NEW MODIFICATIONS IN CHOLINESTERASE TECHNIQUE FOR LONG –TERM PRESERVATION OF STAINING IN FROZEN SECTIONS OF PANCREAS

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ABSTRACT

An investigation was undertaken for the study of the innervation and distribution of acetylcholinesterase substance, in the pancreas of *Lepus europaeus*- Bulgarian Rabbit by a deviced cholinesterase technique, in the Department of Anatomy, Histology and Embryology, Higher Medical Institute**,** Sofia-31 (BULGARIA), in year 1980. After removing, each pancreas was infiltrated with Hyalase injection (1gm.Hyalase ampoule) which was dissolved in 5 ml. of .85% cold saline solution through pancreatic duct. Then the pancreas was treated with Co_2 gas for 15-20 minutes and later on, it was kept in small woolen bag (filled with condensed carbon di-oxide gas), for 10 minutes, and rest of the process was followed as described in 'Materials and Methods'. During microscopic observations of the stained slides (from 1980 to year 2001), it was recorded that the preservation of stain and acetylcholinesterase (AChE) activity were marked in various structures of exocrine and endocrine parts, both, after application of pre-fixation treatment of the pancreas, and in change of parameters such as, pH of buffer solution, incubation period., temperature and fixative etc. The preservation of the stain and the distribution of AChE activity were marked in the neural elements, such as ganglia, nerve cells, nerve fibers, nerve bundles, nerve endings, and other structures, such as islet cells, blood vessels and pancreatic duct. Connective tissue fibres and acinar cells were recorded to be enzyme negative.

Keywords: Preservation, stain, frozen section, Mammalian pancreas.

INTRODUCTION

In the year, 1949, earlier investigator [1] had introduced a histochemical staining method, the cholinesterase method. They have recorded their observations, in the light of recent technique, replacing the old routine histological staining methods based on old silver nitrate [2] and other methods , used for the histological study. Since then, many earlier researchers [3-14] have extensively applied the cholinesterase method for their investigations. This method has become now a day's very important tool, in the analysis of nervous tissues. The value of this method has been greatly increased by the discovery that it can be applied to experimental material to determine the direction of nerve conduction. Now the whole picture of the present investigations has been changed, due to introduction of the cholinesterase method, for the study of the acetylcholinesterase activity (AChE-activity) and their probable role, in various organs in mammals, birds, reptiles and amphibia, and fishes, and for the study of distribution of AChE activity in neural elements and their associated structures in various tissues. The results based on cholinesterase method, could be altered by slight change in the parameters i.e. pre-fixation treatment of the organ, fixation process and their fixative used, various buffer solutions and their pH ranges of the buffer, incubation periods from minutes to hours, and the temperatures from 32 to 37^0C [13, 15-17] and mountant media [13] The acetylcholinesterase (AChE) activity was extensively worked–out by simple microscope [13, 14, 18-24] and by electron microscope [25, 26] and many others. The available literature confirmed that there is no evidence for the preservation of acetylcholinesterase (AChE) activity and retention of the stain, in the exocrine and

endocrine parts of the pancreas, by cholinesterase method.

An experiment was set up in the Department of Anatomy, Histology and Embryology, Higher Medical Institute, Sofia-31, for the revision of cholinesterase method. The pancreas of *Lepus europaeus* - Bulgarian rabbit was used for the study of preservation of stain and distribution of AChE activity, in various structures of the exocrine, endocrine parts, and neural elements such as, ganglia, nerve cells, nerve ending, thick, thin and coarse nerves, nerve bundles which were recorded to be AChE-positive. The pancreatic duct and their ductules and blood Vessels and their capillaries were also enzyme positive, (showed less reaction to AChE activity). The acinar cells and connective tissues were recorded to be enzyme negative i.e. AChE- negative (stained negatively).

MATERIALS AND METHODS

The material and the method for the preparation of the solutions are as follows: Purwar and Petkov, 1990 [12]

(1) 10% cold Neutral formalin solution fixative):- Formaldehyde (35-40%) – 10ml dissolved in 90 ml. of deionised glass distilled water. In the solution, the excess quantity either calcium carbonate powder or marble chips or both, were added for the neutralization of the fixative. The solution was kept in refrigerator at 4 ^{0}C.

(2) SOLUTION "A":

Acetylthiocholine iodide	0.46 gm.
Deionised glass distilled water	2.4 ml.

The acetylthiocholine iodide (either of M/S SIGMA, USA or M/S FLUKA, Swiss) was dissolved in the deionised glass distilled water in 2.4 ml. and on adding the copper sulphate solution (o.8 ml) drop by drop, the brown precipitate was formed which was removed by centrifugation at 7000 rpm .Later on the filtration process was followed and the supernatant was used.

(3) SOLUTION "B":-

It was prepared by adding each according to serials.

i) Copper-Glycine solution	1.2ml.
ii) Magnesium chloride solution	1.2ml.
iii) Anhydrous sodium sulphate solution	20ml.
iv) Buffer solution (Na – acetate buffer)	10ml.

Both, solution **"A**" and solution **"B"** were placed in the refrigerator at 4^{0}C and were added together immediately just before use.

(4) PREPARATION OF STOCK SOLUTIONS:

i) Copper-Glycine (Cu-Gl) solution-3.72gm. glycine and 2.5gm. copper sulphate in 100ml deionised glass distilled water.

ii) Magnesium chloride (MgCl2) solution 9.25 gm. in 100 ml. deionised glass distilled water.

iii) Anhydrous sodium sulphate solution 40gm Na_2So_4, in 100 ml deionised glass distilled water, heated till it dissolved and later on cooled it at room temperature and then mixed

(5) BUFFER-solution:-

i) 2.72gm. Sodium acetate in 100ml deionised glass distilled water.

ii) 1.2ml.Acetic acid in 100ml. deionised glass distilled water.

The buffer solution was prepared by mixing solution "A" and solution "B" and pH 5.2 of the buffer solution was maintained.

PREPARATION OF OTHER SOLUTIONS:

Saline solution (0.85%) - Sodium chloride 0.85 gm. in 100ml deionised glass distilled water was dissolved. The saline solution was placed in a refrigerator at 4^0 C.

Note: 1) Glass deionised distilled water preferably gives better results, rather than the metal deionised distilled water. In this experiment, the glass double distilled water was used.

PROCEEDURE FOR FIXATION AND STAINING OF THE PANCREATIC TISSUE

1. The tissue pancreas was removed -out by opening of the abdominal region, from the adult and healthy animal-

Lepus europaeus - Bulgarian rabbit, after giving anesthesia.

2. After removing, the pancreas, it was kept in woolen bag filled with dry CO_2 (carbon di-oxide) gas and treated with CO_2 gas continuously for 20 minutes, after infiltration with Hyalase ampoule of 1gm. The solution of Hyalase injection was prepared in 8ml. of saline solution (0.85% saline solution). This injection also increased the quick fixation of tissue and preserved the enzymes activity.

3. Later on these treated tissues were transferred in 10% cold neutral formalin for 5 hours for the fixation of the tissue and the enzyme activity, in a refrigerator under control temperature of 4^0C, in the laboratory of the Department of Anatomy, Histology and Embryology, Higher Medical Institute, Sofia-31 (Bulgaria).

4. The frozen sections of 30-35 micron thick were obtained by freezing microtome in the Pathology laboratory.

5. Each section was floated in 0.85 % cold saline water of the petridishes for 15 to 20 minutes for stretching and removing out the formalin from the floated sections. Each section was taken on the slide and allowed to drain-out the saline water from the stretched frozen sections of the slide.

6. After becoming dry at room temperature, these slides were kept in staining solutions "A" and "B" solution, in vertical jars and incubated for 16-hrs. at the temperature of 37^0C, in an oven.

7. Stained slides were developed with 2% cold yellow ammonium sulphide (freshly prepared) for 5 minutes and later on these slides were washed with deionised glass distilled water, for removing extra yellow ammonium sulphide solution.

8. Then the slides were dehydrated, simultaneously in 70%, 90% and absolute alcohol for 10 minutes in each.

9. After removing them from the absolute alcohol, the slides were kept in xylol (xylene) for 15 minutes or till it becomes transparent.

10. Lastly these sections were covered with cover slip, using canada balsom **(**natural/neutral of M/S Sigma, USA) as a mountant.

Note:-1: Indian DPX, mountant media was not found suitable, as it does not preserve the staining of the sections and at times the staining of the sections disappears with in overnight.

2. Biochemicals of M/S SIGMA, USA and M/S FLUKA, SWISS were used in this study, for their purity and genuineness of the product.

RESULTS

MICROSCOPIC OBSERVATIONS

The microscopic observations of the pancreatic sections were recorded and exposed in the year 1980 (May-June); 1983 (September); 1985 (May-June); 1987 (May); 1989 (May); 1991 (May-June); 1992 (October); 1993 (May); 1997 (October); and last in the year, 2001 (May) and the staining difference was recorded in the neural elements i.e. the nerve fibres (both myelinated and non-myelinated), nerve bundles, nerve endings, ganglia, pancreatic duct and ductules, islet cells and pancreatic blood vessels, nerve cells and in the distributed AChE substance in numerous pancreatic structures, as shown in **Table No.-1** and sharpness and contrastness of staining in the structures as shown in **Table No.-2.** The results were based on the observations of the twenty one year's photomicrographs of stained slides in subsequent years. The results were encouraging and promoting to solve the problem of the preservation of the staining, not only in the pancreatic frozen sections but also on other organs.

This paper deals the following points:

1. Pre-fixation treatment of tissue - pancreas.

2. Revision of cholinesterase method and their application in the light of pH of the buffer solution,

temperature during staining of sections and incubation period.

3. Preservation of stain and presence of acetylcholinesterase (AChE) activity in the various structures of the pancreas (exocrine and endocrine, both). The present contribution will be the mile stone for the future researchers.

Table No.1. Distribution and localization of AChE activity in the pancreatic structures.

Name of the tissue	Name of the structure	Staining reaction for AChE substance		Distribution and localization of AChE activity in structure	
		Positive (+)	Negative (-)	Maximum (max)	Minimum (min)
Pancreas	Nerve bundle	+		Max	
	Fine nerves	+		Max	
	Coarse thick nerves	+		Max	
	Ganglia	+		Max	
	Islet cells	+		Max (in granular form)	
	Pancreatic duct and their ductules	+			Min
	Nerve endings	+		Max	
	Nerve cell	+		Max on the periphery	
	Lobular cells		-		Nill
	Connective tissues		-		Nil

DISCUSSIONS

Good number of research papers has been contributed by many previous investigators on the pancreatic innervation in, mammals, birds, reptiles and amphibians, by using cholinesterase method. In reference to mammalian pancreas, their exists two groups. A few investigators have contributed papers only on the basis of old routine histological methods [10, 27] and many others too and on the other hand, a number of investigators have contributed papers on the basis of the cholinesterase method [5, 6, 11, 15, 18, 25-29]. However, in the literature, there exists confusing and conflicting results which are due to the faulty use of cholinesterase method and partly, it may be due to capriciousness in the application and methodology (in preparation of the various solutions).

During this investigation from 1971, till today, and also in the literature, there are variations in pH of the buffer solution and ingredients too, temperature range and incubation period, during the staining of the fresh frozen sections [26, 27, 36] and also slight change in the pre-fixation technique [11].

Table No.2. Preservation of staining in the structures of pancreas (based on microscopic observations in the consequent years from 1980 to the year – 2001) are given below in tabular form.

Name of Tissue	Name of Structure	Staining reaction for (AChE)		Year 1980-1983		Year 1985-1989		Year 1991-1997		Year 2001	
		Negative (-)	Positive (+)	Sharpness	Contrastness	Sharpness	Contrastness	Sharpness	Contrastness	Sharpness	Contrastness
PANCREAS	Acini	-		Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	Connective tissue	-		Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	Coarse thick nerves		+	**	***	**	***	**	***	**	***
	Fine nerves		+	**	***	**	***	**	***	**	****
	Networks of fine nerves on islet cells		+	**	***	**	***	**	***	**	****
	Nerve bundles		+	**	***	**	***	**	***	**	***
	Ganglia		+	**	***	**	***	**	***	**	***
	Islet cells		+	**	***	**	***	**	***	**	***
	Pancreatic duct and their ductule		+	**	***	**	***	**	***	**	***
	Nerve ending		+	**	***	**	***	**	***	**	***
	Nerve cell		+	**	***	**	***	**	***	**	***

- \- AChE-negative (unstained)
- \+ AChE-positive (stained and stain retained)
- ** Sharpness in staining
- *** Contrastness in staining
- **** Contrastness in staining slightly less in the year 2001
- ** Sharpness in staining slightly less , in the year, 2001

A record is the evidence for the tremendous changes in the cholinesterase method, since its introduction in 1949 [1] and other researchers are following the cholinesterase method in their studies with many changes [5-7, 9, 21, 29-38]. A few investigators reported different observations in reference to the staining reaction to islet cells [6, 38]. A few other researcher [38] recorded that islet cells showed positive reaction (AChE-positive) and on the contrary. He recorded that islet cells showed no positive reaction and also reported the absence of

ganglion cells in numerous mammalian pancreas but he found, many groups of ganglion cells in the pancreas of monkey [30] and he could not mention for the staining of connective tissues, blood vessels, pancreatic duct, neural network on the islet cell, nerve ending etc and also [7, 30] made no comment for the preservation of the stain on the various structures and for the study of AChE acivity of the exocrine and endocrine part, in the mammalian pancreas, for a longer period.

However, in the light of present ***observations of twenty one years***, it has become very clear now that the AChE activity was marked in granular form in islet cells. The nerve bundles, fine nerves, ganglia, coarse nerves, nerve endings, nerve cells, and neural network on islet cells, were recorded to be enzyme positive [31]. The pancreatic duct and its ductules and blood vessels and their capillaries, showed less AChE-positive reaction in comparison to ganglia and nerves [31]. Although a few previous researchers have reported the presence of nerve cells in the vicinity of bile duct [28, 29]. However, in the present study, chain of very smaller ganglionic structures, in the vicinity of a few islet cells, usually connected with nerves of periinsular plexus were observed.

It was a very peculiar structure of the rodent's pancreas where AChE-positive activity was marked. Undoubtedly, extensive researches have been carried-out by many investigators [6, 7, 19, 22, 24, 26, 32] and they could not mention in detail about the staining reaction of cholinesterase in various structures of the exocrine and endocrine regions of the pancreas and retention of the stain and AChE activity for a longer period in various structures of the pancreas. However, earlier investigators [6, 7] and many others have left many important details and facts untouched and undecided which have been described and discussed.

Stained slides (from year-1980), were exposed for photomicrography in the year of 1980, 1983, 1985, 1987, 1989, 1991, 1992, 1993, 1997 and 2001), to show the preservation of stain in pancreatic frozen sections of more than 21 years .The staining of fresh frozen sections was done by revised cholinesterase method (Purwar and Petkov., 1990), under maintained pH of buffer solution, 5,2, incubation period 16 hours, and temperature 37 $^{\circ}$C. After the photomicrography, the prints were prepared, later on. The observations are given here in the Table No.1 and Table No.2.

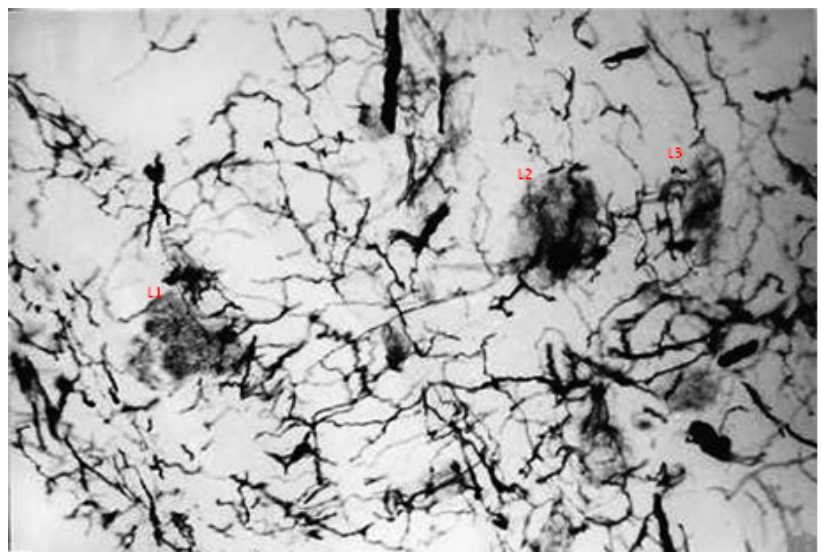

Fig.1. Year 1980. Shows the enlarged part of photomicrograph, where AChE-positive activity could be marked in periductular ganglia (G) and their ganglionic nerves (GNF) and nerve bundle (NB).AChE-positive activity in granular form, can be marked in some nerve bundles,neural networks(NNW) in exocrine region too. X.300.

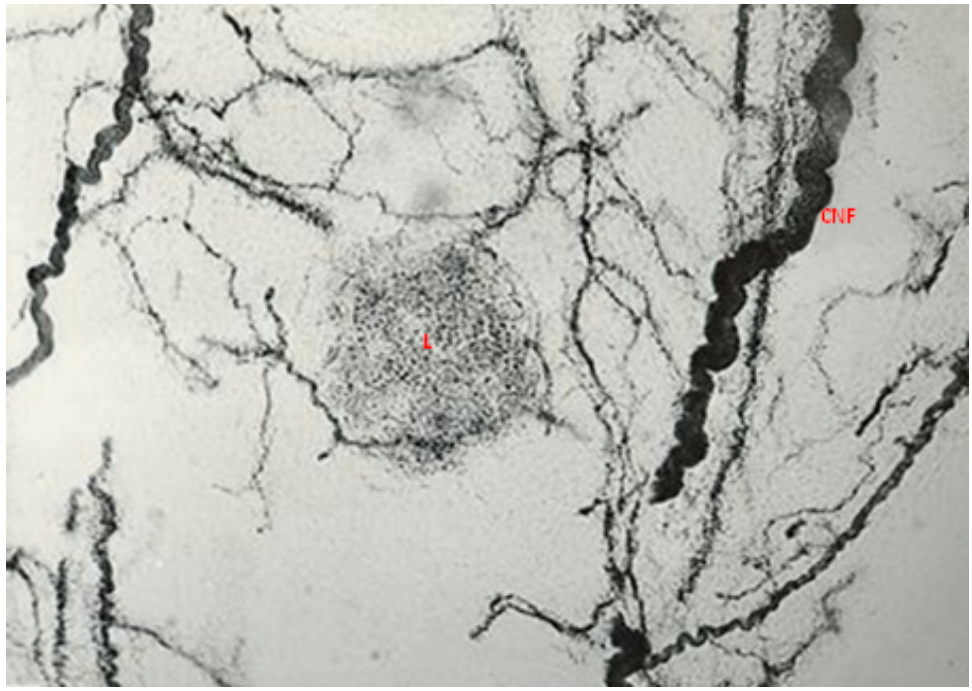

Fig. 2. Year 1980. Shows the AChE-positive activity in granular form in the whole islet cell (L), thick nerves and also in exocrine region. Highly AChE-positive activity was marked in coarse thick nerves (CNF), which were closely related to the nerves of the AChE-positive neural networks (NNW).X.300.

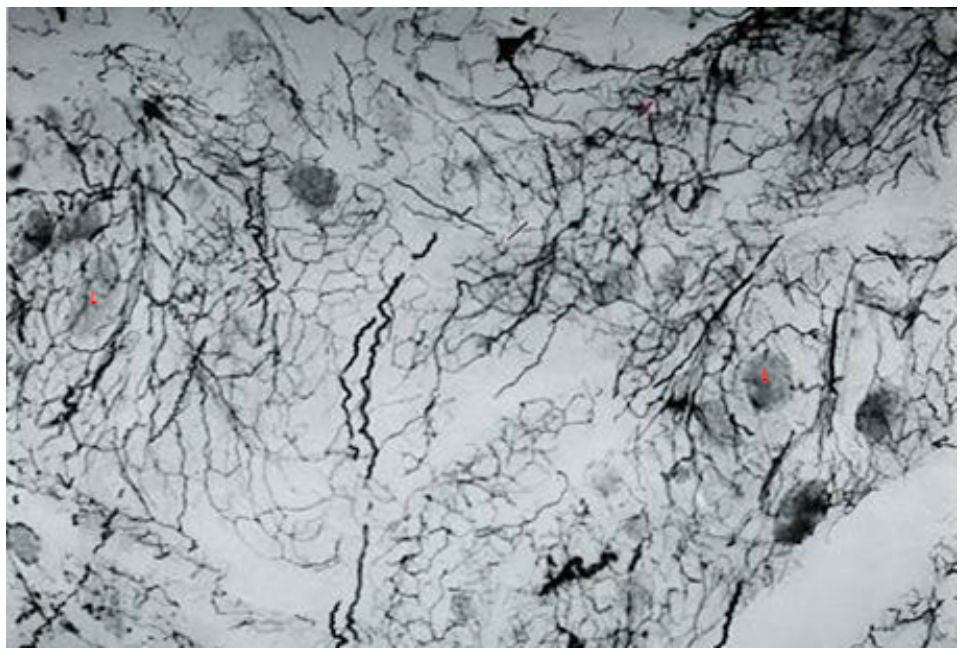

Fig. 3. Year 1983. Shows the distribution of highly AChE-positive nerves and coarse nerves, participating in the formation of vascular network (VNW), over blood vessel (BV), periinsular network (NNW) on islet cell and acinar neural network (NNW) in the exocrine region. The nerves of these plexuses were closely related and associated to each other, forming continuity in the entire region.X.300.

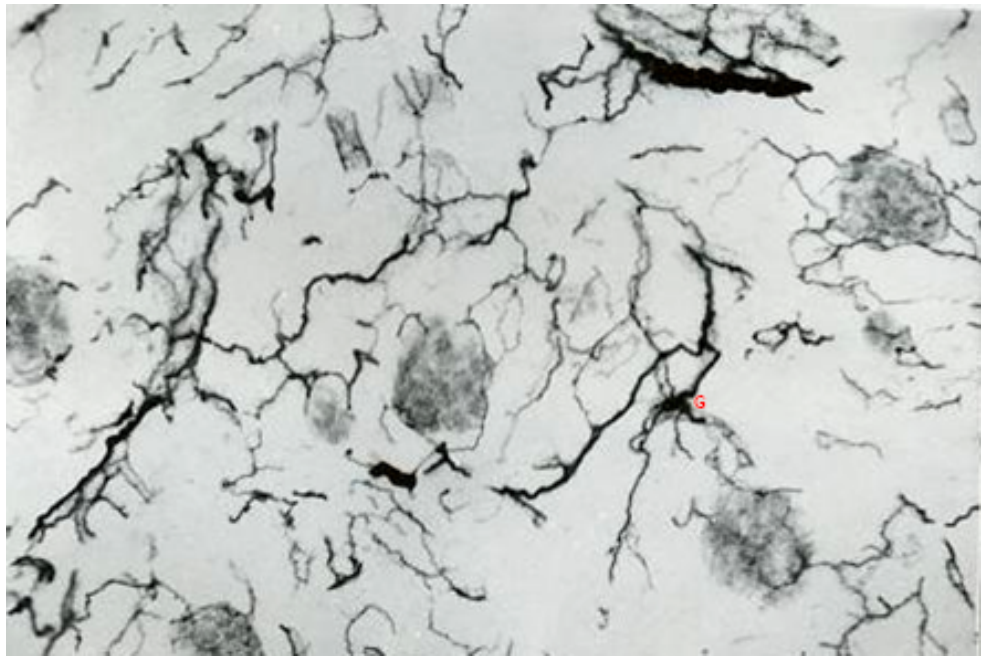

Fig. 4. Year 1985**. S**hows the enlarged part of an islet cell (L), where the distribution of AChE-positive activity can be seen in granular form and AChE-positive coarse nerves formed the periinsular plexus (PL), which probably control the various activities of the islet cell. X400.

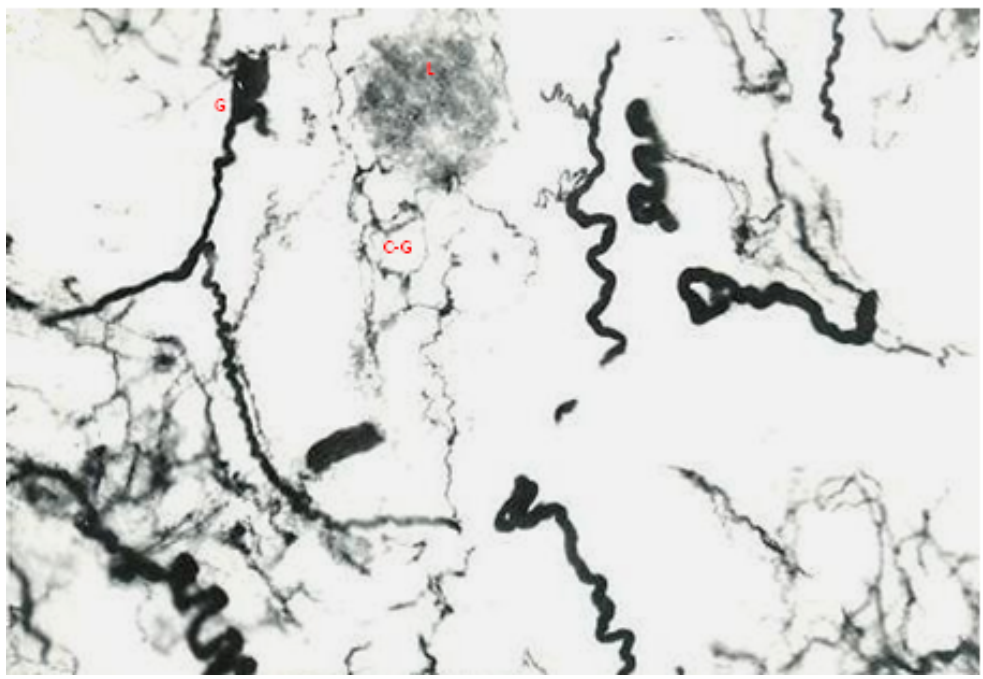

Fig. 5. Year 1987. Shows the distribution of AChE substance in the nerves of the capillary vessels (CV), nerves of the neural networks (NNW) in the acini region and nerve bundle. X250.

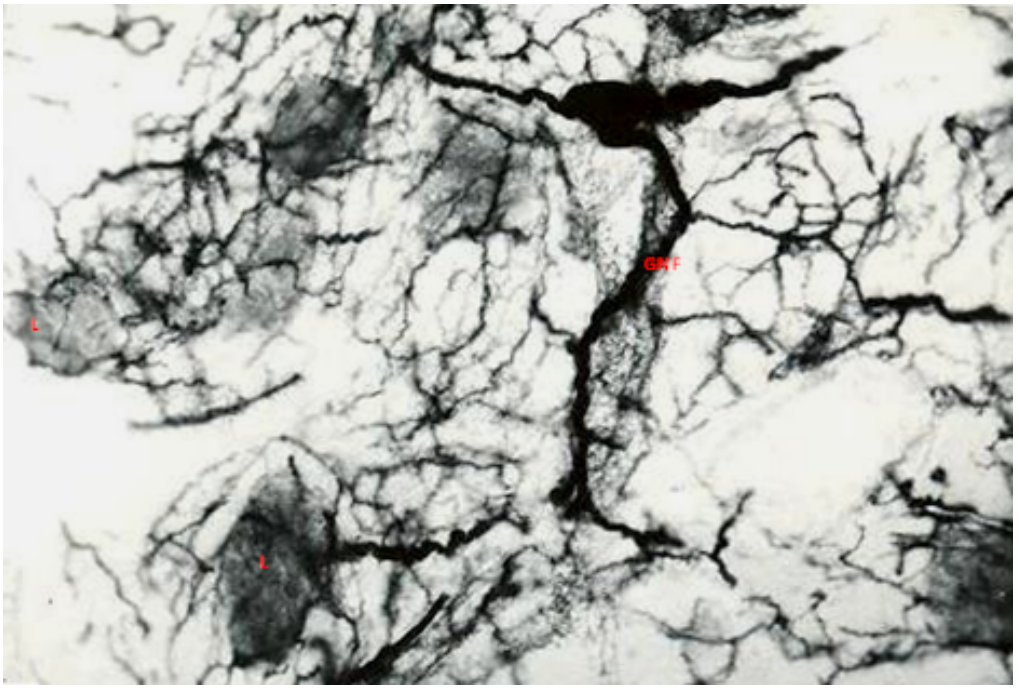

Fig.6. Year 1989. Shows the distribution of AChE activity in the neural networks formed by coarse, fine (non-myelinated) and thick nerves (myelinated), in the exocrine and endocrine region (L_1) where the nerves are seen closely related and associated to each other and at times with the nerves of the other islet cell (IL_2) and AChE-positive nerve bundle (NB). X.250.

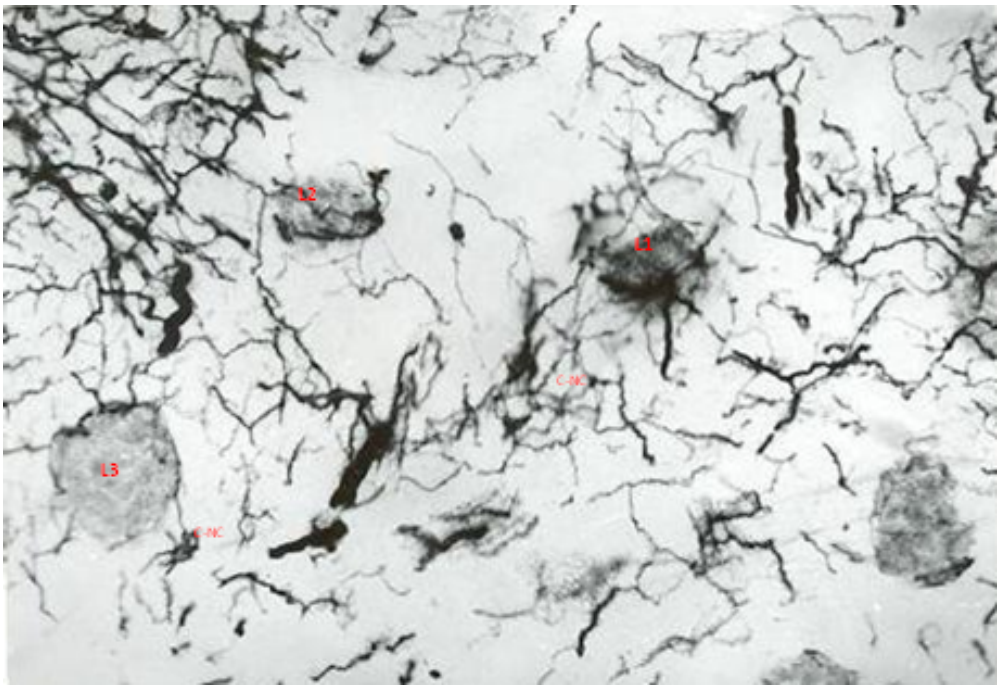

Fig.7. Year 1991. Shows the distribution of AChE activity in the vascular plexus and insular plexus which are formed by common nerves of blood vessel (BV) and islet cells (L_1, L_2). AChE-positive activity was marked in coarse thick nerves and fine nerves .X.250.

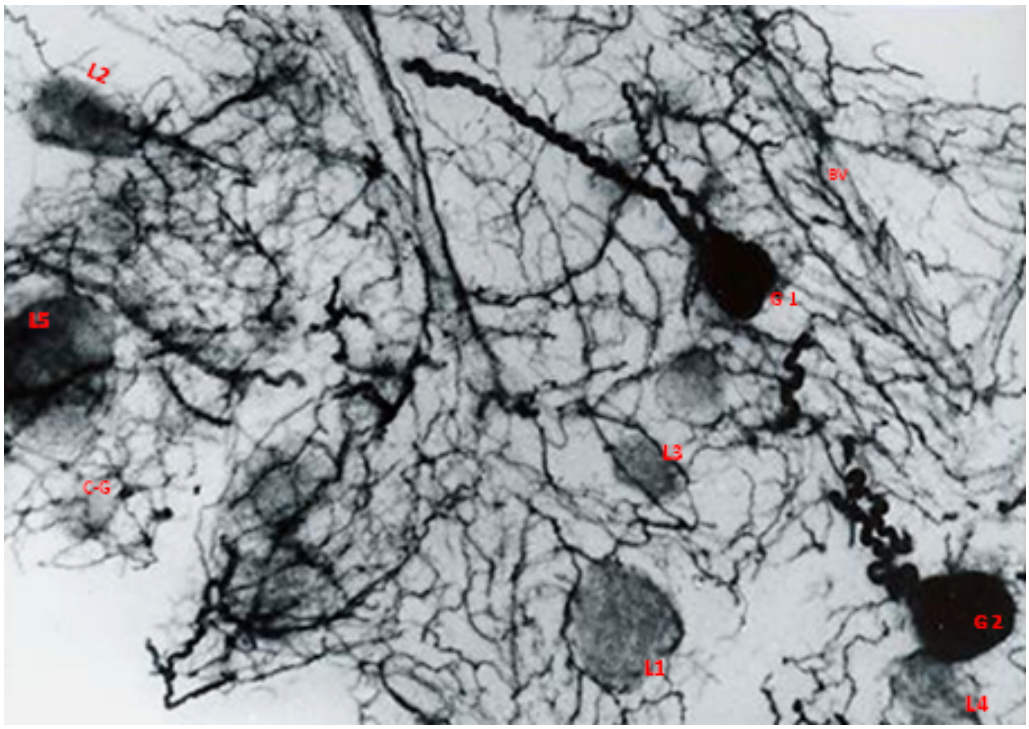

Fig. 8. Year 1992. Shows the distribution of AChE-activity in bipolar ganglia (G_1), in the nerves of the vascular network (formed on blood vessel-BV), and nerves of insular plexus formed near islet cells (L_1, L_2, L_3, L4, L_5). At times, peri-insular ganglia (G_2) was marked on the periphery of the, islet cell (L4) Chain of small and AChE-positive ganglia (C-G) was also marked. X.300.

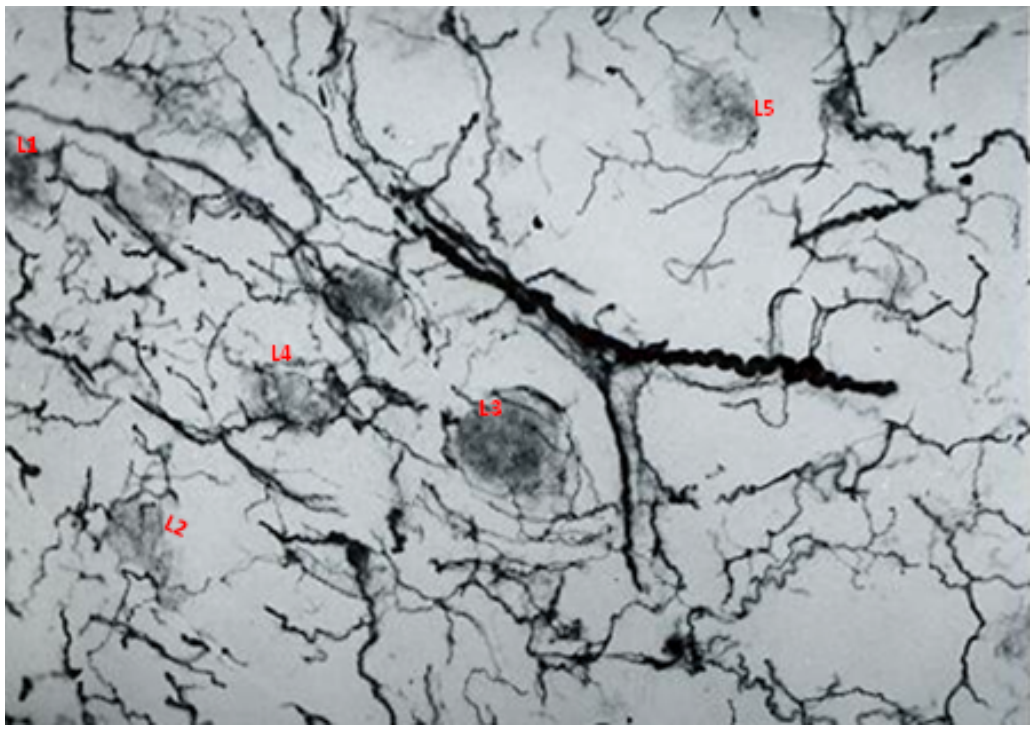

Fig. 9. Year 1993. Shows the distribution of AChE-positive activity in the vascular thick nerves, coarse thick and thin nerves of islet cells (L_1, L_2, L_3, L_4, L_5) . X.250.

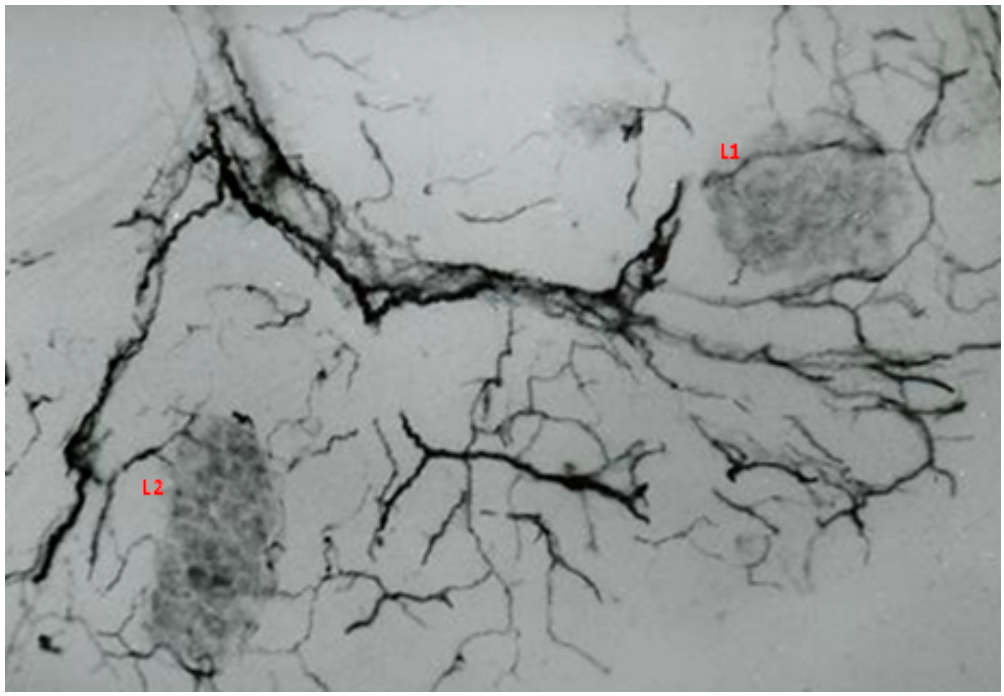

Fig. 10. Shows the distribution of AChE–positive activity, in coarse thick and thin nerves and islet cells (L1, L_2), nerves of vascular plexus, nerves of acini (exocrine region) and nerves of nerve cell which were closely related and associated with each other. X.250.

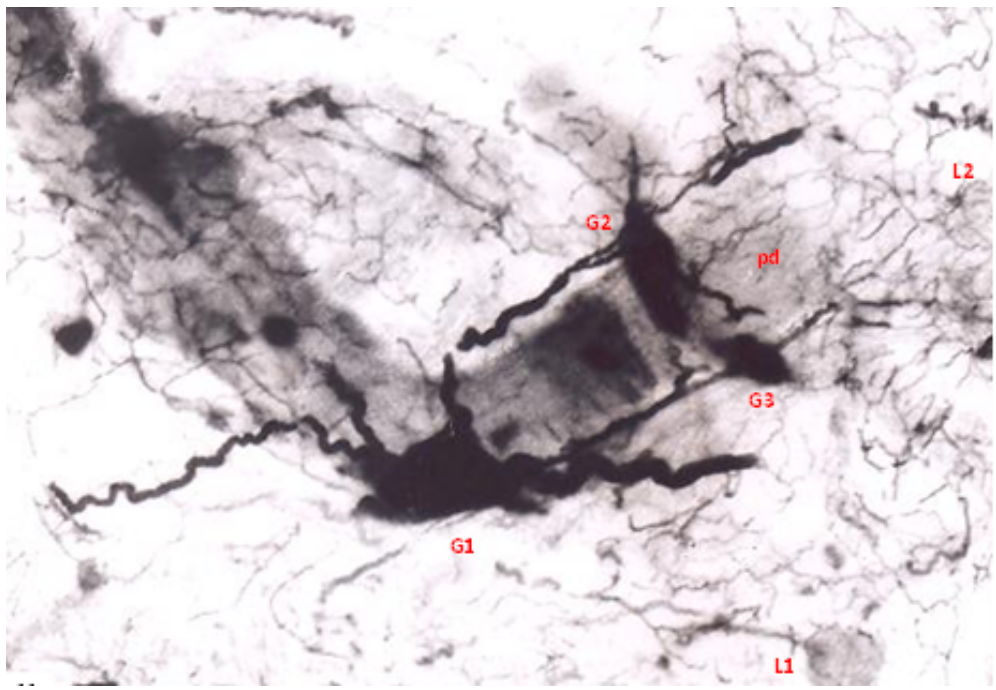

Fig. 11. Year 2001. Shows the distribution of AChE –positive activity in irregular-shaped ganglia. They were arranged in chain-like fashion, and lying on the peripheral surface wall of the major pancreatic duct. AChE-positive activity can be seen in islet cells (L1, L_2), ganglia (G1, G2, G3), and fine nerve fibres. X.300.

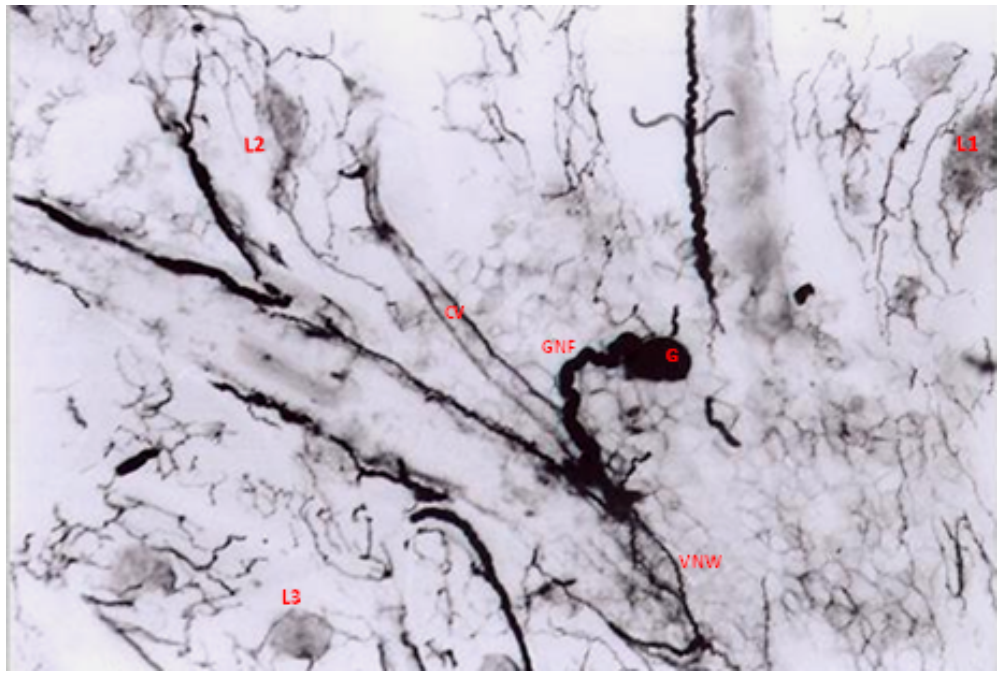

Fig. 12, shows the formation of perivascular network (VNW), over major blood vessel and their capillary branch by ganglia (G) and their ganglionic nerve fibre (GNF), in association with the nerves of capillary vessel (CV), nerves of periinsular plexus, and nerves of acini. A few coarse AChE-positive nerves can be marked running parallel to the peripheral wall of major vessel. AChE- positive activity can be marked in ganglia (G), islet cells (L1, L2, and L3), and fine nerves of the acini, nerve bundle and coarse thick nerves. X.250.

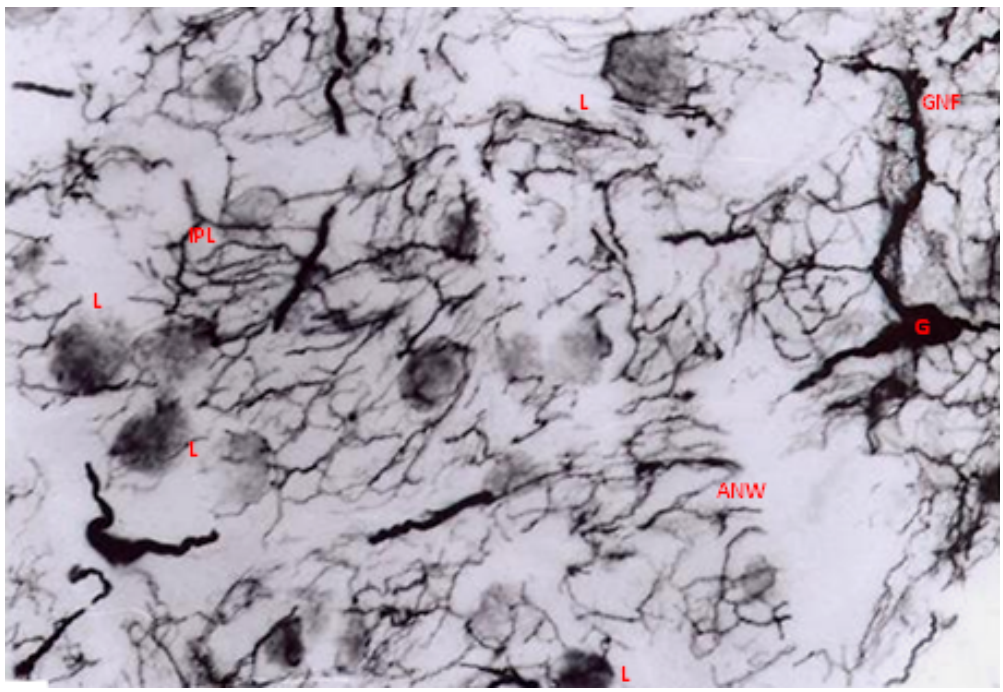

Fig.13. Shows distribution of, numerous AChE-positive islet (L) cells which were closely related and associated with nerves of AChE-positive nerve bundles and nerves of AChE-positive insular plexus (IPL). The nerves of acinar networks (ANW) formed the continuity, with the nerves of the periinsular networks, nerve bundles, and on the other end with preganglionic and postganglionic nerve fibres (GNF) of the AChE-positive ganglia (G). X.250.

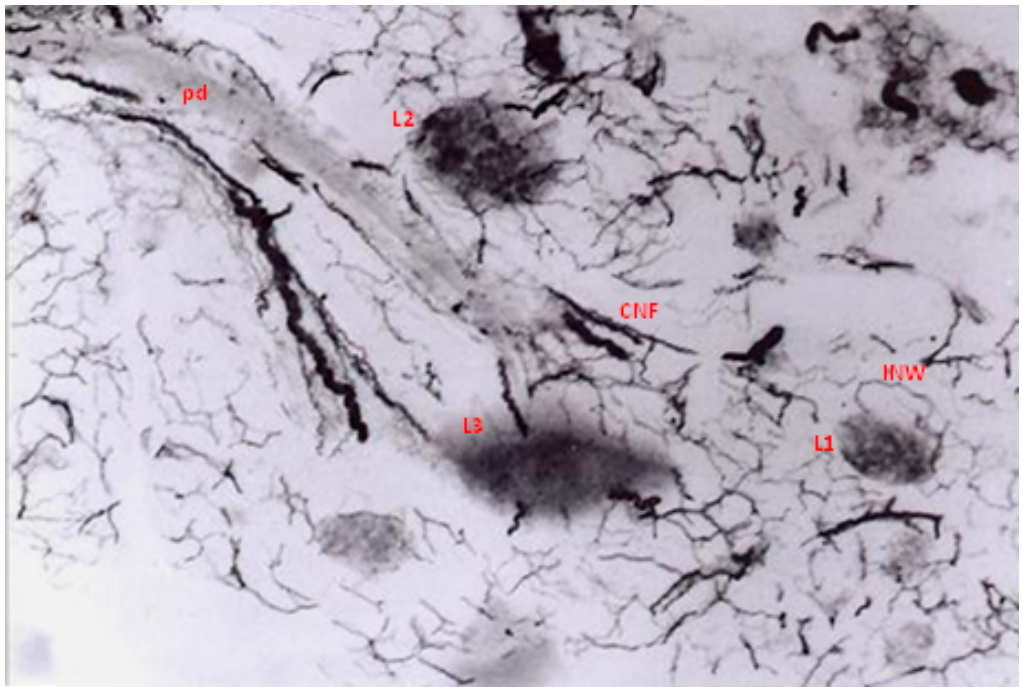

Fig. 14. Shows the distribution of AChE positive activity in pancreatic duct (Pd-in the intralobular region), and islet cells (L) which were recorded closely related and associated with the coarse nerve fibre (NF), nerves of networks of acini and periinsular network (INW) and nerves of peripheral wall of pancreatic duct (Pd). X.250.

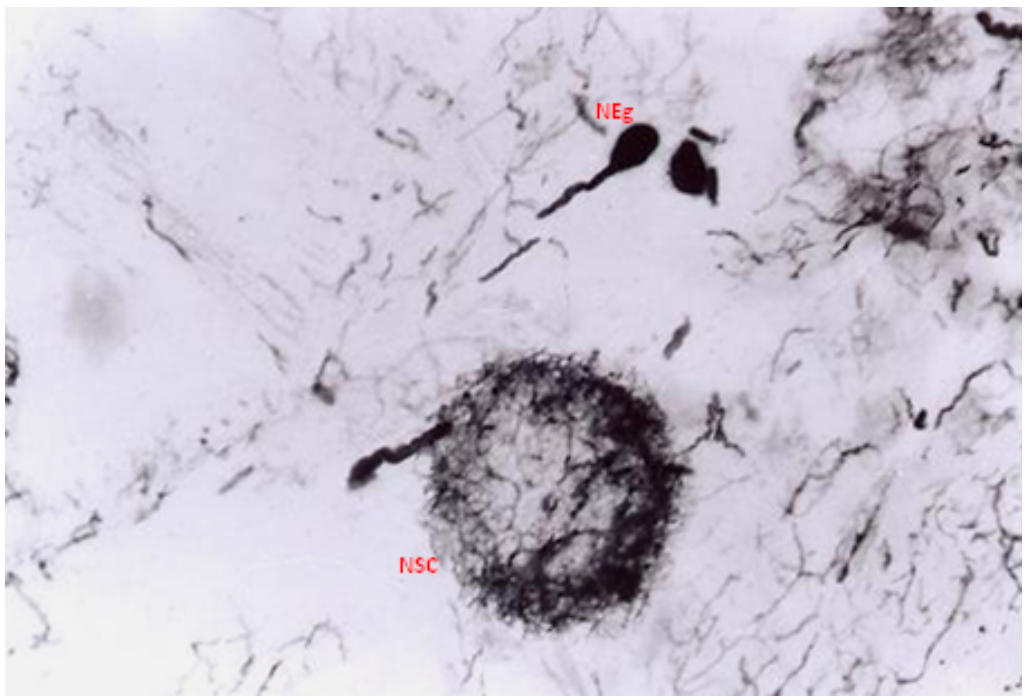

Fig.15. Shows the distribution of AChE-positive activity in the bulb-like nerve ending (NEg), nerve bundle and fine nerves and a rounded structure perhaps neuro-sensory centre (NSC). This centre was encircled by numerous nerves of myelinated and non-myelianted nature. They may act as 'neuro-transmitter centre' in the pancreas. X.250.

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