, 1910, 1918; McCrady, 1938) a thick albuminous coat is laid down on the zona pellucida as the egg traverses the oviduct. The investigations of Hammond (1934) on the fertilization of rabbit ova in relation to time of insemination show that the albuminous coat deposited on the tubal ovum plays an important part in preventing fertilization.

While the early terms 'chorion' and 'pro-chorion' expressed a certain mistaken belief in the probable functional significance of this albuminous covering of the rabbit ovum, until recently neither the true chemical nature nor the probable role played in the physiology of the developing egg by this coat has been the

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subject of a detailed study by modern histochemical methods. In fact, only Braden (1952) in his full investigation of the nature of the zona pellucida of the rat and rabbit eggs has made any observations on the composition and staining reactions of the 'albumen' layer of rabbit eggs.

The pioneer researches of Dalcq (1952 *a*, *b*, *c*) and Dalcq & Massart (1952) on the histochemistry of mammalian ova give details of the vital staining reactions of a number of rodent's eggs and blastocysts, but their interest is focused almost exclusively on the cytoplasmic constituents of the ovum, and consequently their findings will not come within the scope of the present paper, which is concerned mainly with the vital staining reactions of the albumen coat of the rabbit ovum.

MATERIAL AND METHODS

Ten animals were used in the experiment. The animals were killed at varying intervals, ranging from 20 hours to 5½ days, after mating. In 7 instances the aim was to obtain tubal ova, while in 3 cases blastocysts from the uterine horns were recovered. The ova and blastocysts were obtained by flushing either the uterine tubes or the uterine horns respectively with Ringer's fluid containing 0·01–0·05 per cent. toluidin blue. In the case of the tubal eggs the Fallopian tubes were straightened by dissecting away the mesosalpinx and the tubes were flushed from the uterine end towards the fimbriae. To obtain the uterine ova the horns were separated at the cervical junction and were flushed by inserting a cannula at the tubal end. The washings were collected in watchglasses. The ova and blastocysts either appeared in the washings already vitally stained or became stained in the first few minutes after collecting them. They were easily visible to the naked eye and any subsequent manipulation could be carried out without any additional visual aid. The majority of the ova were observed in Ringer's fluid in the living state and were photographed under the Leitz's Panphot on panchromatic plates and on Kodachrome films. Some tubal eggs and also some uterine blastocysts were fixed by adding Heidenhein's Susa fixative drop by drop to the Ringer's solution. Afterwards the eggs were embedded in agar using the technique described by Samuel (1944). In the agar the eggs were dehydrated in slow stages and carried into paraffin wax. Sections, after treating them with iodine-alcohol, were stained in 0·5 per cent. aqu. toluidin solution, dehydrated and enclosed in D.P.X.

DESCRIPTION

Even in the earliest specimens examined in the present series (20 hours after mating) the corona radiata cells were virtually absent, or if occasional clusters of them were left attached to the zona pellucida, they became buried under the gradually thickening albuminous coat. The ultimate fate of these corona radiata cells is still open to speculation. After vital staining the albuminous coat acquired

a distinct reddish colour (γ metachromasia) while the segmenting egg and the zona pellucida itself appeared blue (Plate, fig. 1). Careful observation under suitable lighting conditions revealed a delicate, concentric stratification of the albuminous coat, thus indicating its appositional growth. This stratification is even more obvious on sections of fixed and embedded specimens (Plate, fig. 2).

In many cases occasional villus-like processes could be observed, probably the result of an unequal deposition of the albuminous matter round the ovum while the ovum remained stationary for a short period in one of the culs-de-sac of the tube. It is conceivable that these villus-like processes are partly responsible for Barry's term 'chorion' applied to the albuminous coat.

The examination of the vitally stained specimens suggests that the albuminous matter is deposited by the tubal epithelium and is not a product of the ovum. Occasionally an albuminous coat is deposited on a small cluster of cumulus cells or around the fragments of detached tubal villi. Frequently smaller, and even larger, spherules of albuminous matter were observed without any central cellular core whatsoever (Plate, fig. 4).

Our vitally stained tubal eggs revealed an astonishing frequency of early prenatal death. In one instance 44 hours after mating 8 ova were recovered from the tubes. Only 1 of them appeared to be normal and later sectioning revealed that this ovum was at the 16-cell stage of cleavage. In the remaining 7 metachromatically stained spherules there was an intensive, diffuse basophil colouring reaching almost, but not quite, to the surface. There was no evidence of blastomeres (Plate, fig. 3). Examination of the ovaries revealed the presence of 8 fully formed corpora lutea. On the basis of this evidence one must assume that these ova have died and undergone disintegration at some stage of their development prior to the 16-cell stage.

In all of the specimens recovered from the uterine horns a gradual thinning of the albuminous coat was observed as the blastocysts enlarged. In these expanding blastocysts the more superficial layers of the albuminous coat still show intense metachromasia, but in the deep layers this is replaced by a distinct blue colour which reaches its greatest intensity at, or adjacent to, the zona pellucida. These staining characteristics are most marked in the more advanced blastocysts and these observations seem to indicate that the albuminous coat is undergoing a progressive physico-chemical change in consequence of which it is losing its metachromatic properties.

In a 5½ days' specimen five large blastocysts were recovered from one uterine horn; a corresponding number of corpora lutea were found in the ovary of the same side. In the other horn, which was dissected, only one blastocyst was found, although there were three corpora lutea in the corresponding ovary. This single blastocyst was attached to the antimesometrial side of the uterus and was fixed *in situ* for histological study. The other five blastocysts were vitally stained and revealed under the microscope an intact zona pellucida, but a complete absence of the albuminous coat.

DISCUSSION

'Except for certain notable investigations of ovarian dynamics, there has been no extensive inquiry into the physiology of living mammalian ova. . . . Experimentation has lagged presumably because of the difficulty of handling living ova' (Pincus, 1936). Since these words were written much has been achieved in this comparatively neglected field of embryology. Nevertheless the emphasis is still on the study of non-mammalian eggs and there is a tacit assumption that conditions which obtain in non-mammalian ova would also hold good for mammalian eggs.

The technique used for securing mammalian ova has changed very little since Cruikshank carried out his pioneer experiments in 1797, and, as already stated, until recently few histochemical methods have been used in the study of mammalian eggs. In the present investigation our interest has centred on the albuminous coat of the rabbit ovum, and for this purpose toluidin blue was the obvious vital dye to employ. After the vital staining of the albuminous coat the rabbit ova were, so to speak, enlarged and brought well within the limits of naked-eye visibility. Since these experiments were started some years ago, many other vital dyes have been used while searching for human and other mammalian eggs, and undoubtedly this procedure makes the finding and handling of these ova very much easier.

To base conclusions as to the true nature of the albuminous coat entirely on the results of toluidin blue staining may be open to criticism. Nevertheless, regarding metachromasia obtained after toluidin blue staining, Baker (1950) states: 'The reaction is so precise that it is a reliable histochemical test.' Pearse (1953), though admitting that 'there is a fair controversy about the specificity of metachromatic staining reaction obtained with toluidin blue', comes to the conclusion that 'strong gamma (red) metachromasia appears to indicate presence of acid mucopolysaccharides'. Consequently we feel that at the present stage of our study this simple method—applicable to the living ovum—is satisfactory.

According to Holmgren & Wilander (1937) and also Hess & Hollander (1947) the intensity of metachromasia after toluidin blue staining is a reliable indication of the degree of sulphuration of the molecule. Heparin, a poly-sulphuric acid ester of the polysaccharide group, is most strongly metachromatic, epithelial mucus and chondro-mucoid are both mono-sulphuric acid esters and are optical isomers. Epithelial mucus, however, is noticeably more metachromatic in its staining response to toluidin blue than is chondroitin sulphuric acid, the latter remaining unstained at the concentrations of toluidin blue we have employed in our vital staining experiments.

In view of the above considerations, and also because of the support received from the detailed chemical and histochemical studies of Braden (1952), there seems to be little doubt that the albuminous coat covering the tubal eggs of the rabbit contains considerable quantities of sulphurated mucopolysaccharides of

epithelial origin, and we agree with Braden that there is every justification for the plea that instead of 'albuminous' it should be designated as a 'mucous' or 'mucopolysaccharide' coat.

The enlargement of the blastocyst cavity was described by Assheton (1894), who came to the conclusion that during this period fluid collects in the blastocyst and that it is the increased hydrostatic pressure which is responsible for the enlargement. Heuser & Streeter (1929) are of the opinion that the formation of the segmentation cavity is the result of the precocious differentiation and functional activity of the trophoblast. Pincus (1936) observed that the formation and enlargement of the blastocyst cavity does not take place in rabbit ova cultured on artificial nutritive media, and he more or less postulated the necessity of a uterine factor in the initiation of the expansion. Gregory (1930) observed that while the mucous coat of the tubal egg is viscous and tends to adhere to the bottom of the dish and readily accumulates foreign particles, that surrounding the early uterine blastocyst is less viscous. He believes that the mucus gives up its water to the expanding ovum, or that it is digested and absorbed by the egg, which is probably aided in this process by the uterine secretions.

In our series the vitally stained young uterine blastocysts showed a gradual reduction in the thickness of the mucous coat around the expanding ovum. Furthermore, it was observed that in the deeper layers, adjoining the zona pellucida, there was a distinct loss of metachromasia. The resulting blue staining of this area would suggest an enzymatic hydrolysis and a gradual depolymerization of the mucous coat. The 'spreading response' which accompanies this process should and could provide the necessary hydrodynamic force for the expansion of the blastocyst. The hydrolysis starting in the deepest, juxta-ovular layer of the mucous coat seems to indicate that the enzyme is discharged by the ovum and its production is probably the function of the trophoblast, but on the other hand the observations of Pincus on cultured rabbit ova quoted above strongly suggest the precipitating, or initiating, influence of an additional and so far unknown uterine factor.

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EXPLANATION OF PLATE

FIG. 1. Morula of rabbit 20 hours after mating. Vitally stained with toluidin blue and photographed as a whole mount in Ringer's fluid. Kodachrome.

FIG. 2. The same morula fixed in Susa, embedded by the agar method in paraffin, sectioned and stained with toluidin blue. Kodachrome.

FIG. 3. Rabbit ova recovered from the uterine tube 44 hours after mating and vitally stained with toluidin blue. Observe that only one (at top of picture) is normal; in the others the diffuse basophilia indicates an early prenatal death. Kodachrome.

FIG. 4. Expanding blastocysts recovered from the uterine horns 86 hours after mating, and vitally stained with toluidin blue. Observe the gradual outward disappearance of the metachromasia of the albuminous coat. Kodachrome.

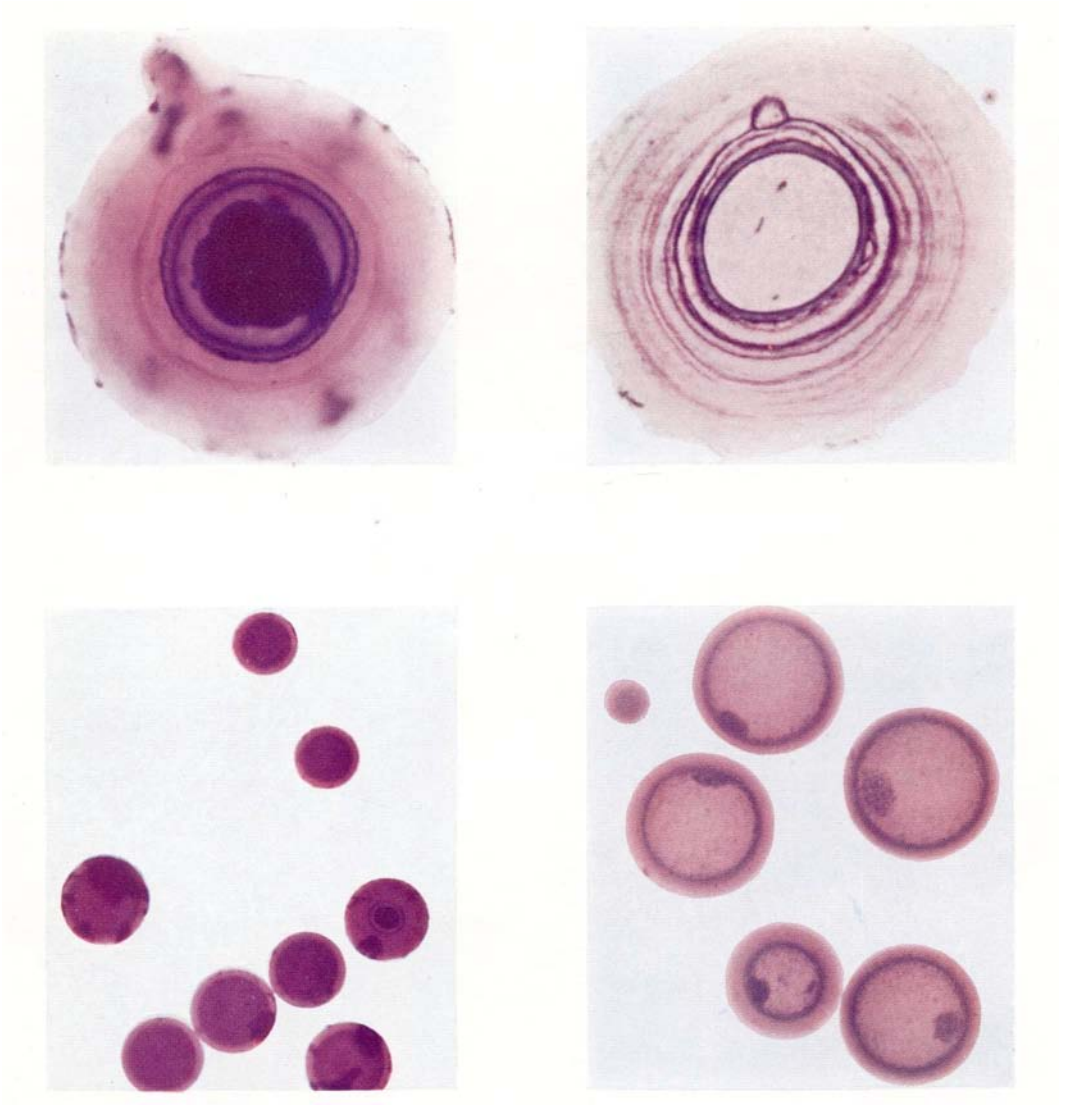

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Plate 1