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Mucopolysaccharide histochemistry of the oviduct of the toad, *Bufo melanostictus*, before and during ovulation

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Summary. In both non-ovulating and ovulating toads of the species, *Bufo melanostictus*, the fimbrium stained only weakly for mucopolysaccharides (MPs) whereas the infundibulum stained strongly for neutral MPs and also for glycogen. In the non-ovulating toad, only neutral and sulphated MPs were detected in the goblet cells of the upper magnum, whereas sulphated, neutral and sialomucins were detected in the glands. In the middle magnum, sulphated and sialic acid-containing carboxylated MPs were detected in both the goblet cells and glands. In the lower magnum, neutral, sulphated, and sialic acid-containing MPs were detected in the goblet cells and only sulphated and sialic acid-containing MPs were detected in the glands. In the isthmus and ovisac, only sulphated MPs were present in the goblet cells. During ovulation, there was no change in the distribution of sulphated MPs throughout the oviduct. Sialic acid-containing MPs could not be detected in many of the goblet cells of the upper and lower magnum nor in most of the glands of the lower magnum.

Key words: Toad oviduct, *Bufo melanostictus*, Mucopolysaccharides

Introduction

Histochemical studies of mucopolysaccharides (MPs) in the amphibian oviduct have been carried out in *Triturus* (Kambara, 1956; Humphries and Hughes, 1959), some species of *Rana* (Lee, 1967; Shivers and James, 1970; Suvarnalatha et al., 1975) and *Bufo* (Low et al., 1976). These studies have shown not only variation in the mucopolysaccharide content of different regions of the oviduct (Kambara, 1956; Low et al., 1976) but also seasonal changes (Kambara, 1956). Variations in the type of MPs in different species have also been demonstrated (Humphries and Hughes, 1959; Lee, 1967; Pereda, 1969; Suvarnalatha et al., 1975).

The present paper describes the results of a comparative histochemical study of MPs in the oviduct of the ovulating and non-ovulating toad.

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Materials and methods

The sex and size of toads, assessment of the status of the ovaries and artificial induction of ovulation have been described previously (Tan and Chen, 1992). All toads were sacrificed by vascular perfusion through the cardiac ventricle with calcium acetate formalin (Lillie, 1965). Three toads were used for each experiment. The infundibulum (IF), upper magnum (UM), middle magnum (MM), lower magnum (LM) and ovisac (OV) were identified, as described by Tan and Chen (1992), and isolated. Except for the fimbrium (FI), which leads into the ostium of the oviduct, and the OV, each segment was further subdivided into three parts (labelled as IF1, IF2, IF3, etc); the isthums was divided into two parts. Each segment was then embedded in paraffin and 10 µm-thick sections were cut in the transverse plane.

1) Periodic acid-Schiff (PAS) reaction (McManus and Mowry, 1960)

This reaction was used to demonstrate the presence of carbohydrates, including glycogen. The paraffin sections were divided into two groups. After dewaxing and hydration, Group I sections were oxidised in 0.5% periodic acid followed by staining with Schiff's reagent. Group II sections were subjected to digestion with 1% aqueous solution of diastase (BDH Chemicals Ltd., England) for 45 minutes at 37 °C, after which they were washed and treated as described above with periodic acid and Schiff reagent. Periodate-reactive substances were coloured a bright magenta.

2) Best's carmine method (Best, 1906)

This is an empirical stain for glycogen. For this experiment, the sections were celloidinised and divided into 2 groups: those in Group I were stained in Harris' haematoxylin and the carmine staining solution followed by differentiation in Best's differentiator for 5-60 seconds. Sections in Group II were digested with

diastase after celloidinisation before they were stained. Glycogen granules were stained bright red.

3) Alcian Blue techniques

a) Alcian blue 8GX at pH 2.5 (Bancroft and Stevens, 1983). This dye was used to stain acid mucins. The technique described by Bancroft and Stevens (1983) was used.

b) Combined Alcian blue-PAS technique (Mowry, 1956). The sections were stained in alcian blue 8GX (pH 2.5) for 5 minutes and then with PAS (McManus and Mowry, 1960). Four types of colouration could be obtained: 1) magenta red (R); 2) red-blue (RB), where the magenta predominates over the blue colour of alcian blue; 3) blue-red (BR), where the blue predominates over the magenta of PAS; and 4) blue (B). This combined technique was used to distinguish between vic-glycol and acid groups. Acid mucins were stained bright turquoise blue (alcianophilia).

c) Combined high temperature methylation-saponification technique (Spicer and Lillie, 1959). This method was employed to distinguish between sulphated and carboxylated mucins. The sections were celloidinised and three replicates of each, labelled A, B and C, were treated as follows. Sections A and B were first treated with the methylation agent (99.2% methanol and 0.8% concentrated hydrochloric acid). Section A was subsequently treated with the saponification agent (70% alcohol and 1% potassium hydroxide) while section B was kept in 70% alcohol. Section C was used as the control and was initially incubated in water and then in 70% alcohol. At the end of the experiment, the sections were decelloidinised and were stained with alcian blue 8GX and counter-stained with 0.1% eosin. Absence of alcianophilia following methylation alone indicated the presence of sulphated and/or carboxylated MPs. If methylation was restored by saponification, then carboxylated acid MPs was present.

d) Sulphuric acid hydrolysis techniques (Lamb and Reid, 1969). This method identifies enzyme-labile and enzyme-resistant sialomucins. The sections were celloidinised and incubated in 0.1N sulphuric acid at 60 °C while control sections were heated at the same temperature and for the same period of time in distilled water. After decelloidinisation, the sections were stained with alcian blue and counterstained with eosin. Removal of alcianophilia by mild acid hydrolysis indicated the presence of sialomucins, i.e. sialic acid-containing carboxylated MPs.

Results

1) Fimbrium

In non-ovulating toads, the FI was only weakly positive for PAS; this was not affected by diastase digestion. It was negative for alcian blue. This indicated the presence of only small amounts of neutral MPs. In ovulating toads, it was negative for both PAS and alcian blue, indicating a depletion of the neutral MPs.

2) Infundibulum

Table 1 shows the distribution of MPs in the goblet cells in the three parts of the IF in ovulating and non-ovulating toads.

In non-ovulating toads, the epithelial cells were moderately PAS-positive and the intensity of staining was not reduced by diastase digestion. The luminal ends of some epithelial cells stained bright red with Best's carmine stain; none were stained after diastase digestion. The cells were not stained with alcian blue and in the combined alcian blue/PAS technique, they were stained magenta-red. The results indicated the presence of neutral mucins.

In ovulating toads, the cells in IF2 and IF3 were also periodate-reactive (Table 1) and were resistant to diastase digestion. Most of these cells were located at the bases and sides of the pits between adjacent epithelial folds. The cells in IF1, however, were not periodate-reactive. None of them stained with alcian blue.

Table 1. Distribution of MPs in the goblet cells in the infundibulum.

	IF1	IF2	IF3
PAS	2+(-)	2+(3+)	2+(3+)
PAS/diastase	2+(-)	2+(1+)	3+(2+)
Best carmine	1+(-)	1+(1+)	1+(1+)
Best carmine/diastase	-(-)	-(-)	-(-)
Alcian blue/PAS	R(-)	R(R)	R(R)
Alcian blue	-(-)	-(-)	-(-)
Alcian blue/methylation	-(-)	-(-)	-(-)
Alcian blue/methylation-saponification	-(-)	-(-)	-(-)
Control	-(-)	-(-)	-(-)
Alcian blue/acid hydrolysis	-(-)	-(-)	-(-)
Control	-(-)	-(-)	-(-)

-: negative; 1+: weakly positive; 2+: moderately positive; 3+: strongly positive; R: magenta-red; ( ): ovulating toad.

Fig. 1. LM of a non-ovulating toad. The goblet cells (arrow) and glands (G) are PAS positive. L= lumen. PAS stain. x 84

Fig. 2. UM of a non-ovulating toad. The goblet cells (arrow) and glands (G) are strongly alcianophilic. L= lumen. Alcian blue stain. x 200

Fig. 3. MM of a non-ovulating toad. Combined alcian blue/PAS technique. Arrow= goblet cell. G= gland. x 200

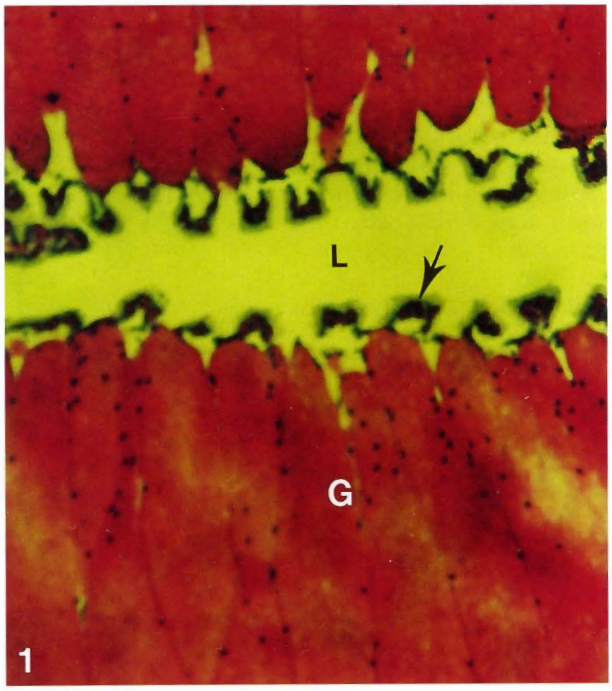
Fig. 4. UM of a non-ovulating toad showing moderate alcianophilia after saponification. Arrow= goblet cell. G= gland. x 200

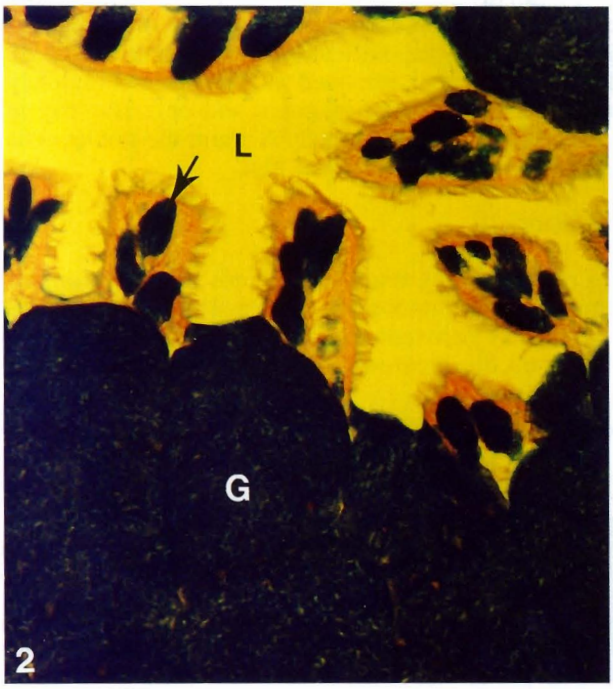
3) Magnum

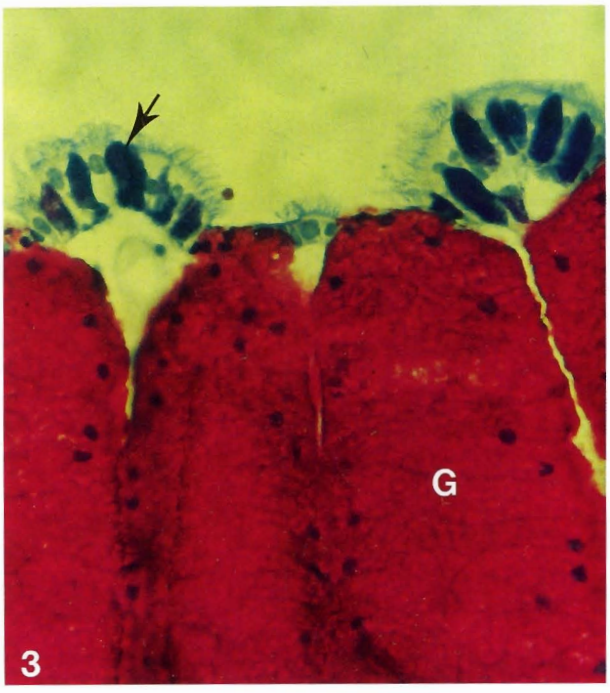
a) Goblet cells

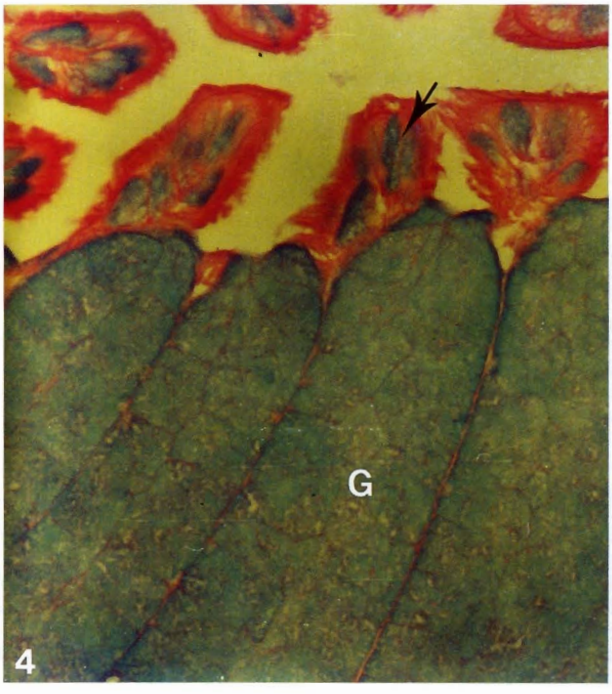
Table 2 shows the distribution of MPs in the goblet cells and glands of the UM, MM and LM of non-ovulating and ovulating toads.

In non-ovulating toads, the goblet cells of all regions of the magnum were strongly PAS-positive (Fig. 1) and were unaffected by diastase digestion. They stained weakly with Best's carmine stain, but were all unstained after diastase digestion. They showed alcianophilia (Fig. 2) which was removed by methylation, indicating the presence of sulphated and/or carboxylated MPs. With the combined alcian blue/PAS stain, there was a predominance of magenta-red staining in the UM and LM but predominance of blue in the MM (Fig. 3). In the MM and LM they showed moderate alcianophilia after saponification (Fig. 4) whereas those in the UM were only weakly alcianophilic, indicating the presence of









carboxylated acid MPs. After mild acid hydrolyxis, the alcianophilia in these cells was reduced in all regions of the magnum, indicating the presence of sialic acid-containing carboxylated MPs.

During ovulation, these cells were still periodate-reactive and diastase resistant and they stained weakly with Best's carmine, which was abolished after diastase digestion. The intensity of the blue staining with alcian blue, however, was weak in UM1, moderate in UM2, UM3 and MM1 and strong in the rest of the magnum (Fig. 3). Methylation abolished the alcianophilia in the goblet cells throughout the magnum. But restoration of alcianophilia after saponification was variable (Table 2). Mild acid hydrolysis removed alcianophilia completely in the cells of the upper magnum and only partially in the rest. With the alcian blue/PAS stain, the goblet cells of the MM were mainly stained blue.

b) Glands

In non-ovulating toads, the glands of all regions of the magnum were PAS-positive (Fig. 1) and the granules were diastase resistant. Staining with Best's carmine, which was unaffected by diastase digestion, was moderately intense only in the uppermost and lowermost parts of the magnum. Except for the uppermost part of the magnum, the glands in all other parts of the magnum showed alcianophilia (Fig. 2), which was removed by methylation but was restored by subsequent saponification (Fig. 4). The alcianophilia was also reduced slightly by mild acid hydrolysis in comparison with control sections. With the combined alcian blue/PAS stain, the glands of the LM were stained blue. In contrast, the upper regions of the magnum showed a predominance of magenta-red over blue staining (Fig. 4).

In ovulating toads, the glands of the magnum were all periodate-reactive and resistant to diastase and they stained a weak red with Best's carmine. Except for the uppermost region of the magnum, they were all alcianophilic (Fig. 3). The alcianophilia was reduced by methylation but was restored by saponification. With mild acid hydrolysis, the alcianophilia was markedly reduced.

3) Isthmus and ovisac

Table 3 shows the distribution of MPs in the goblet

Table 2. Distribution of MPs in the goblet cells and glands of the UM, MM and LM.

	UM1	UM2	UM3	MM1	MM2	MM3	LM1	LM2	LM3
PAS									
Goblet cells	3+(3+)	3+(3+)	3+(4+)	4+(4+)	4+(4+)	4+(4+)	4+(4+)	4+(4+)	4+(4+)
Glands	4+(3+)	3+(4+)	3+(4+)	3+(4+)	3+(4+)	3+(4+)	3+(4+)	3+(2+)	3+(2+)
PAS/diastase									
Goblet cells	3+(3+)	3+(3+)	4+(4+)	4+(4+)	4+(4+)	4+(4+)	4+(4+)	4+(4+)	4+(4+)
Glands	4+(3+)	3+(4+)	3+(4+)	3+(4+)	3+(3+)	3+(4+)	3+(3+)	3+(2+)	3+(2+)
Best carmine									
Goblet cells	1+(tr)	1+(tr)	1+(tr)	1+(tr)	1+(tr)	1+(1+)	1+(1+)	1+(1+)	1+(-)
Glands	2+(2+)	2+(1+)	tr(1+)	tr(1+)	tr(tr)	tr(1+)	tr(1+)	tr(1+)	2+(1+)
Best carmine/diastase									
Goblet cells	-(1+)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)
Glands	2+(1+)	2+(1+)	tr(1+)	tr1(+)	tr(1+)	tr(1+)	tr(1+)	tr(1+)	2+(1+)
Alcian blue/PAS									
Goblet cells	RB(BR)	RB(B)	RB(B)	B(B)	B(B)	R(B)	RB(B)	RB(B)	BR(B)
Glands	B(R)	BR(RB)	RB(RB)	RB(B)	B(B)	B(B)	B(B)	B(RB)	B(R)
Alcian blue									
Goblet cells	2+(1+)	3+(2+)	3+(2+)	3+(2+)	3+(3+)	3+(3+)	3+(3+)	3+(3+)	3+(3+)
Glands	-(-)	2+(2+)	2+(2+)	2+(2+)	2+(2+)	2+(2+)	2+(2+)	2+(2+)	2+(1+)
Alcian blue/methylation									
Goblet cells	-(-)	-(-)	-(-)	-(-)	1+(-)	1+(-)	tr(-)	-(-)	-(-)
Glands	-(-)	-(1+)	-(tr)	-(1+)	1+(tr)	1+(1+)	tr(1+)	tr(tr)	1+(1+)
Alcian blue/methylation-saponification									
Goblet cells	tr(-)	tr(tr)	tr(-)	1+(tr)	2+(tr)	3+(2+)	2+(-)	2+(tr)	3+(2+)
Glands	-(-)	2+(2+)	2+(2+)	2+(3+)	2+(2+)	3+(3+)	2+(2+)	2+(2+)	2+(2+)
Control									
Goblet cells	3+(1+)	4+(2+)	4+(3+)	4+(3+)	4+(3+)	4+(4+)	4+(3+)	4+(4+)	4+(4+)
Glands	-(-)	3+(3+)	4+(3+)	4+(3+)	4+(4+)	4+(4+)	4+(4+)	4+(4+)	4+(3+)
Alcian blue/acid hydrolisis									
Goblet cells	1+(-)	2+(-)	1+(-)	2+(1+)	2+(1+)	3+(2+)	3+(2+)	2+(3+)	2+(2+)
Glands	-(-)	1+(1+)	2+(2+)	2+(2+)	2+(2+)	2+(2+)	2+(3+)	2+(2+)	2+(-)
Control									
Goblet cells	2+(2+)	3+(2+)	4+(3+)	4+(3+)	4+(4+)	4+(4+)	4+(3+)	4+(4+)	4+(4+)
Glands	-(-)	3+(3+)	4+(3+)	3+(4+)	4+(4+)	4+(4+)	3+(3+)	3+(3+)	4+(1+)

-: negative; tr: trace; 1+: weakly positive; 2+: moderately positive; 3+: strongly positve; 4+: very srongly positive; R: magenta-red; B: blue; BR: blue predominance over magenta-red; RB: magenta-red predominance over blue; ( ): ovulating toads.

cells of the isthmus and OV were weakly periodate-reactive and resistant to diastase digestion. They were alcianophilic; the alcianophilia was abolished with methylation but was weakly restored after saponification. Mild acid hydrolysis had little effect on the alcianophilia. With the combined alcian blue/PAS staining, all the secretory cells were stained blue.

In ovulating toads, the epithelial secretory cells of the isthmus were weakly periodate-reactive while those of the first two-thirds of the OV were moderately so. In both regions, the reaction was diastase resistant. Staining with Best's carmine was very weakly positive and this was abolished by diastase digestion. All were moderately alcianophilic; the alcianophilia was dramatically reduced by methylation but was slightly restored by saponification. Acid hydrolysis did not reduce the alcianophilia. These cells thus contained sulphated acid MPs, and perhaps traces of carboxylated acid MPs. The results also indicated the absence of sialomucins.

Table 3. Distribution of MPs in the goblet cells of the isthmus and OV.

	IS1	IS2	OV1	OV2	OV3
PAS	1+(1+)	1+(1+)	1+(2+)	1+(2+)	1+(3+)
PAS/diastase	1+(1+)	1+(1+)	1+(2+)	1+(2+)	1+(3+)
Best carmine	1+(tr)	1+(tr)	1+(tr)	1+(tr)	2+(-)
Best carmine/ diastase	-(-)	-(-)	-(-)	-(-)	-(-)
Alcian blue/PAS	B(B)	B(B)	B(B)	B(B)	B(BR)
Alcian blue	3+(3+)	3+(2+)	3+(2+)	3+(2+)	3+(3+)
Alcian blue/methylation	-(tr)	-(tr)	-(tr)	-(tr)	-(tr)
Alcian blue/methylation-saponification	1+(1+)	1+(1+)	1+(1+)	2+(1+)	1+(2+)
Control	4+(3+)	4+(3+)	4+(3+)	4+(3+)	4+(2+)
Alcian blue/acid hydrolysis	3+(3+)	3+(3+)	3+(3+)	4+(3+)	3+(2+)
Control	3+(3+)	3+(3+)	3+(3+)	4+(2+)	3+(2+)

-: negative; tr: trace; 1+: weakly positive; 2+: moderately positive; 3+: strongly positive; 4+: very strongly positive; B: blue; BR: predominance of blue over magenta-red; ( ): ovulating toads.

4) Ciliated epithelial cells

In non-ovulating toads, though these cells were non-secretory, in all regions of the oviduct they often contained bright red glycogen granules which were stained by Best's carmine at their luminal ends.

During ovulation, few of these cells appeared to show the presence of glycogen granules with Best's carmine when compared to the non-ovulatory oviduct.

5) Distribution of MPs in the glands and goblet cells

Table 4 summarises the distribution of MPs in the different regions of the oviduct before and during ovulation.

Discussion

Ciliated cell

In a previous study of the oviduct of the toad, *Bufo melanostictus*, the ciliated cells bearing long cilia which were densely crowded together on the luminal surface of the cell were described (Tan and Chen, 1992). Since the oviduct has only a very thin layer of smooth muscle, it was suggested that ciliary action might play an important role in the propulsion of the eggs down the tract during ovulation (Low et al., 1976; Tan et al., 1992). The present histochemical study has demonstrated the presence of glycogen in the ciliated cells of non-ovulating toads. In ovulating toads, glycogen was not detected in these cells. This suggests that glycogen could be depleted in these cells during ovulation and may be related to the activity of the cilia. In human fallopian tube, Fredricsson (1959) has shown not only an increase in ciliary activity during ovulation, but also a decrease in

Table 4. Summary of the distribution of MPs in the magnum, isthmus and OV.

REGIONS	GOBLET CELLS			GLANDS		
	Sulphated MPs	Sialic acid-containing MPs	Neutral MPs	Sulphated MPs	Sialic acid-containing MPs	Neutral MPs
UM1	+(+)	+(-)	-(+)	-(-)	-(-)	-(-)
UM2	+(+)	-(+)	+(+)	+(+)	+(+)	+(+)
UM3	+(+)	-(-)	+(-)	+(+)	+(+)	+(+)
MM1	+(+)	-(+)	-(-)	+(+)	+(+)	+(-)
MM2	+(+)	+(-)	-(-)	+(+)	+(+)	-(-)
MM3	+(+)	+(+)	-(-)	+(+)	+(+)	-(-)
LM1	+(+)	+(-)	+(-)	+(+)	+(-)	-(-)
LM2	+(+)	+(-)	+(-)	+(+)	+(+)	-(-)
LM3	+(+)	+(+)	+(-)	+(+)	+(+)	-(-)
IS1	+(+)	-(-)	-(-)			
IS2	+(+)	-(-)	-(-)			
OV1	+(+)	-(-)	-(-)			
OV2	+(+)	-(-)	-(-)			
OV3	+(+)	-(-)	-(-)			

+: MPs present; -: MPs absent; ( ): denotes presence or absence of MPs in ovulating toads.

glycogen.

Goblet cells and glands

The oviducts of both non-ovulating and ovulating toads are rich in MPs throughout their entire length except for the FI, which contains only traces of MPs. The magnum contains the most carbohydrates while the proximal IF contains less and the isthmus and OV the least. All the carbohydrates are diastase resistant and are therefore not glycogen. Some differences were noted in the distribution of these MPs during ovulation.

In both the non-ovulating and ovulating toads, the infundibulum has been found to secrete neutral MPs. Most of the cells which secrete the MPs are located at the bases of the epithelial folds. It has been suggested that the cells at the bases of such folds in the IF of *Xenopus laevis* and *Bufo bufo japonicus* secrete a substance which causes a structural transformation of the vitelline coat, to assist in sperm penetration and fertilization (Grey et al., 1977; Katagiri et al., 1982). From the results of the present study, it may be suggested that such substances might be neutral MPs.

In the magnum of both the non-ovulating and ovulating toads, regional differences were noted in the distribution of neutral and acid (sulphated and carboxylated) MPs. In the goblet cells of non-ovulating toads, sulphated MPs were found in all regions of the magnum, neutral MPs only in the UM and LM and sialic acid-containing carboxylated MPs mainly in the MM and LM. This contrasts with the glands in which sulphated and sialic acid-containing carboxylated MPs were found throughout the magnum, except for its uppermost part, and neutral MPs were confined only to the UM. In the ovulating toad, there was no change in the distribution of sulphated MPs. However, neutral and sialic acid-containing carboxylated MPs could not be detected in either the goblet cells or glands of the LM,

Little attention has been focused on the isthmus and OV in other studies of the anuran oviduct. The present study has shown that sulphated MPs are present in the isthmus and OV of both non-ovulating and ovulating toads. In a previous scanning electron microscopic study of the oviduct of *Bufo melanostictus*, Tan and Chen (1992) have shown that the surface of the isthmus and OV are covered with an amorphous substance during ovulation. The function of this substance is not known. From the results of the present histochemical study, it may be suggested that this amorphous substance is sulphated MP. The possibility that sulphated MPs even at such a distal part of the oviduct may have an effect on the fertilizability or hatchability of the eggs cannot be totally ruled out as it has been reported that eggs taken from these regions in *Bufo melanostictus* had a higher fertilizability and hatchability than anywhere else along the whole oviduct (Low et al., 1976).

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