

# イヌ肥満細胞プロテアーゼの分離・精製と 測定法の開発

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## 研究成果報告書

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研究代表者 堀井 洋一郎  
(宮崎大学農学部教授)

## はしがき

アレルギー反応、特に即時型アレルギー反応における肥満細胞の果たす役割の重要性についてはヒトを含む多くの動物において、すでに良く知られている。最近、イヌのアレルギー疾患が増加傾向にあり、小動物臨床においてその重要性が増している。また、イヌでは肥満細胞腫の発生が他の動物に比較して多いこともよく知られている。しかし、イヌにおける肥満細胞の分布やその本来の生理的意義や機能については、まだあまり良く知られていないのが現状である。このことから、イヌにおける肥満細胞関与の疾病的理解や治療に、さまざまな混乱や困難が生じていることは想像に難くない。我々の予備的調査によれば、正常犬の組織中にも非常に多くの肥満細胞が存在しており、特に消化管粘膜には他の動物では見られないほど多くの肥満細胞が分布していることがわかった。この結果はイヌにおいて肥満細胞に起因する疾病が他の動物に比較して多く見られることの一因となっている可能性を示唆するものと考えられる。

肥満細胞はセリンプロテアーゼ（キマーゼ、トリプターゼ）を豊富に有しており、多くの動物で、他の組織や細胞中のものとは性状が異なる肥満細胞特異的なプロテアーゼが見つかっている。したがって、本研究では予備調査に基づき、正常犬の全身の組織中における肥満細胞の分布と、その性状を詳細に調べ、特にキマーゼやトリプターゼの存在を確かめた。次に肥満細胞が非常に多く分布する消化管粘膜を手始めの材料とし、肥満細胞固有のプロテアーゼの分離・精製を試みた。

イヌの肥満細胞は全身の臓器に観察され、得に腸管粘膜では1平方mmあたり800～1000個の肥満細胞がみられた。正常動物の消化管粘膜にこれほど多くの肥満細胞が観察された報告はみあたらず、イヌは他の動物に比較して肥満細胞の豊富な動物であるといえる。イヌでは他の動物に比較して肥満細胞腫の発生が多いが、肥満細胞の絶対数が多いことと何らかの関連がある可能性が考えられる。また肝臓にも多くの肥満細胞の分布が見られたが、これもマウス、ラット、ウサギなどの実験動物やヒトにおける分布と明らかに異なっている。これらの肥満細胞の組織化学的性状は、プロテオグリカンの染色性に及ぼすホルマリンや電解質濃度の影響の度合いから、他の動物と同様に結合組織型と粘膜型の大きな区分はあるものと考えられる。またそれらの肥満細胞におけるプロテアーゼの分布をみるために、特異基質を用いて *in situ* で酵素活性を調べたところ、全ての肥満細胞でトリプターゼ、キマーゼいずれの活性も認められた。

陽イオン交換クロマトグラフィーを用いて、消化管粘膜の肥満細胞からのキマーゼ及びトリプターゼの分離精製を試みたが、現時点では収量が少なく酵素の特異性を検討するまでに至らなかった。精製過程の検証のために行ったマウス肥満細胞由来のMMC P-1の精製は非常にうまくいき、特異性の高い抗体の作成に成功した。この抗体はイヌの肥満細胞プロテアーゼには反応せず、Mus属ネズミの肥満細胞プロテアーゼとのみ反応した。

本研究の最終目標は、このプロテアーゼに対する抗体を得て、免疫組織化学的手法による組織内肥満細胞の分布や性状の検索、また病理組織標本中の肥満細胞の診断・検索に、さらにELISA法に応用して血液中や組織中のプロテアーゼ量を測定することであったが、今回そこまで到達できなかった。しかし、イヌにおける肥満細胞の基本的な性状や全身における分布が詳細に検討されたことにより、本研究は小動物臨床、特にイヌのアレルギー疾患の理解に貢献できたと考える。

## 研究組織

研究代表者：堀井洋一郎（宮崎大学農学部教授）

研究分担者：牧村 進（宮崎大学農学部教授）

## 研究経費

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## 研究発表

### (1) 学会誌等

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2. NOVIANA, D., TIURIA, R., WIDODO, S. & HORII, Y. : Distribution and histochemical characterizations of dog's mast cells. *Media Veteriner*, 5, 21-22, 1998.

## 研究成果

### I. INTRODUCTION

Derived from pluripotent hemopoetic stem cells and their phenotype is bidirectionally changeable under the regulation of "micro environment" (Macy, 1986) mast cells occur in all vertebrate classes from fishes to mammals, but wide variation exist in their distribution, numbers, and intracellular constituent (Macy, 1986 and Madewell *et al.*, 1985). However, not been proven to derived from a common stem cell in either animals or human (Denburg, 1992).

They are classified into two subtypes, mucosal type (MMC) and connective tissue type (CTMC), based on their histochemical properties (Enerback, 1986), reactivity to secretagogues (Shanahan *et al.*, 1985), type of granule protease's (Miller *et al.*, 1989), and also of their growth factor dependency (Smith and Weis, 1996 and Hamaguchi *et al.*, 1987). MMC are widely distributed in the gastrointestinal and respiratory mucosa, and CTMC are commonly seen in the skin and tongue (Bienenstock *et al.*, 1985) their distribution varies among species and sites. Their primary function appears to concern defense mechanism, particularly the induction of acute inflammatory reactions and participation in immune responses (Galli, 1993). The subsequent response to antigen or other micro environmental changes can be modified or influenced by the behavior of the enteric nervous system, and ultimately by the central nervous

system (Hamaguchi *et al.*, 1987). It is well known that dog mast cells contain an impressive array of physiologically active component (Mc Kay and Bienenstock, 1994).

Here we further describe the distribution and histochemical characterizations of mast cells in various sites including gastrointestinal of dogs. The result show, that mast cells were observed in the whole organ that examined and the number were varied among their sites.

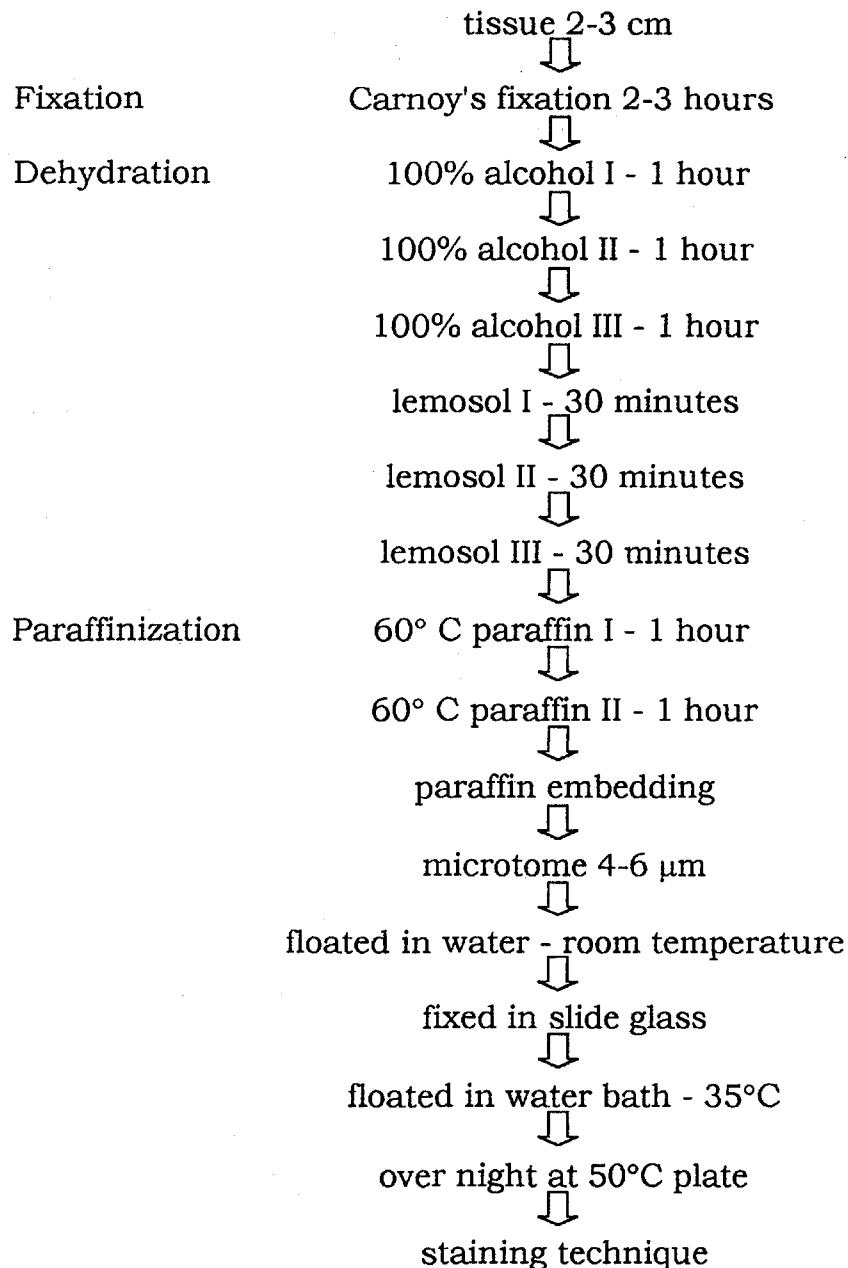
## **II. MATERIALS AND METHODS**

### **II.1. Animals**

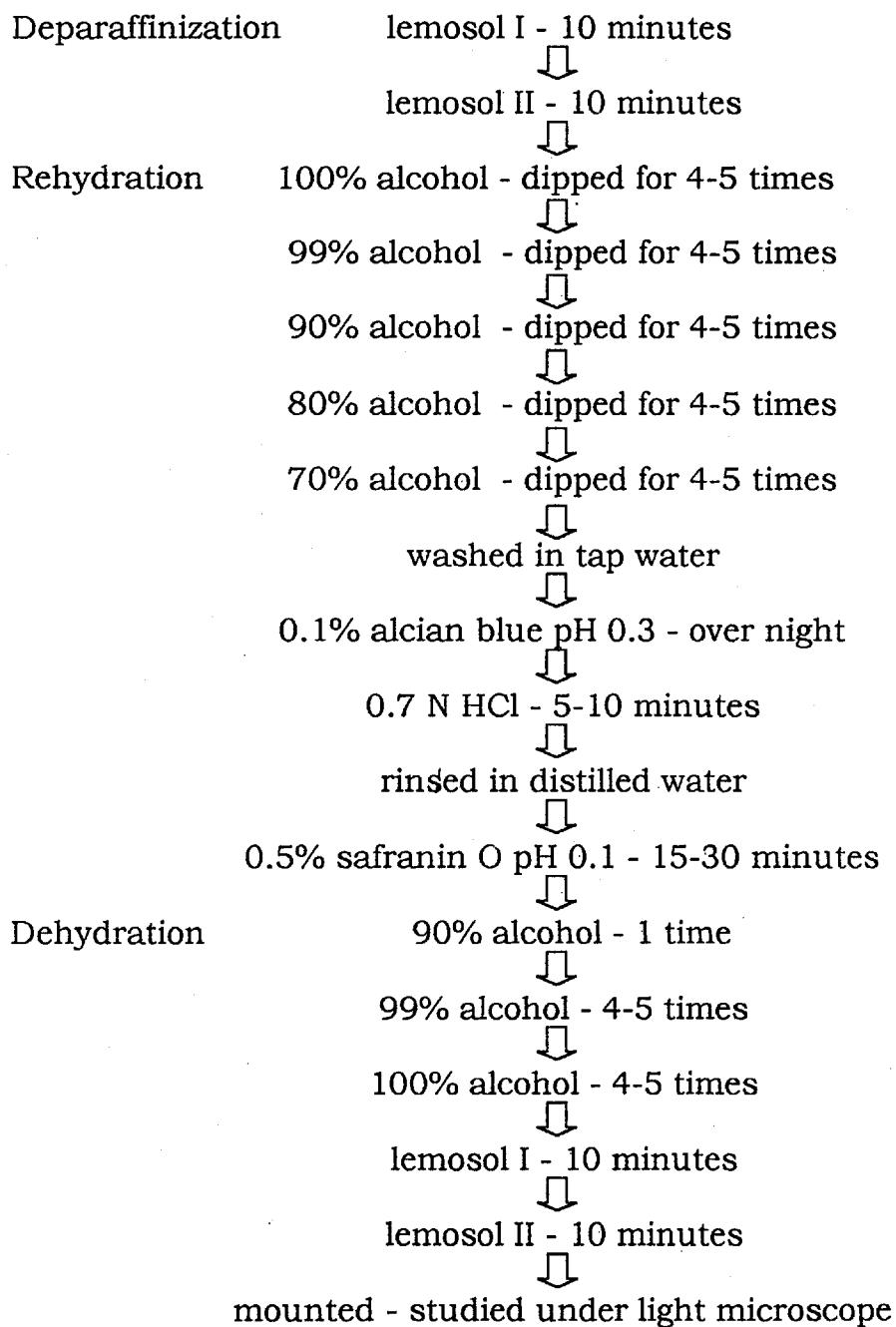
All animals used in this study were clinically normal adult dogs (2 male and 1 female). The organs that observed were ear (skin), tongue, lung, heart, lymphoglandula bronchiole, lymphoglandula mesentery, spleen, kidney, peritoneum, liver, stomach, duodenum, jejunum, ileum, caecum, colon and rectum.

In order to served animals as controls, 7 weeks old Wistar rats were infected by subcutaneous inoculation with 25.000 third-stage infective larvae ( $L_3$ ) of *Strongyloides venezuelensis* and duodenum examined 3 weeks after infection.

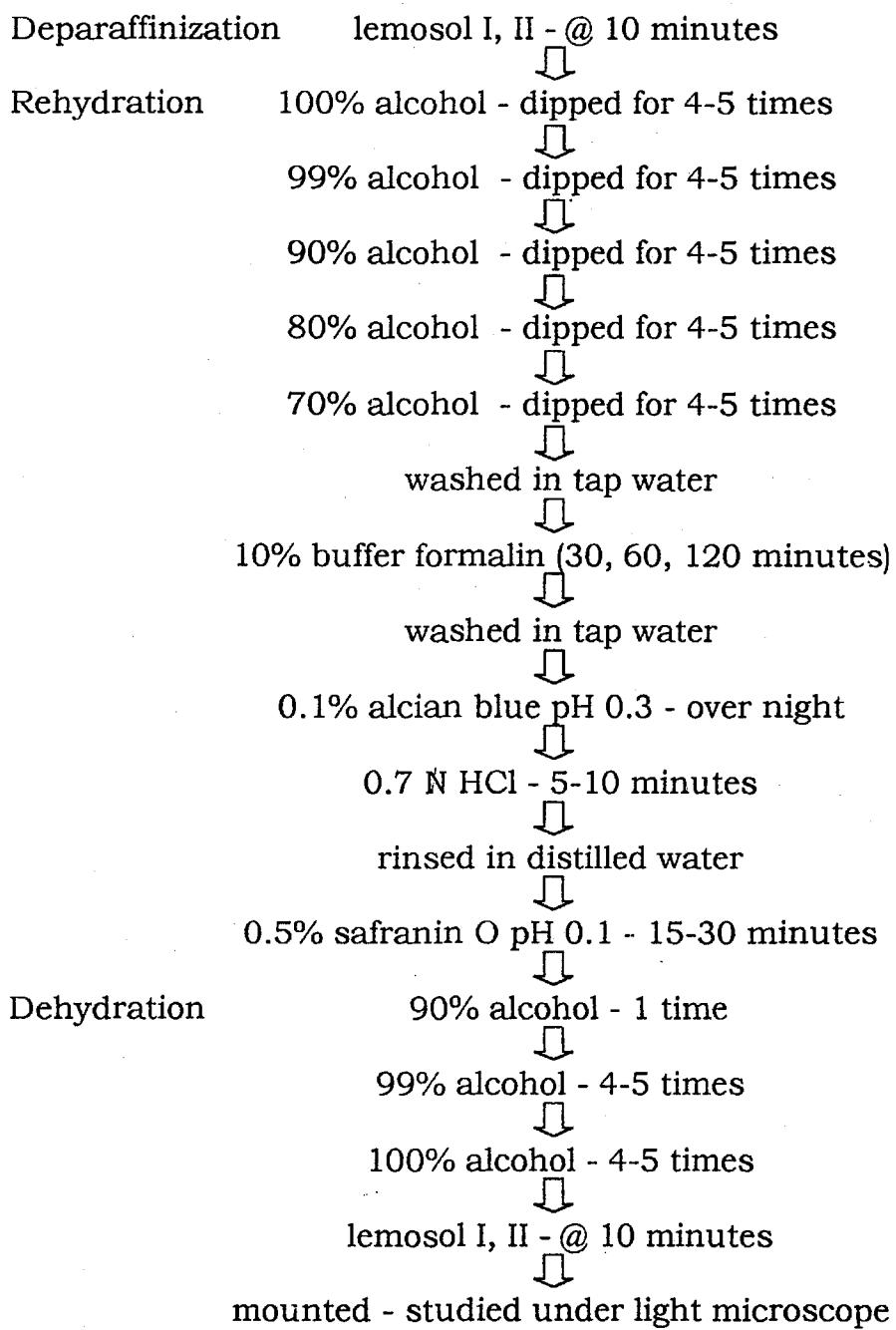
## II.2. Tissue Processing



**II.3. Staining Process for Distribution of Mast Cells (Nawa *et al*, 1994)**

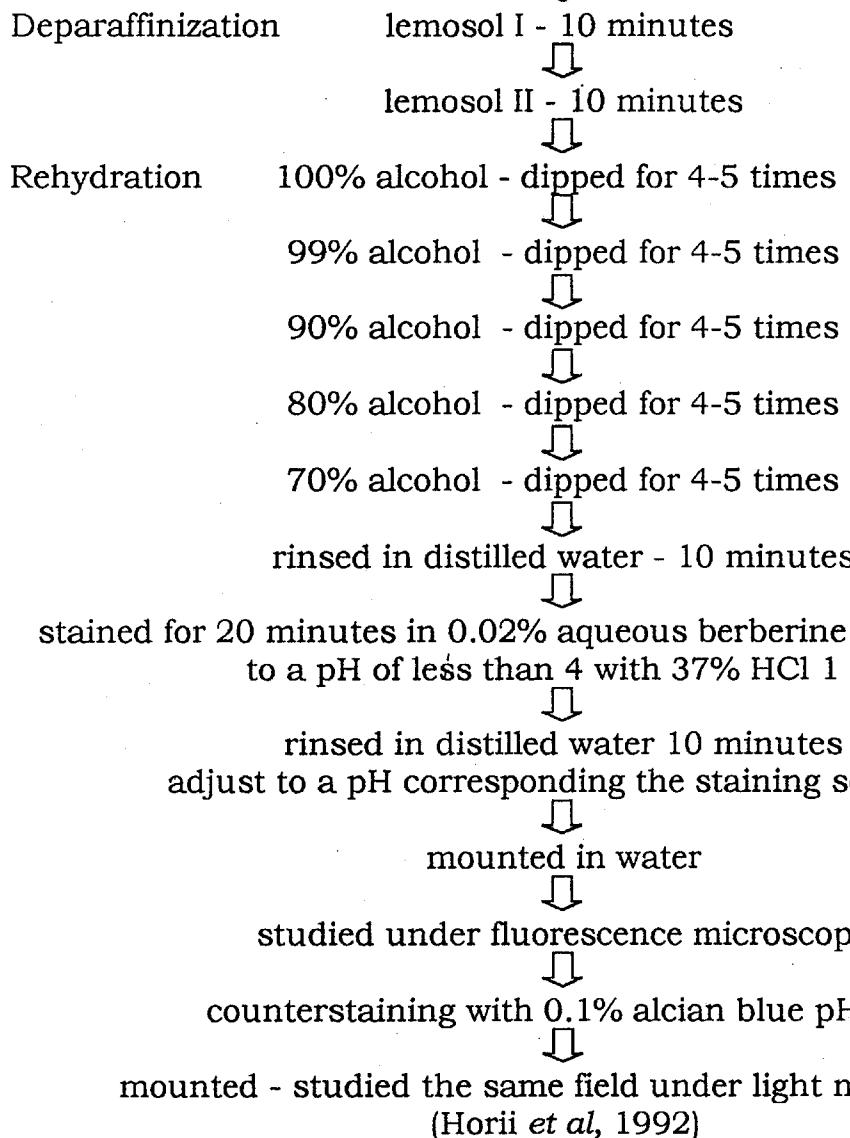


**II.4. Staining Process for Mast Cells Stainability Against Formalin Fixation (Nawa *et al*, 1994)**



**II.5. Berberine Sulfate Staining Technique to Confirm the Presence of Heparin (Enerback, 1974)**

slide glass from tongue, liver, jejunum, colon from dog  
and duodenum from rat



**II.6. Critical Electrolyte Concentration (CEC) Staining Technique to Define Glycosaminoglycans In Situ (Scott and Dorling, 1965)**

6  $\mu$ m serial sectioned tongue, liver, jejunum, colon from dog and duodenum from rat

Deparaffinization      lemosol I, II - @ 10 minutes

Rehydration      100% alcohol - dipped for 4-5 times

                        99% alcohol - dipped for 4-5 times

                        90% alcohol - dipped for 4-5 times

                        80% alcohol - dipped for 4-5 times

                        70% alcohol - dipped for 4-5 times

washed in tap water

0.05% alcian blue in 0.005 M buffer acetate contain various concentration of MgCl<sub>2</sub>

rinsed in distilled water

Dehydration      90% alcohol - 1 time

                        99% alcohol - 4-5 times

                        100% alcohol - 4-5 times

lemosol I, II - @ 10 minutes

mounted- studied under light microscope

The CEC was defined as the electrolyte concentration at which the staining of 50 % of mast cells was extinguished.

### **III. RESULTS**

Mast cells in different parts of the body appear to have different morphologic, biochemical and functional characteristic (Rogers, 1996). As shown in Table 1, mast cells were distributed in the whole organ that examined and the number were varied among their sites. It stained with alcian blue and safranin O had blue granules against a pale red background (Strobel and Miller, 1981). They distributed throughout connective tissue that consist of collagen, elastic and reticular fibers, adjacent to blood or lymphatic vessels (Figure 1a). There were no significant decreased after fixed in buffered formalin in comparison with section that fixed in Carnoy's fluid (Figure 1b). Even for some organs (tongue, heart, kidney, liver, and spleen) seen the constant number of mast cells. Alcian blue and safranin O stainability of mast cells against formalin fixation in ear of dogs were showed in Table 2. All of them were stained mixture (both alcian blue and safranin O) after fixed in Carnoy's fluid and several mast cells were stained with safranin O after 60 and 120 minutes fixed in buffered formalin. From these results mast cells that distributed in those organs were formalin resistant.

**Table 1.** Distribution and formalin sensitivity of mast cells in various sites of dogs.

Tissue	Mast cells / mm square in sections fixed in			
	Buffered formalin			Carney's fluid
	30 minutes	60 minutes	120 minutes	
Ear	54.4±7.6	54.4±7.6	54.4±7.6	54.4±7.6
Tongue	41.7±1.0	41.7±1.0	41.7±1.0	41.7±1.0
Heart	14.4±2.6	14.4±2.6	14.4±2.6	14.4±2.6
Lung	102.6±7.1	102.4±7.1	102.4±7.1	102.7±7.2
Lgl.bronchiale	80.1±4.8	80.1±4.8	80.1±4.8	80.2±4.8
Lgl.mesentery	43.3±4.8	43.3±4.8	43.1±4.5	43.4±4.8
Kidney	1.1±0.1	1.1±0.1	1.1±0.1	1.1±0.1
Liver	95.7±4.1	95.7±4.1	95.7±4.1	95.7±4.1
Spleen	12.2±3.1	12.2±3.1	12.2±3.1	12.2±3.1
Peritoneum	13.2±0.1	13.2±0.1	13.2±0.1	13.3±0.2

Figures represent mean ± Standard Deviation from 3 animals.

**Table 2.** Alcian blue and safranin O stainability of mast cells against formalin fixation in ear of dogs.

	Mast cells / mm square in sections fixed in			
	Buffered formalin			Carney's fluid
	30 minutes	60 minutes	120 minutes	
Alcian blue	0	0	0	0
Safranin O	0	9.7±0.4	13.3±4.7	0
Mix	54.4±7.6	44.6±7.4	41.1±3.0	54.4±7.6

Figures represent mean ± Standard Deviation from 3 animals.

Numerous mast cells were observed in the gastrointestinal (Table 3), primary in villous lamina propria and the great number those were in jejunum and rectum (Figure 2c). No intraepithelial mast cells were present in the villous lamina propria from dog on the normal condition, whereas distribution of mast cells in the duodenum of *Strongyloides venezuelensis*-infected rats were commonly seen in the mucosal lamina propria and occasionally

intraepithelial. Those numbers in villous lamina propria after 30 and 60 minutes fixed in buffered formalin were less compare after fixed in Carnoy's fluid (Figure 2d), whereas for muscularis mucosa, submucosa, muscle layer and serosa seen the constant number of mast cells after fixed in buffered formalin. The same condition with mast cells that seen in the other organs that examined (Table 1,2) mast cells in muscularis mucosa, submucosa, muscle layer and serosa were formalin resistant and several of them safranin O-positive cells.

In order to see whether mast cells of dogs contain heparin in their granules, Carnoy's fixed tissue section were first examined fluoromicroscopically by berberine sulfate staining and subsequently the same sections were stained with alcian blue and safranin O. Strongly berberine sulfate fluorescence positive mast cells of jejunum and colon of dogs present in muscularis mucosa, submucosa, muscle layer and serosa, whereas very few in villous lamina propria (Table 4) (Figure 3a). There were no berberine sulfate fluorescence positive mast cells in villous lamina propria from duodenum rats (Table 4). This point indicated that mast cells in muscularis mucosa, submucosa, muscle layer, and serosa are of the heparin containing type. Practically all mast cells in tongue and liver were exclusively berberine-positive (Table 4) (Figure 4a) indicating that, they contain

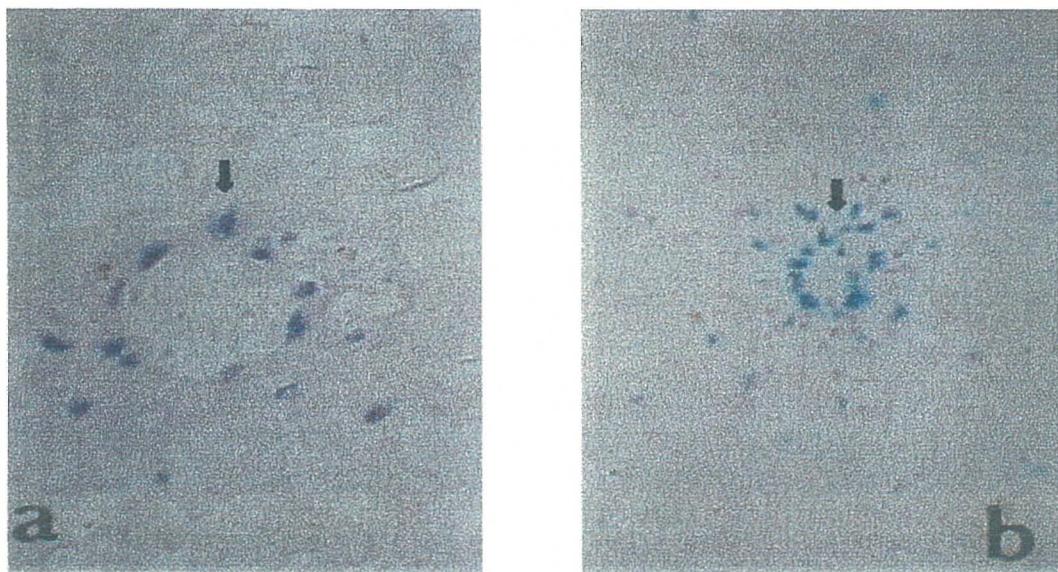
heparin in their granules moreover these mast cells were formalin resistant.

**Table 3.** Alcian blue and safranin O stainability of mast cells against formalin fixation in the gastrointestinal of dogs.

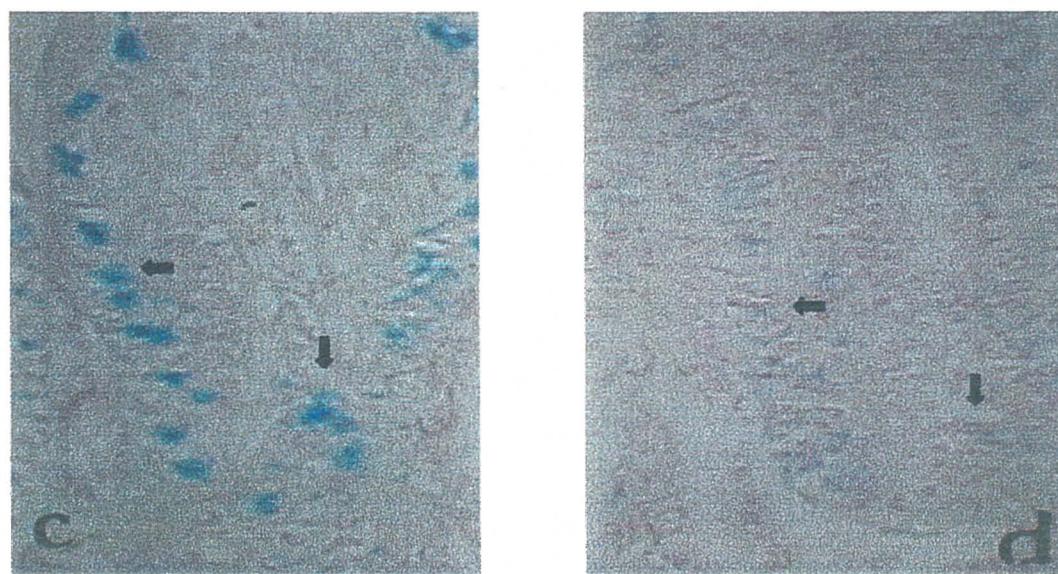
Animals and tissue	Mast cells / mm <sup>2</sup> in sections fixed in											
	Carriey's fluid			Buffered formalin			Formalin			SO <sub>2</sub>		
	AB+	SO+	Mixed	AB+	SO+	Mixed	AB+	SO+	Mixed	AB+	SO+	Mixed
<i>Dogs</i>												
Duodenum	VLP	518.9±47.2	0	0	0	0	372.7±24.2	0	0	288.5±44.6		
	MM+SM	159.4±4.9	0	3.9±2.2	0	26.6±6.8	135.1±1.5	0	39.5±15.5	121.4±12.8		
	ML+S	234.3±65.9	0	3.9±2.8	0	11.3±4.4	226.4±63.7	0	31.6±17.8	205.4±48.4		
Jejunum	VLP	607.1±69.0	0	0	0	0	582.4±63.0	0	0	535.8±65.9		
	MM+SM	210.2±16.3	0	5.4±3.9	0	31.6±5.7	184.6±17.7	0	32.3±4.8	183.9±17.3		
	ML+S	220.2±21.6	0	8.5±1.1	0	0	228.2±21.3	0	2.5±2.0	225.8±19.4		
Ileum	VLP	561.6±35.7	0	0	0	0	328.5±70.8	0	0	219.9±84.8		
	MM+SM	101.1±5.4	0	2.2±1.6	0	15.0±5.0	86.4±4.8	0	21.2±3.9	79.9±6.1		
	ML+S	136.7±52.5	0	7.5±1.6	0	0	144.0±51.5	0	4.4±1.2	139.6±51.0		
Cecum	VLP	507.0±40.3	0	0	0	0	327.9±17.1	0	0	264.3±35.5		
	MM+SM	145.6±13.9	0	1.1±0.9	0	26.3±1.5	120.9±12.9	0	43.6±9.1	103.6±20.7		
	ML+S	104.8±6.8	0	6.0±1.9	0	3.0±0.6	107.6±8.0	0	16.3±4.3	94.3±6.5		
Colon	VLP	475.4±27.4	0	0	0	0	192.4±81.0	0	0	191.0±81.6		
	MM+SM	147.1±45.6	0	3.3±2.6	0	9.2±1.7	141.1±42.4	0	33.3±4.9	117.0±38.7		
	ML+S	83.3±12.5	0	6.2±0.8	0	0.9±0.1	88.3±13.0	0	10.6±2.4	78.6±14.1		
Rectum	VLP	627.5±14.2	0	0	0	0	351.6±17.1	0	0	201.5±32.7		
	MM+SM	140.0±21.6	0	3.4±2.6	0	25.1±7.7	119.3±17.6	0	45.3±11.0	99.2±10.9		
	ML+S	92.0±13.0	0	5.5±2.3	0	2.9±0.5	94.2±15.1	0	12.9±3.5	84.2±13.9		
Stomach	VLP	319.3±104.1	0	0	0	0	164.6±54.1	0	0	132.1±74.2		
	MM+SM	71.0±8.2	0	5.4±0.9	0	3.5±1.4	72.9±9.0	0	10.0±4.2	66.4±11.6		
	ML+S	52.7±14.8	0	2.4±1.3	0	1.4±0.5	53.6±14.7	0	4.0±1.1	51.0±15.3		
<i>Rats</i>												
Duodenum <sup>a</sup>	VLP	2036.9±146.3	0	0	0	0	486.4±123.6	0	0	0		
	MM+SM	852.2±102.0	0	0	0	0	844.3±90.9	0	89.2±12.1	755.0±79.0		
	ML+S	173.3±33.0	0	5.6±1.4	0	67.5±6.1	114.4±27.4	0	159.1±32.0	19.8±10.4		

Figures represent mean ± standard deviation from 3 animals. AB+ = alcian blue positive; SO+ = safranin O positive; VLP = villous lamina propria; MM+SM = muscularis mucosa and sub mucosa; ML+S = muscular layer and serosa.

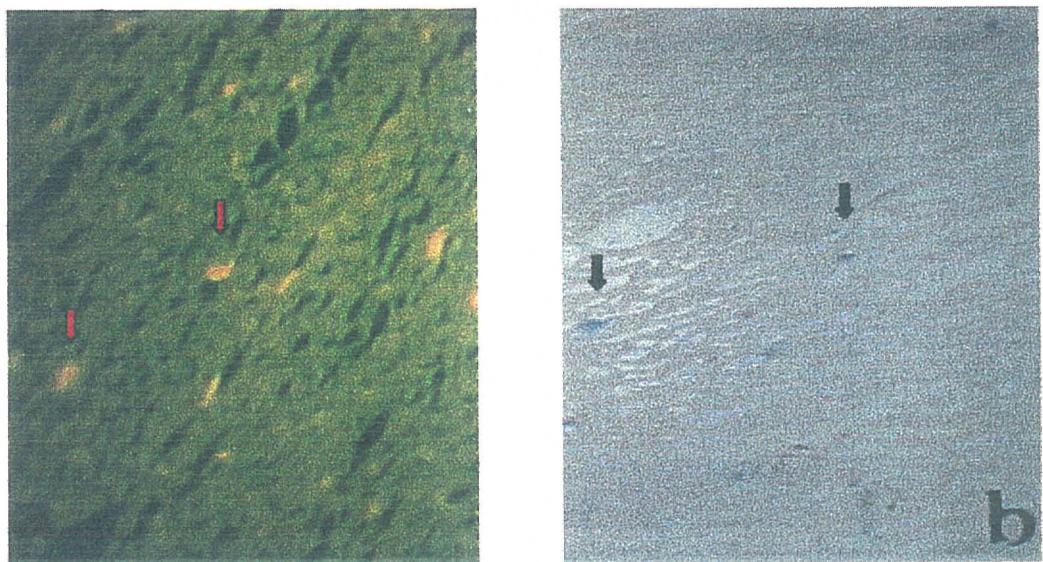
<sup>a</sup> Duodenum tissue were obtained from rats 3 weeks after infection with 25.000 *Strongyloides venezuelensis* as a control.



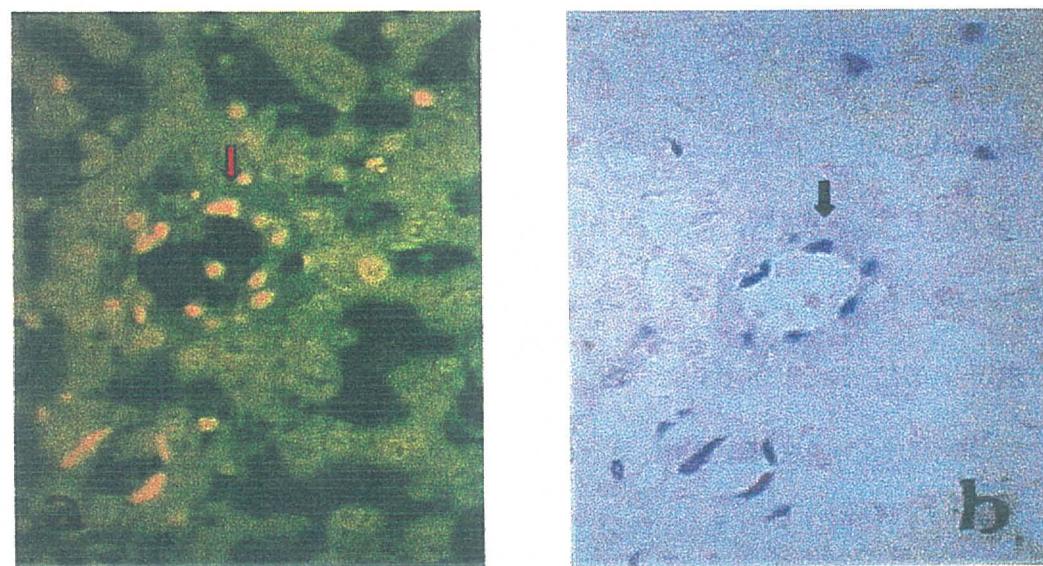
**Figure 1.** Mast cells in the dog's liver adjacent to blood vessel after fixed in Carnoy's solution (a) and after fixed in buffer formalin solution for 60 minutes (b).



**Figure 2.** Mast cells in the villous lamina of duodenum from dog after fixed in Carnoy's solution (c) and after fixed in buffer formalin solution for 60 minutes (d).



**Figure 3.** Successive staining of mast cells with berberine sulfate and alcian blue-safranin O in the inner muscle layer of jejunum. Sections were first stained with berberine sulfate, photographed under a fluorescence microscope (a). The sections were then washed and stained with alcian blue-safranin O, photographed under a light microscope at the same field (b).



**Figure 4.** Staining of mast cells with berberine sulfate and alcian blue-safranin O in the liver of dog. Sections were first stained with berberine sulfate (a) and then stained with alcian blue-safranin O (b).

**Table 4.** Berberine sulfate stainability of mast cells in various sites of dogs.

Animals and tissue	Berberine positive		
	VLP	MM+SM	ML+S
Dogs			
Jejunum	+	+++	+++
Colon	+	+++	+++
Rats			
Duodenum <sup>a</sup>	-	+	++

Animals and tissue	Berberine positive		
	VLP	MM+SM	ML+S
Dogs			
Tongue	++++ (practically all)		
Liver	++++ (practically all)		

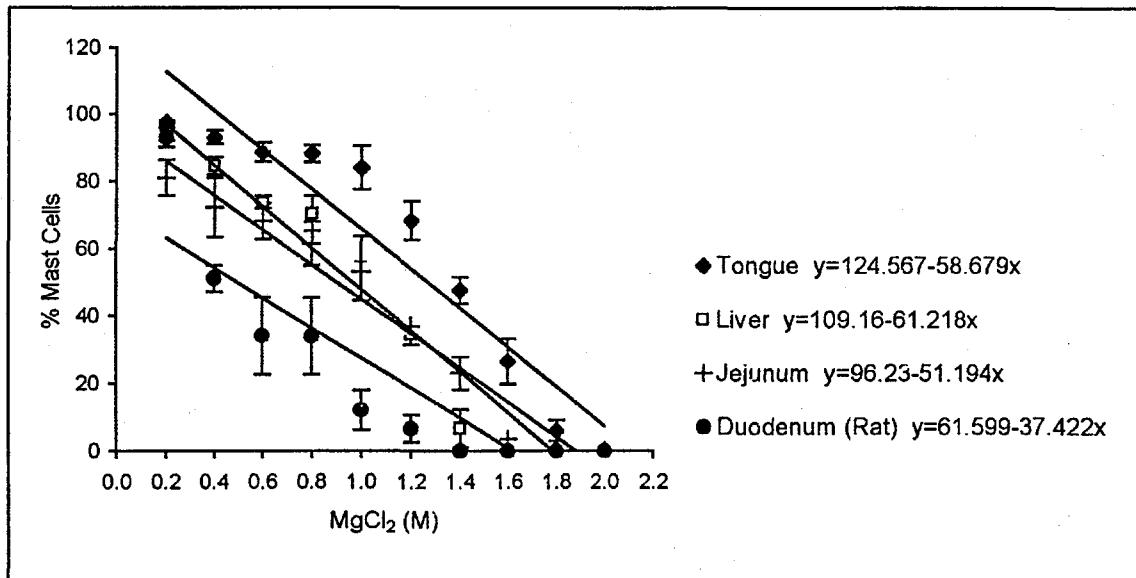
VLP=villous lamina propria; MM+SM=muscularis mucosa and sub mucosa; ML+S=muscular layer and serosa; + = berberine sulfate fluorescence positive; - = berberine sulfate fluorescence negative.

<sup>a</sup> Duodenum tissues were obtained from rats three weeks after infection with 25.000 *S. venezuelensis* L<sub>3</sub> as a control.

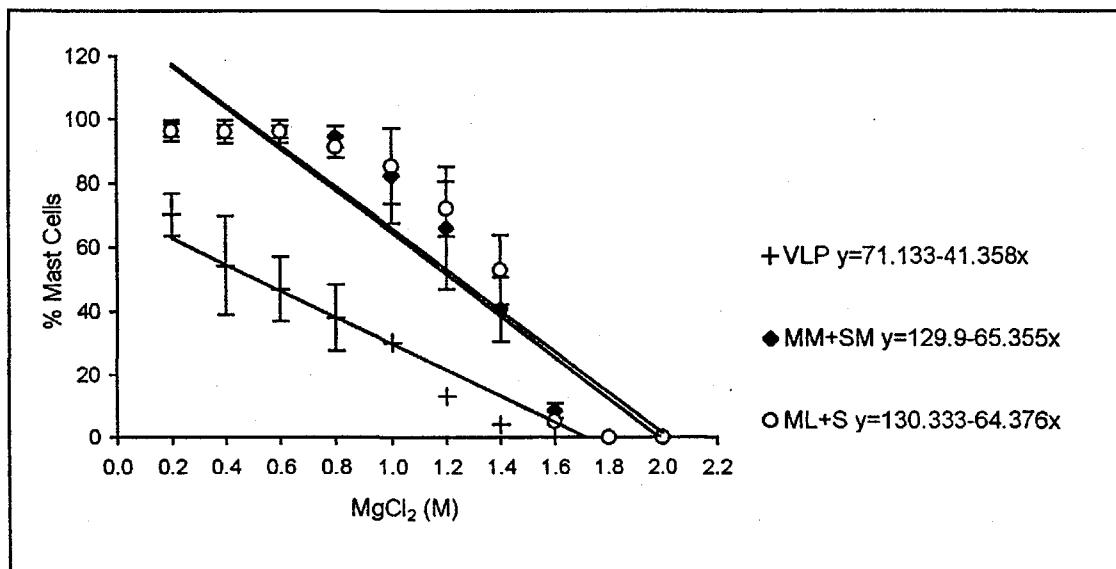
To further confirm these findings, the CEC which is the concentration of MgCl<sub>2</sub> that caused 50% reduction of alcian blue staining was determined for mast cells in dog. From fifth Figure, the CEC of mast cells in the tongue and liver of dogs were about 1.3 M and 1.0 M. All these values were far more higher than that of duodenum rats mast cells about 0.5 M indicating that these mast cells contained heparin in their granules. And the CEC of mast cells in the jejunum of dogs was about 0.9 M; this point was for whole part of jejunum, including villous lamina propria until serosa (Figure 5). As shown in Figure 6, the CEC of mast cells in the villous lamina

propria was 0.5 M and for those in (muscularis mucosa and submucosa), (muscle layer and serosa) were about 1.2 M and 1.2 M. If these findings were connected with the berberine sulfate staining data (Table 3), those mast cells in muscularis mucosa, submucosa, muscle layer and serosa were strongly berberine sulfate fluorescence, whereas that complex were very few in villous lamina propria. Both situations indicated that jejunum mast cells from muscularis mucosa until serosa contained heparin in their granules.

**Figure 5.** Critical electrolyte concentration staining of tongue ( $\blacklozenge$ ), liver ( $\square$ ) and jejunum ( $+$ ) mast cells of dogs in comparison with duodenum mast cells of rats ( $\bullet$ ). Each point and vertical bar represent means value  $\pm$  standard deviation of three animals.



**Figure 6.** Critical electrolyte concentration staining of VLP (+), MM+SM ( $\blacklozenge$ ) and ML+S ( $\circ$ ) jejunum mast cells of dogs. Each point and vertical bar represent means value  $\pm$  standard deviation of three dogs. VLP=villous lamina propria; MM+SM=muscularis mucosa and sub mucosa; ML+S=muscular layer and serosa.



#### IV. DISCUSSION

Armed with specific IgE antibodies, and strategically positioned adjacent to blood vessels, lymphatic, and nerves in the epidermal and mucosal surfaces, the mast cells is in an ideal position to act as an 'antigen sensor' or surveillance cell (Galli, 1990). In several species, there appear to be at least two distinct classes of tissue mast cells that differ in their content of intragranular amines, proteoglycans, and proteases (Katz *et al.*, 1985). In clinically normal dogs, a few to several mast cells may be encountered in smear of lymphonode aspirate, rare in smears of bone marrow aspirate, and absent from smears of buffy coat (Bookbinder *et al.*, 1992) but found in buffy coat preparation from any dog with an inflammatory skin disease and not to disseminated mast cell tumor (Cayatte *et al.*, 1995). Based on morphologic, ultrastructural and histology criteria, Enerback (1966) has shown mast cell heterogeneity in different sites of rats. Following this, histochemical method to demonstrate proteoglycans has been practically used to distinguish them in situ rats and mice (Enerback *et al.*, 1986). Staining mast cells populations with alcian blue followed by safranin O, two cationic dyes believed to bind differentially granular proteoglycans (Katz *et al.*, 1985) give good result after fixed with Carnoy's or Motas's, whereas some fixatives such as neutral buffered formalin preserve CTMC granules but not MMC granules in

histology sections (Enerback, 1966). Optimal fixation will result in precipitation of glycosaminoglycans leaving polyanionic sites available for binding of dye (Strobel and Miller, 1981).

Mast cells observed in various sites of dogs except those present in villous lamina propria were formalin resistant and several of them stain mixture between alcian blue and safranin O. Distributed throughout connective tissue which consist collagen, elastic and reticular fibers, adjacent to blood or lymphatic vessels. To destine specially mast cells in ear, we counted separate between mast cells which stained only alcian blue or safranin O, or mixture (both alcian blue and safranin O) whereas several of them safranin O positive cells after buffered formalin fixation (Table 2). Fujimaki *et al* (1992) reported that no significant differences on morphology of mast cells after treated with formaldehyde were see using transmission electron microscope.

In order to stain mast cells granules that present in villous lamina propria, alcian blue lost it's stainability against buffered formalin fixation (Table 3) (Figure 2d) indicating formalin sensitive granules. Furthermore these mast cells bind only alcian blue after Carnoy's fixation, whereas some mast cells in the other part of gastrointestinal bind both alcian blue and safranin O, indicating these granules contain proteoglycans with less sulfated

glycosaminoglycans than do the granules of mast cells in muscularis mucosa, submucosa, muscle layer and serosa. Strobel and Miller (1981) suggested that failure of dye binding in intestine mucosal mast cells may be due to alterations induced by formaldehyde in the relationship between glycosaminoglycans and basic proteins of the granules. Considering such variance, further comparative histochemical and functional characterization such as reactivity to secretagogues (Shanahan *et al.*, 1985) is necessary to determine whether formalin-sensitive mast cells in dog are abundant in gastrointestinal mucosal tissue, those present in villous lamina propria termed as mucosal mast cells (MMC). Whereas formalin-insensitive/resistant mast cells are widespread throughout connective tissue, termed as connective tissue mast cells (CTMC).

The dye berberine sulfate forms a strong fluorescent complex with heparin, so that it has been used for the detection of CTMC (Enerback, 1974). Strongly berberine sulfate fluorescence positive of jejunum and colon dogs present in muscularis mucosa, submucosa, muscle layer and serosa, whereas very few in villous lamina propria (Table 4). Furthermore, exclusively berberine positive mast cells present in tongue and liver (Table 4), these two points indicating are of the heparin containing type, moreover these mast cells were formalin resistant.

In addition to the berberine fluorescence method, determination of the critical electrolyte concentration, the principle of which was introduced by Scott and Dorling (1965) has also been used for the detection of mast cells subtypes (Enerback *et al.*, 1986). In rats the CEC of MMC is substantially lower (about 0.6 M) than that dermal CTMC (about 1.0 M), reflecting chondroitin sulfate and heparin as their respective granular proteoglycans (Enerback, 1987 and Miller *et al.*, 1972). The CEC of mast cells in the tongue and liver of dogs were about 1.3 M and 1.0 M, indicating contained heparin in their granules (Figure 5). As shown in Figure 6, the CEC of mast cells in the villous lamina propria was 0.5 M and in the (mucosalis mucosa and submucosa) and (muscle layer and serosa) were about 1.2 M and 1.2 M. These findings supported that liver, tongue and muscularis mucosa until serosa mast cell granules contained highly sulfated glycosaminoglycans which is heparin, than do the granules of mast cells in villous lamina propria.

## **V. CONCLUSION**

As an overall conclusion dog also have mast cells that distributed in the connective tissue of ear (skin), tongue, lung, heart, lymphoglandula bronchiole, lymphoglandula mesentery, spleen, kidney, peritoneum, liver, stomach, duodenum, jejunum, ileum, caecum, colon and rectum. Like other species dog's mast cells have two subtype, MMC, and CTMC, which can be discriminated by their location and histochemical characterizations.

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