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MUCINOGENESIS AND ENZYMOMORPHOLOGY OF RAT VAGINA;  
PHOSPHORYLASE,  $\beta$ -GLUCURONIDASE, DPNH-  
DIAPHORASE AND PHOSPHATASES

By

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A B S T R A C T

Vaginal epithelium of ovariectomized rats following the subcutaneous injection of oestrogenic hormone was analyzed by enzymomorphology and particular attention was given to mucin formation. Localization and intensity of enzymes examined in this study such as amylophosphorylase, amyo-1,4 → 1,6-transglucosidase, alkaline and acid phosphatases, 5-nucleotidase, esterase, DPNH-diaphorase, and  $\beta$ -glucuronidase varied depending on the stage of hormonal stimulation. Mucified cells contained the various enzymes examined, being especially rich in phosphorylase, alkaline phosphatase, 5-nucleotidase,  $\beta$ -glucuronidase and DPNH-diaphorase. These seemed to be related to the intracellular metabolism in the mucinous cells of epithelial layers. One of these, amylophosphorylase was demonstrated only in mucified cells within whole epithelial layers in mucified stage, and a close relationship between it and mucin formation was observed. The location of amyo-1,4 → 1,6-transglucosidase resembled the phosphorylase finding and suggests a similar physiologic function.

It is known that the vaginal epithelium changes from the squamous to the mucous type during pregnancy and pseudopregnancy and that mucification is produced by some hormones of follicular fluid or corpus luteum (Mayer & Allen 1933).

Also, it was previously reported that vaginal epithelium contained various

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enzymes such as  $\beta$ -glucuronidase, acid phosphatase, alkaline phosphatase, esterase and diphosphopyridine nucleotide (DPNH-) diaphorase, each showing a characteristic localization in the epithelial layers and stroma. Next, the action of gonadal hormones on  $\beta$ -glucuronidase activity in the basal germinal layer was noted in human (Fishman & Mitchell 1959) and rat (Hayashi & Fishman 1961) vagina. Succinic dehydrogenase and DPNH-diaphorase were also strongly active in vaginal epithelium during mucinogenesis in the ovariectomized rat following treatment with oestradiol and progesterone (Rosa & Velardo 1959). In view of these facts, mucinogenesis and the activity of certain enzymes in vaginal epithelium seem to be closely related with physiologic function as influenced by hormonal regulation.

Phosphorylase, the enzyme which catalyzes formation and splitting of 1,4-linkage of glucosidic bonds in polysaccharides was demonstrated in vaginal epithelium (Takeuchi *et al.* 1955), but the physiological significance was not clear. Accordingly, an investigation has been undertaken to relate the vaginal enzymmorphology of phosphorylase with a number of other enzymes and with the process of mucinogenesis. These experiments and the subsequent results and conclusions are the subject of the present communication.

#### MATERIALS AND METHODS

Three-month-old female rats of Wistar strain were ovariectomized and, beginning with the seventh day after operation, were injected subcutaneously 0.025  $\mu$ g of oestradiol- $17\beta$  once daily for three days. Vaginas were removed immediately, frozen on dry ice and then cut separately into three blocks. One provided a source of frozen sections of unfixed tissue, a second block was fixed in cold acetone and the third in chloral hydrate-formol solution after Fishman & Baker (1956).

Amylophosphorylase, amylo-1,4  $\rightarrow$  1,6-transglucosidase, alkaline and acid phosphatases, 5-nucleotidase, esterase, DPNH-diaphorase, and  $\beta$ -glucuronidase were the enzymes demonstrated histochemically in this study. Takeuchi's (1956) method in which alcohol was added to the Takeuchi & Kuriaki's (1955) original substrate mixture at  $p_H$  5.7 was used for demonstration of amylophosphorylase. Potassium glucose-1-phosphate was used as the substrate. In this method, carbohydrate synthesized from glucose-1-phosphate with amylophosphorylase was stained blue with iodine reaction and red purple with periodic acid Schiff's reagent following positive digestion by amylase or human saliva. The original substrate mixture without alcohol at  $p_H$  6.2 for demonstration of amylo-1,4  $\rightarrow$  1,6-transglucosidase was used according to Takeuchi's (1958) method. Carbohydrate synthesized from the substrate with amylo-1,4  $\rightarrow$  1,6-transglucosidase via amylophosphorylase was stained violet or red violet with iodine and purple with periodic acid Schiff's reagent. Frozen sections of vaginal tissue were utilized for these two methods. Alkaline glycerophosphatase was demonstrated by the calcium-silver method using Takamatsu's (1939) original substrate with magnesium chloride added (Takeuchi 1944). The method of Farber *et al.* (1956) for demonstration of DPNH-diaphorase and Fishman & Baker's (1956) technique for demonstration of  $\beta$ -glucuronidase were used with chloral hydrate formol fixed sections. For the demonstration of vaginal mucins, the periodic acid Schiff (PAS) reaction was employed

with frozen sections following salivary digestion. Alcian blue stain also was employed according to Steedman's (1950) technique.

Enzyme digestion tests on synthesized polysaccharides and on mucopolysaccharide in tissue sections were performed.  $\alpha$ -Amylase (Nutritional Biochemicals Co.),  $\beta$ -amylase (Nutritional Biochemicals Co.),  $\beta$ -glucuronidase (Ketodase, Warner-Chilcott Lab.) and testis hyaluronidase (Sigma Chemical Co.) were the enzyme reagents employed. Digestion tests by  $\alpha$ -amylase and  $\beta$ -amylase in tissue sections were performed at  $p_H$  5.5 and  $p_H$  5.0 respectively. For the  $\beta$ -glucuronidase digestion, the tissue sections were incubated for 2 to 24 hours at 37° C in a mixture of  $\beta$ -glucuronidase preparation (1 mg/ml) in 0.85 per cent saline solution adjusted to  $p_H$  5.2 by acetate buffer. Thereafter, they were stained with periodic acid Schiff's reagent. For hyaluronidase treatment, the sections were incubated for 3 to 24 hours at 37° C in a solution of testis hyaluronidase preparation (1 mg/ml) in 0.85 per cent saline. After washing the sections in distilled water, they were stained with periodic acid Schiff's reagent.

## RESULTS

The castrated rats examined following the administration of oestrogenic hormone did not always exhibit mucin in the superficial cell layer. In some animals the tissue had been transformed into a cornified epithelium. The present observations concern mainly vaginal epithelial structures containing mucus but also include a description of the cornified stage.

*Table 1.*

Localization and intensity of vaginal histochemical and enzymorphologic reactions.

Localization	Early Mucification			Mucification stage			Cornified stage		
	L	I	B	L	I	B	L	I	B
Enzyme Reactions									
PAS (Mucin)	++	—	—	+++	—	—	—	—	—
Alcian blue	+	—	—	++	—	—	—	—	—
Amylophosphorylase	++	—	—	+++	—	—	—	((+))	(+)
Amylo-1,4 → 1,6-trans-glucosidase	+	--	—	++	—	—	—	—	(+)
Alkaline phosphatase	+++	++	-(+)	+++	++	-(+)	-(+)	+	-(+)
Acid phosphatase	++	++	++	++	++	++	+++	++	++
5-Nucleotidase	+++	(+)	(+)-	+++	(+)	+—	—	+	(+)
Esterase	+	+	++	++	++	++	+++	+	(+)
DPNH-diaphorase	++	++	+++	+++	+++	+++	—	+	++
$\beta$ -Glucuronidase	++	+	+++	+++	++	+++	—	+	+++

L: Luminal layer; I: Intermediate layer; B: Basal layer; +++ Intense reaction; ++ Moderate reaction; + Weak reaction; (+) Very weak reaction; ((+)) Weakest reaction.

The following is an account of enzymorphology during mucinogenesis. Thus, in vaginal epithelium showing mucin formation in the luminal layer, it was noted that phosphorylase activity was intensely active only in mucinous cells while it was not visible in the other epithelial cells at all (Table 1, Fig. 2). The amylo- $1,4 \rightarrow 1,6$ -transglucosidase activity was evident to a lesser extent at the same location (Fig. 3). 5-Nucleotidase activity was much more intense in luminal mucified cells than in the other epithelial cells. This finding for 5-nucleotidase seemed to be very similar to that for amylophosphorylase except for the positive reaction of the former enzyme in capillaries of the superficial area in the mucous membrane (Table 1, Fig. 11). Although the  $\beta$ -glucuronidase, DPNH-diaphorase and alkaline phosphatase evidently were intense in the superficial epithelium at the mucification stage, they were also present in the other layers. The  $\beta$ -glucuronidase reaction appeared extremely strong in the basal layer (Fig. 6) while little or no alkaline phosphatase activity was observed in the basal layer (Table 1, Fig. 10). The esterase and acid phosphatase activities were present in the whole epithelium, but no specific correlation with mucinogenesis of the epithelium was found (Table 1, Fig. 12).

In the vaginal epithelium with cornification, high activity of acid phosphatase and esterase were recognized in the cornified layer (Table 1). No evident correlation of the other enzymes examined in this study with cornification was seen in any layers of vaginal epithelium. In this stage, little amylophosphorylase activity was seen in primitive cells in the lower intermediate layer (Table 1, Fig. 15).

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*Figs. 1-6.*

The vaginal epithelium exhibiting full mucification.

Serial sections of the tissue block removed from the same origin.

*Fig. 1.* Control picture for amylophosphorylase (Iodine reaction technique) in a frozen section. No reaction.

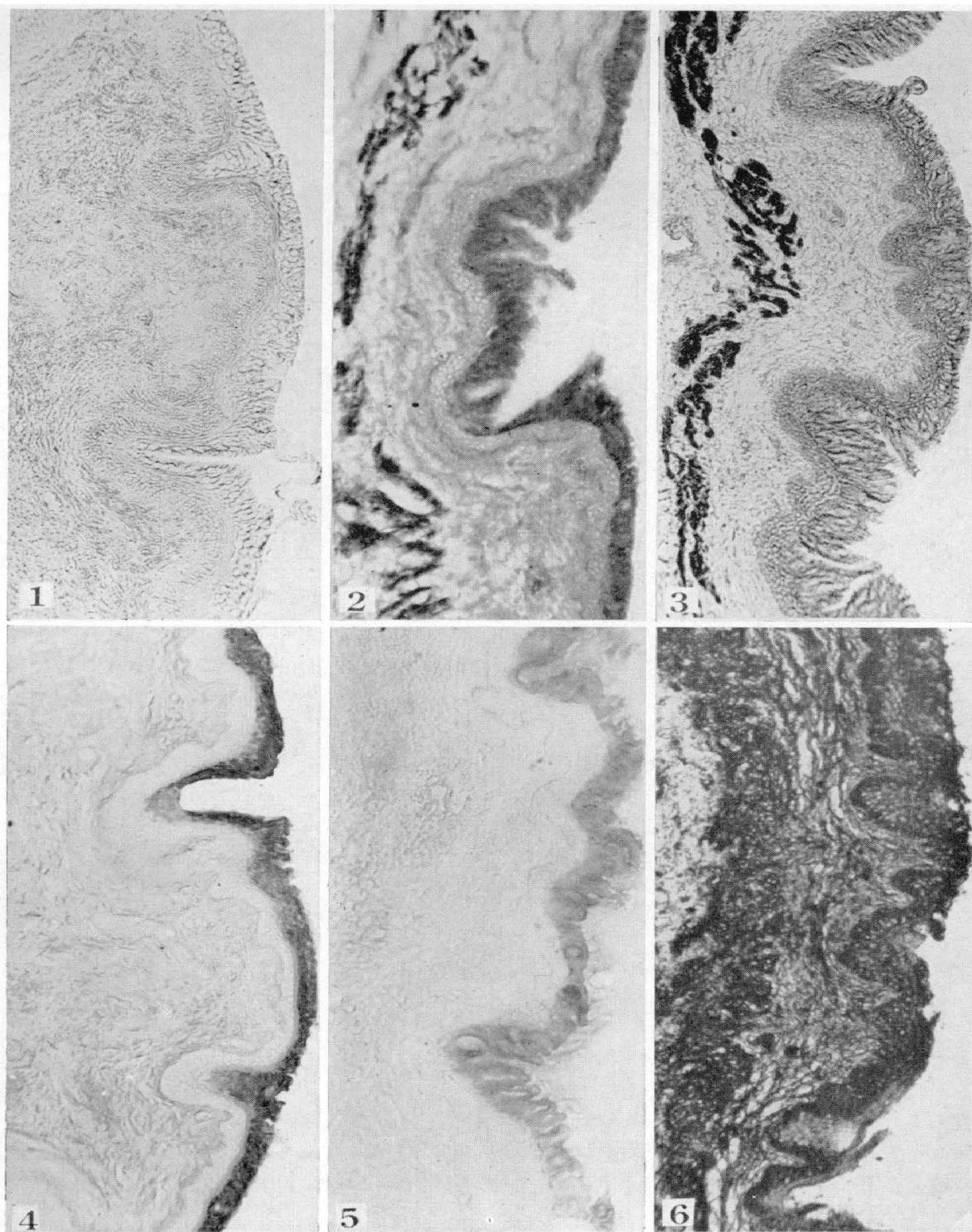
*Fig. 2.* Amylophosphorylase. Note marked activity in mucified epithelial cells and in smooth muscle fibers of muscle layer.

*Fig. 3.* Amylo- $1,4 \rightarrow 1,6$ -transglucosidase. A weak reaction (red purple colour in slide) is evident in mucified cells of luminal layer. The other epithelial cells in the intermediate and basal layers do not contain the enzyme, although the picture is made darker by yellow colour of the negative iodine reaction (black and white photographs give a false impression of the actual activity of the enzyme reaction). Smooth muscle fibers are much more reactive.

*Fig. 4.* Mucins (PAS reaction without counterstain). PAS-positive substance is shown only in mucified cells.

*Fig. 5.* Alcian blue stain without counterstain. PAS-positive substance in mucified cells stains moderately with alcian blue.

*Fig. 6.*  $\beta$ -Glucuronidase. All the cells of each layer in the vaginal mucous membrane show a positive reaction of varying intensity. Much more intense reaction is evident in the basal and mucified layers, particularly in basal cells.



The post-cornified or premucified epithelium devoid of mucin exhibited a thinner layer populated by a great number of polynuclear leucocytes showing intense reactions of some enzymes (Fig. 17), particularly of alkaline and acid phosphatases, 5-nucleotidase and phosphorylase. The weak reaction for amylo-phosphorylase was observed in epithelial cells of the intermediate layer (Table 1, Fig. 13).

There were two kinds of polysaccharides newly synthesized from glucose-1-phosphate by enzymes in rat vagina by use of the methods for demonstrating amylophosphorylase and amylo- $1,4 \rightarrow 1,6$ -transglucosidase. Thus, one seemed to be a linear 1,4-linked polysaccharide formed by amylophosphorylase which stained blue with iodine and was digested by  $\alpha$ -amylase,  $\beta$ -amylase and human saliva. The other one must be a branched polysaccharide synthesized from glucose-1-phosphate by the amylo- $1,4 \rightarrow 1,6$ -transglucosidase via amylophosphorylase, which stained red purple with iodine and was digested by  $\alpha$ -amylase and saliva but not completely by  $\beta$ -amylase (Table 2). These polysaccharides could be synthesized either in muscle fibers or in the vaginal epithelium, and differed histochemically from mucopolysaccharide in the epithelium. Mucopolysaccharide in the vaginal epithelium could not be digested with  $\alpha$ - and  $\beta$ -amylase or human saliva, could be stained with alcian blue but not with iodine solution (the dilute Gram's solution was used in this experiment), while polysaccharides synthesized histochemically from glucose-1-phosphate by the enzymes could be apparently digested with human saliva and  $\alpha$ - or  $\beta$ -amylase, stained with iodine, but not with alcian blue (Table 2). Both were able to stain purple red with PAS reaction.

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*Figs. 7-12.*

The vaginal epithelium with a low content of mucin at an early stage of mucification.

*Fig. 7.* PAS reaction. Epithelial cells in the superficial layers contain a small amount of mucin stained weakly or moderately with periodic acid Schiff's reagent.

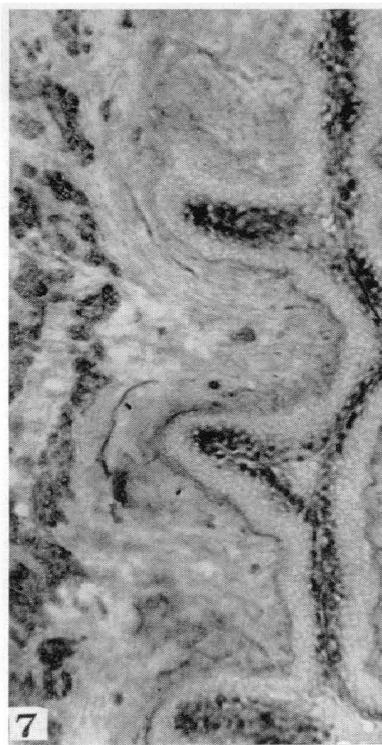
*Fig. 8.* Alcian blue stain. Alcian blue stain in the region of PAS-positive substance is weaker in the luminal layers which do contain a few moderately positive cells.

*Fig. 9.* Diphosphopyridine nucleotide diaphorase. Both mucosa and muscle layer are reactive, particularly the epithelium.

*Fig. 10.* Alkaline glycerophosphatase. The superficial layer, containing few mucified cells of the epithelium, the upper and middle-intermediate layers of the epithelium and the capillaries in the lamina propria show an intense reaction.

*Fig. 11.* 5-Nucleotidase. Much more intense reaction is exhibited by the superficial mucified layer. Capillaries are also positive. Smooth muscle fibers of the muscle layer are also moderately reactive (compare with Fig. 2, Fig. 7 and Fig. 10).

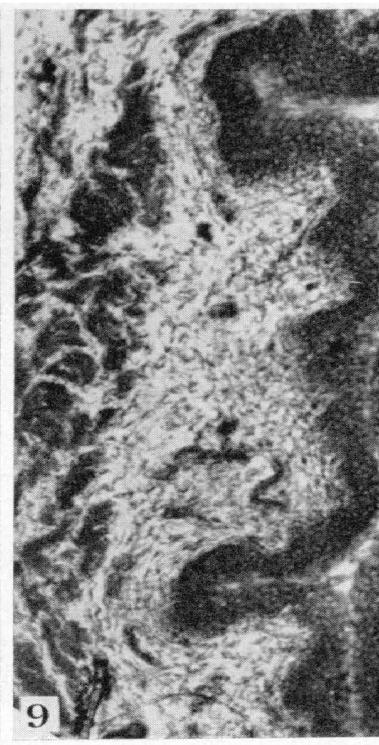
*Fig. 12.* Acid  $\alpha$ -naphthyl phosphatase. The activity is present in all layers of the epithelium. Slight diffusion can be seen in subepithelial connective tissue.



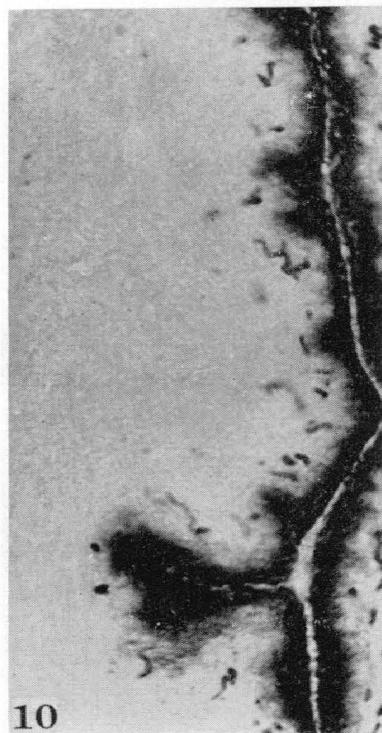
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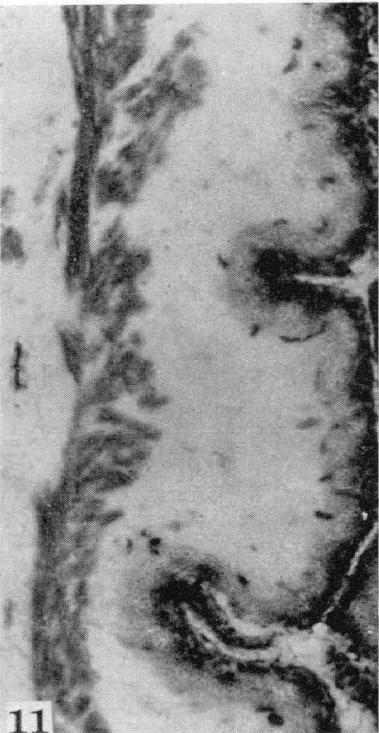
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9



10



11



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Table 2.

Test distinguishing between vaginal mucopolysaccharide and polysaccharide synthesized histochemically.

Histochemical test	PAS-stain	Iodine reaction	Alcian blue	$\alpha$ -Amylase	$\beta$ -Amylase	$\beta$ -Glucuronidase	Hyaluronidase
Polysaccharide							
Mucins in Epithelium	+++	—	++	—	—	—	—
1,4-Linked Polysaccharide	+++	+++	—	+	+	—	—
Branched Polysaccharide	+++	++	—	+	—	—	—

Figs. 13-18.

The vaginal epithelium at the cornified stage and afterwards.

Fig. 13. Amylophosphorylase (Iodine reaction technique). The primitive epithelium appearing after the cornified stage is very weakly reactive, giving a light blue with iodine reaction technique. Smooth muscle layer is rich in enzyme, showing a deep blue reaction with iodine.

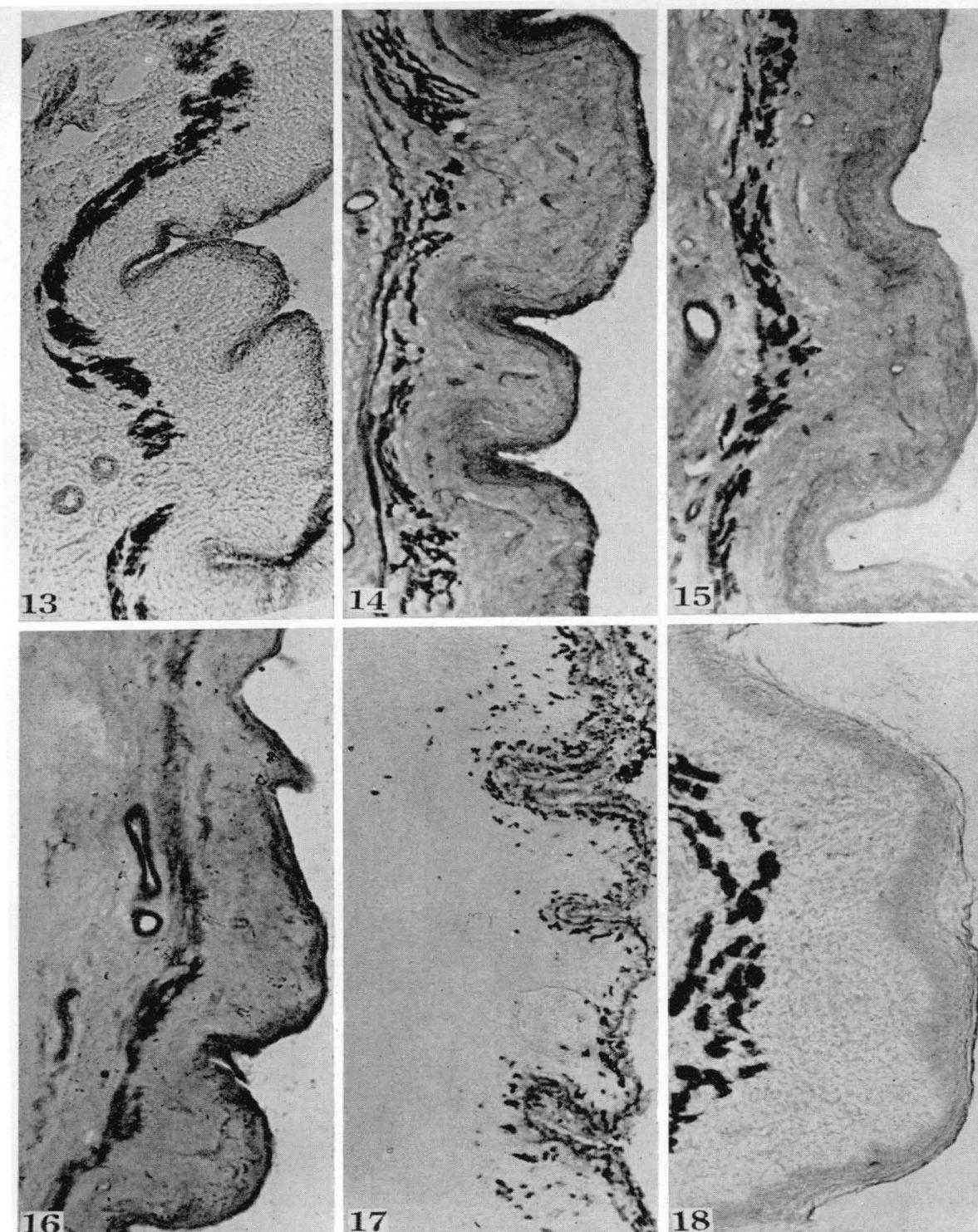
Fig. 14. Amylophosphorylase (PAS-stain technique). Moderate reaction appears in superficial layer with mucinogenous cells at the early stage of mucification. Smooth muscle layer is intensely reactive.

Fig. 15. Amylophosphorylase (PAS-stain technique). Very weak reactions showing fine granules of carbohydrate are present in basal and lower intermediate layers of the epithelium at the cornified stage.

Fig. 16. Amylophosphorylase (PAS-stain technique). A similar finding to Fig. 13 stained with iodine reaction.

Fig. 17. Alkaline glycerophosphatase. An intense reaction is exhibited by capillaries and leucocytes seen in early stage after cornification. Weak reaction is present in the epithelium, especially in the intermediate layer.

Fig. 18. Amylophosphorylase (Iodine reaction technique). Weak positive reaction seen in the PAS-stain technique in Fig. 15 is difficult to see in the black and white photograph, but the positive reaction in smooth muscle fibers is evident.



## DISCUSSION

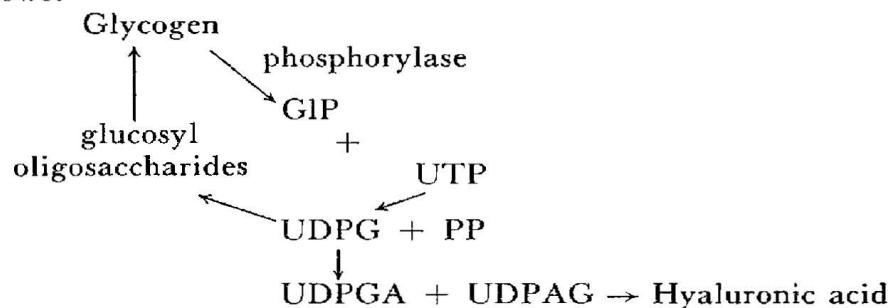
According to a current view, amylophosphorylase catalyzes, in general, the release and formation of 1,4-linkages in polysaccharide, especially in glycogen of animals. It is, however, unknown yet physiologically whether this enzyme functions only in relation to energy metabolism or for other unrelated physiological purposes. According to *Takeuchi et al.* (1955), the phosphorylase activity was demonstrated in skeletal and heart muscle fibers in various organs; liver cells, collecting tubules of kidney, sweat gland, nervous tissue, retina, cartilage cells, leucocytes, embryonal epithelial cells in various organs and so forth. In mature animals phosphorylase was richer in muscle fibers, liver cells, nervous tissue etc., while in embryonal stages it was also evidently higher in the epithelial cells such as epidermis, squamous epithelium and the other epithelium in organs. Secretory cells in various mature glands contain no or little phosphorylase enzyme activity. The physiologic significance of embryonal phosphorylase was thought to be different from that of phosphorylase in muscles, liver cells and nerve cells in the mature individual. Moreover, the physiologic significance of phosphorylase in nerve cells and cartilage cells must also be different from that of muscle and of liver phosphorylase, respectively. Thus, the physiological significances of phosphorylase in tissue cells seem to be connected to the specific function and development of each tissue (*Takeuchi & Kuriaki* 1955; *Takeuchi et al.* 1955; *Takeuchi* 1958; *Takeuchi* 1960).

With regard to oestrogen-vaginal enzyme relationships, it was previously found that  $\beta$ -glucuronidase, diphosphopyridine nucleotide diaphorase, alkaline and acid phosphatases and esterase enriched the vaginal epithelium and that the characteristic distribution of each enzyme in the epithelium was influenced by the hormonal environment (*Fishman* 1959; *Fishman & Mitchell* 1959; *Fishman* 1961; *Hayashi & Fishman* 1961). In the experiments by *Rosa & Velardo* (1959), the succinic dehydrogenase and DPNH-diaphorase systems tended to behave characteristically both in localization and intensity of reaction, depending on the type and dosage of hormone administered. Also, in general these systems, especially DPNH-diaphorase, were relatively intense in the vaginal epithelium in mucinogenous stage resulting from the administration of a minimal amount of oestrogen. However, in this case, the latter enzyme activity was demonstrated not only in the mucinogenous cells of luminal layer but also in the nonmucified cells of the intermediate and basal layers as seen in our experiment also.

It was already noted that rich phosphorylase activity was occasionally demonstrated in vaginal epithelium, especially in pregnancy and also in vagina of newborn (*Fujio et al.* 1956). In our experiments, it was found that the phosphorylase activity resulted from the influence of the hormonal administration and it appeared to be closely connected with mucification of vaginal

epithelium. Similar to the above finding also was the amylo-1,4 → 1,6-trans-glucosidase activity which was somewhat weaker.

It is possible to relate phosphorylase to mucopolysaccharide formation as follows:



Thus, it is probable that glycogen is the source of the glucose-1-phosphate (G1P) via phosphorylase. G1P plus uridine triphosphate (UTP) combine by the action of uridyl transferase to form one molecule of uridine diphosphate glucose (UDPG) and pyrophosphate (PP). The specific dehydrogenase for UDPG converts it to uridine diphosphate glucuronic acid (UDPGA) which together with uridine diphosphate N-acetyl glucosamine (UDPAG) represent precursors of hyaluronic acid. It is conceivable that the amount of glycogen present at any time could be small, but that it is being continually replenished as the demand for UDPGA for mucopolysaccharide synthesis increases.

#### A C K N O W L E D G E M E N T S

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