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Energy Dispersive X-ray Microanalysis of Zymogen Granules of Mouse Pancreas Using Fresh Air Dried Tissue Spread

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Summary. Electron microscopy of fresh air dried pancreas spreads revealed a well preserved ultrastructure of pancreatic exocrine cells. The zymogen granules were identified as homogeneously electron dense round bodies of approximately 300 nm in diameter, the same as were seen in fresh frozen dried sections. Energy dispersive X-ray microanalysis of zymogen granules and spots 200 nm distant from the granules used as controls disclosed peaks for chlorine, potassium and sulfur in all of five granules examined and in three of them, phosphorus was found in addition to the above elements. The results seem to suggest that pancreatic juice may be contributed from the electrolyte content of secretory granules in contrast to the general assumption that enzymes and electrolytes are separately excreted in pancreatic juice.

To know the distribution of diffusible substances in tissues at the ultrastructural level, X-ray microanalysis and dry mount autoradiography can be applied. For these methods, however, a new approach is required to prepare the ultrathin sections. In the conventional electron microscopic sections most of these substances are lost during section preparation, especially in fixation and dehydration.

Fresh frozen dried ultrathin sections appear ideal for this purpose and many attempts have been made to prepare them (CHRISTENSEN, 1969, 1971; APPLETON, 1969, 1974; TAKAYA, 1974). However, the artifacts, probably due to the ice crystal formation, are a disadvantage of the method although efforts have been made to avoid them with some success (TAKAYA, 1974).

The finding that fresh air dried tissue spread relatively well preserves the ultrastructure of cells has prompted the author to examine the intracellular distribution of elements using an energy dispersive X-ray microanalyzer. In our preliminary report presented in the Third Symposium of Scanning Electron Microscopy in Biology and Medicine (at Kuju, Japan; September 10, 1974) it was demonstrated that the preparation provided interesting information on the distribution of minerals within cells. In the present paper some results concerning pancreatic zymogen granules will be reported. It will be discussed that the results give a better understanding of the mechanism of electrolyte secretion in the exocrine pancreas.

Materials and Methods

Pancreas of albino mice (about 25 g) of both sexes were used as materials. Pieces of fresh pancreas tissue were spread on the collodion membrane covered copper grids (400 meshes) on the slide glass by pressing and pulling them with the edge of the cover glass. The spread was immediately dried in the air. The tissue spreads on the copper grids were observed by the electron microscope (Hitachi HS-7) with

acceleration voltage at 50 kV to examine the ultrastructure of cells without fixation, embedding or staining and to identify the zymogen granules. They were examined by the energy dispersive electron probe spectrometer (Nuclear Diodes, EDAX) with the scanning transmission apparatus (ASID) attached to an electron microscope (JEM-100C) with acceleration voltage at 60 kV. Five zymogen granules and five control spots about 200 nm distant from the margin of the respective granules were analyzed in this study. The beam spot was about 200 Å in diameter and the integrated pulse count was about for 180 sec.

Fig. 1. Fresh air dried spread of exocrine pancreatic cell cytoplasm as observed by the scanning transmission apparatus (ASID) attached to the electron microscope (JEM-100C) at acceleration voltage, 60 kV. Zymogen granules of various sizes are seen homogeneously electron dense. There are streaks of break surrounding the zymogen granules attributable to the shrinkage by dehydration. Mitochondria with electron dense granules are recognized. There are small electron dense granules of the size of about 800 Å in diameter which are probably the intracisternal granules. $\times 13,000$

Fig. 2-11. Spectrograms of the spots on the granules (**Fig. 2, 4, 6, 8 and 10**) and the ones ca. 200 nm distant from the margin of the respective granules (**Fig. 3, 5, 7, 9 and 11**), using the energy dispersive type spectrometer (Nuclear Diodes, EDAX) at acceleration voltage, 60 kV.

Observations and Results

Electron microscopy of the fresh air dried pancreas spreads disclosed that in very thin areas of the spread, the zymogen granules could be recognized clearly as homogeneously electron dense round bodies of the size of about 300 nm in diameter as were seen in the fresh frozen dried sections (CHRISTENSEN, 1971; TAKAYA, 1974). The limiting membrane of the granules usually could not be seen. Mitochondria were identified to have abundant electron dense granules of 300–600 Å in diameter which were dispersed within the homogenous content of medium electron density. Mitochondria showed quite various shapes from rounded to elongated. Spaces among these structures appeared clear and contained abundant small electron dense structures of the size of 100–200 Å in diameter. They showed in many places the arrangement of rough endoplasmic reticulum although the lines corresponding to membranes could not be seen. The nucleus appeared more electron dense than the surrounding cytoplasm and it contained electron dense heterochromatin at its periphery.

Electron microscopy of spreads by the scanning transmission apparatus (ASID) clearly revealed the zymogen granules and mitochondria containing peculiar dense granules. The zymogen granules were often found surrounded by a streak of transparent break attributable to shrinkage by dehydration (Fig. 1). Electron probe microanalysis by the energy dispersive type spectrometer disclosed peaks for sulfur, chlorine, potassium and phosphorus in three of five granules examined (Fig. 2, 8, 10) and sulfur, chlorine and potassium in one of them (Fig. 4) and only sulfur and chlorine in the other (Fig. 6). Five spots about 200 nm apart from the respective zymogen granules examined showed no peaks for any elements (Fig. 3, 5, 7, 11), except one in which the spot indicated an occurrence of phosphorus and chlorine (Fig. 9).

Discussion

Energy dispersive X-ray microanalysis does not give exact quantitative results but it provides a good survey of elements in tissue spreads and sections when the elements are present in relatively high concentrations.

It is difficult to decide the chemical combination of the elements which were detected in the zymogen granules by the present electron probe analysis. Most of the sulfur containing compounds found in the animals are organic compounds (DZIEWIAKOWSKI, 1962). Potassium and chlorine and presumably some phosphorus are ions and in the fluid phase in intact granules. Most of phosphorus is probably contained in the organic compounds.

Sulfur detected in the zymogen granules of the pancreatic acinar cell may be explained by the occurrence of sulfur containing enzymes in the secretory granules. Sulfur may be attributed in some part to mucopolysaccharides. By electron microscopic autoradiography using sulfate S^{35} , BERG and YOUNG (1971) showed that the Golgi apparatus of the pancreatic acinar cells is concerned with the manufacture of sulfated materials, probably sulfated mucopolysaccharides, from the inorganic sulfate, which are packed, together with the enzymes, in zymogen granules and released with them into the pancreatic secretion. In the previous study reported in the Symposium on Scanning Electron Microscopy, sulfur was detected in the secretory granules of various tissues including pituitary anterior lobe and posterior lobe as well as

neutrophil myelocytes of the bone marrow in the fresh air dried tissue spreads and in fixed embedded unstained sections.

It is interesting to consider the mineral elements detected in the zymogen granules in connection with the secretory process of pancreatic juice. It is generally believed that electrolytes and water are secreted separately from the enzymes. This assumption was reinforced by the dissociation in the secretion of electrolytes containing water and of the enzymes found in the juice of the pancreas stimulated by pancreozymin and secretin (HARPER, 1967). Enzymes are secreted in the acinar cells by the exocytotic process by opening the zymogen granules into the lumen of the acinus (PALADE et al., 1962; ICHIKAWA, 1965) and the electrolytes are generally accepted to be released from a different site by a different mechanism. JANOWITZ (1967) deduced chiefly from the experiments by secretin stimulation that the intercalated duct and centroacinar cells are concerned with the secretion of electrolytes and water.

The analysis of the micropuncture of the acinar fluid has provided an explanation of the mechanism of pancreatic juice secretion. MARGOS and McSHERRY (1971) reported the acinar fluid of the rat and the rabbit pancreas contained 149.0, 157.4 meq/l sodium, 6.7, 3.1 meq/l potassium, 11.6, 42.9 meq/l chloride and 37.8, 111.4 meq/l bicarbonate ions respectively. They demonstrated that concentrations of sodium and potassium ions are the same as in the plasma and each is independent of the flow rate of the juice. It was also shown that chloride and bicarbonate are exchanged in the course of the juice excretion. The result also confirmed the assumption of THAYSEN (1960) and JANOWITZ (1967) that the initial fluid is chloride-containing, and has suggested the acinar cells are also concerned with the secretion of electrolytes (HARPER, 1967). According to the secretion mechanism of the zymogen granules by electron microscopy (PALADE et al., 1962) and the present electron probe analysis of the zymogen granules, it is conceivable that chlorine and potassium could contribute to the initial fluid. However, further study is needed to decide whether these ions are secreted only by the exocytotic process of the zymogen granules or in conjunction with the other mechanism.

Mineral content of zymogen granules of the exocrine pancreas was studied by microincineration. The granules were found ash free in spodograms by most authors (KURSZYNSKI, 1966). Some authors reported heavy deposits in the zymogen granules in the sections prepared by the freeze-drying method and incineration using a specially devised electron induced micro-incineration apparatus. Heavy deposits were attributed to calcium and magnesium. Sodium, potassium and chlorine were believed to have been lost due to the heat and electron bombardment (see the review by KURSZYNSKI, 1966). Calcium and magnesium which were not detected in the present experiment may be disclosed by increasing the counting time and using the improved spectrometers.

As to sodium, the peaks at the energy level of sodium specific X-ray in two of five zymogen granules do not necessarily indicate the presence of sodium in the granules, although no peaks were demonstrated in the rest of the granules and in the controls. Half of the sodium specific X-ray fails to pass the beryllium window to be detected by the diodes. The difficulty in the interpretation of the peaks for sodium is increased by the fact that the indifferent pulse or noise from the white X-ray shows higher peaks at the elements of lower atomic number. Copper L alpha line also

disturbs the identification of peaks for sodium. Use of other grids than copper may solve the problem.

The uneven thickness of the spread in different places may disturb the interpretation of the X-ray microanalysis. The granules probably remain as thickened structures, whereas the areas between them might be stretched very thin. Electron beams can activate more mass in the thick places than in the thin portions so that more elements are liable to be detected in zymogen granules than in the thin portions. Therefore the exact distribution of the elements could not be obtained by this method. Analysis using the fresh frozen dried ultrathin sections will provide more exact information on the distribution of the elements.

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新鮮伸展乾燥標本を用いた膵外分泌顆粒のエネルギー分散型 X線マイクロアナライザーによる分析

高 屋 憲 一

膵臓外分泌組織を新鮮な状態で伸展して空気中で急速に乾燥させると、微細構造が良く保存され、いろいろな構造が観察可能であった。外分泌顆粒は均質な高密度の内容をもつ円形の直径約 300 nm の構造物として認められ、それは新鮮凍結超薄切片での所見と同じである。エネルギー分散型X線マイクロアナライザーを用いて 5 つの外分泌顆粒とその縁から 200 nm はなれた点を対照として分析すると、顆粒には S, Cl, K にピークが見られ、いくつかの顆粒には P も認められた。対照には特殊な元素のピークはほとんど見られなかった。外分泌顆粒は開口分泌によりその内容を腺腔に放出するが、電解質は酵素とは別に分泌されると従来考えられている。しかし顆粒に K や Cl が検出されることは、これらの電解質も顆粒の内容と共に腺腔に分泌されることを示唆するものと考えられる。

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