Virchows Arch. A Path. Anat. and Histol. 362, 231—245 (1974)
© by Springer-Verlag 1974

The Significance of Calcium in Insulin Secretion

Ultrastructural Studies on Identification and Localization of Calcium in Activated and Inactivated B Cells of Mice*

H.-J. Schäfer and G. Klöppel

Institute of Pathology, University of Hamburg (Director: Prof. Dr. G. Seifert)

Received November 2, 1973

Summary. Calcium plays an important role in the release of insulin. When the GBHA [glyoxal bis (2-hydroxyanil)] reaction is employed, calcium can be clearly demonstrated by light microscope in pancreatic islets of mice. The specificity of this finding has now been proved by elemental X-ray analysis. Electron microscopically, certain cations can be visualized by a precipitation technique using potassium pyroantimonate as the precipitating agent. In the B cell of mice this technique reveals a characteristic precipitation pattern. Elemental X-ray analysis suggests that the precipitates contain high amounts of calcium. The pattern of the precipitates changes dependent on the functional state of the B cell. In normoglycemia the deposits are mainly associated with the granule membranes, the cell membranes and the cytoplasmic matrix. In hypoglycemia there is a shift of precipitates into the endoplasmic reticulum and the mitochondria, which are thought to be storage organelles for intracellular calcium. The deposits within the halos of the numerous secretory granules, are diminished. In hyperglycemia there is a marked ion translocation across the cell membrane to its inner surface and particularly into the halos of the secretory granules, while the deposit content of mitochondria and endoplasmic reticulum is decreased. Within the saccules of the secretory granules, the deposits sometimes seem to impregnate a filamentous network, which encloses the secretory granule and cannot be seen by conventional electron microscopical preparations. The morphological data suggest that emiocytosis of hormone granules is associated with a release of cellular calcium. The presented observations in treated and untreated animals extend and support the conceptions on the specific role of calcium within the insulin releasing mechanism of the B cell.

Zusammenfassung. Calcium spielt eine wichtige Rolle bei der Insulinsekretion. Lichtmikroskopisch läßt sich unter Anwendung der GBHA [Glyoxal bis (2-hydroxyanil)]-Methode Calcium eindeutig in den Pankreasinseln der Maus nachweisen. Die Spezifität dieses Befundes wurde durch die Röntgenelementaranalyse gesichert. Elektronenmikroskopisch können bestimmte Kationen durch eine Präzipitationstechnik mit Hilfe von Kaliumpyroantimonat sichtbar gemacht werden. In der B-Zelle der Maus ergibt diese Technik ein charakteristisches Verteilungsmuster von Präzipitaten. Die Röntgenelementaranalyse zeigt, daß die Präzipitate große Calciummengen enthalten. Das Verteilungsmuster der Niederschläge verändert sich in Abhängigkeit von dem funktionellen Zustand der B-Zelle. Bei Normoglykämie treten die Ausfällungen hauptsächlich in Verbindung mit den Membranen der Hormongranula, den Zellmembranen und der Matrix des Cytoplasmas auf. Bei Hypoglykämie zeigt sich eine Verschiebung der Präzipitate in das endoplasmatische Retikulum und in die Mitochondrien, die als Speicherorganellen für das intracelluläre Calcium angesehen werden. Die Ausfällungen innerhalb der Halos der zahlreichen Sekretgranula sind vermindert. Bei Hyperglykämie ergibt sich eine erhebliche Ionenverlagerung durch die Zellmembran zu deren innerer Oberfläche und besonders in die Halos der Sekretgranula, während die Präzipitatmengen in Mitochondrien und endoplasmatischem Retikulum vermindert sind. Innerhalb der Vesikel der Sekretgranula scheinen die Präzipitate manchmal ein filamentöses Netzwerk zu imprägnieren,

* Supported by SFB 34, Hamburg.

welches das Sekretgranulum umhüllt und bei konventioneller elektronenmikroskopischer Präparation nicht sichtbar ist. Die morphologischen Befunde deuten darauf hin, daß die Emiocytose der Hormongranula mit einer Ausschleusung von cellulärem Calcium einhergeht. Die vorliegenden Beobachtungen an intakten Tieren erweitern und bestätigen die Konzeptionen über die spezifische Rolle des Calciums im Insulinsekretionsmechanismus der B-Zelle.

Introduction

Grodsky and Bennett (1966) and Milner and Hales (1967) first stated that extracellular cations, particularly sodium and calcium, are essential for the glucose-induced insulin release. Further studies on this field (Curry *et al.*, 1968; Hales and Milner, 1968; Dean and Matthews, 1970) pointed to the importance of calcium for the secretory process within the B cells. Investigating the dynamics of calcium in isolated islets placed in perifusion chambers, Malaisse, Malaisse-Lague and coworkers (Malaisse-Lague *et al.*, 1971; for further literature see Malaisse, 1973) have recently established that the glucose stimulus provokes an uptake of ^{45}Ca in B cells. It has therefore been suggested that accumulation of calcium in B cells possibly represents a link between the postulated glucoreceptor at the cell membrane and the release of insulin. Rasmussen (1970) assumed that intracellular calcium translocations and altered calcium sensitivity might induce a contraction of the microtubular system (Lacy *et al.*, 1968), which in turn may lead to the displacement of the beta granules to the cell surface and finally to emiocytosis. Malaisse (1973) and coworkers concluded from their recent studies that insulin release following B cell stimulation proceeds with an efflux of cellular calcium.

In order to elucidate the proposed involvement of intracellular calcium in the secretory process it is of interest to study the localization of calcium within the B cells, and to investigate the possible association to certain ultrastuructural cell sites.

The pyroantimonate technique, originally described by Komnick and Komnick (1963) for sodium detection, has also been found suitable for electron dense calcium precipitation. For this purpose it has been employed to several soft tissues (Legato and Langer, 1969; Yarom and Meiri, 1971; Thureson-Klein and Klein, 1971; Spicer and Swanson, 1972; Schäfer, 1973; Schäfer and Otto, in preparation). In some cases the lack of ion specificity can be compensated by combining with other methods and biochemical data. With respect to the B cell this method was employed by Herman *et al.* (1973) in an in vitro study with isolated pancreatic islets. They observed precipitates particulary in association with the beta granules and the cell membranes. By electron microprobe analysis calcium was found to be predominant in these preciptates. Following the addition of glucose to the incubation medium the precipitates appeared to be increased.

Extending these recent investigations we studied the distribution of calcium within B cells of intact normo-, hypo- and hyperglycemic mice. In a previous paper (Schäfer and Klöppel, 1973), employing the GBHA reaction for specific light microscopical demonstration of mobile calcium, we were able to show that the islets contain much intracellular calcium. Furthermore it could be demonstrated that the total amount of calcium within the islets obviously depends on the functional state of the B cells. Using the EDAX system for elemental X-ray analysis the specificity of these findings has now been further supported. The present ultrastructural studies on ion distribution by means of the pyroantimonate technique revealed characteristic precipitation patterns within the B cells, which also showed an association with different functional states of the cells.

Material and Methods

19 male Wistar mice, weighing 25–30 g, were kept on constant conditions and fed an Atromin® standard diet and Aq. fontana ad libitum. They were divided in three groups.

Group 1. 4 normoglycemic mice served as untreated controls.

Group 2. 4 mice were made hypoglycemic by injection of 0.5 mg crystalline bovine insulin (Hoechst, Frankfurt) i.p. They were sacrificed 30 minutes later.

Group 3. 6 mice were made hyperglycemic by intravenous injection of guinea-pig anti-insulin-serum (GPAIS). They were sacrificed 90, resp. 180 minutes later.

Blood samples were collected for determining the blood glucose levels (for details see Schäfer and Klöppel, 1973). Immediately after sacrifice the pancreas was removed and divided in three major parts. The first and second portion served for light microscopical examination, which included demonstration of B cell granulation and of mobile cellular calcium using the glyoxal bis (2-hydroxyanil) (GBHA) technique (Kashiwa, 1966). For details see Schäfer and Klöppel (1973).

The third portion of the pancreas was fixed for electron microscopical ion detection by rapid immersion into an ice-cold solution of 3% glutaraldehyde and 2% potassium-pyroantimonate (Gomba *et al.*, 1972). The pH of the solution had been adjusted to 7.3 by dropwise addition of 0.01 N acetic acid. After immersion the tissue was immediately cut into small pieces for better penetration of the fixation fluid. The fixation time was 2 hours. After rinsing in a solution of 2% potassium pyroantimonate and 5% sucrose (pH 7.3) the tissue was postfixed for 1 hour in a solution containing 1% OsO_4 and 2% potassium pyroantimonate as well as some drops of 0.01 N acetic acid for adjustment to pH 7.3. After rinsing in 7% sucrose for three times the tissue was dehydrated in ethanol and propylene oxide and embedded in Epon 812. 0,5 µ sections were stained with 1% toluidine blue for light microscopic observation. Ultrathin sections were cut on a Reichert ultramicrotome OM U2 by diamond knives, mounted on copper grids and visualized either unstained or stained with uranylacetate, resp. uranyl-acetate and lead citrate on a Zeiss EM 9 electron microscope.

X-ray analysis was performed in some paraffin and Epon sections. For comparison we used two systems: an energy-dispersive system (EDAX, Chicago) and a wavelength-dispersive system (JEOL JXA-50 A). Both systems were combined with a JEOL 50 A scanning electron microscope. The paraffine sections of pancreatic tissue were treated with GBHA for retention and light microscopic staining of mobile calcium (Schäfer and Klöppel, 1973). The sections were mounted on carbon slides. Orientation and direction of the electron beam was performed by light optical control. Sections of epoxy embedded material (golden interference colour), which was treated with pyroantimonate as described above, were mounted on copper grids and remained unstained. Orientation was performed by scanning transmission mode.

Results

Blood Glucose Values, Histopathological Findings and GBHA Staining

These results are briefly summarized (for details see Schäfer and Klöppel, 1973). In the untreated animals (Group 1) the blood glucose levels ranged from 120 to 170 mg/100 ml. The islets showing a distinct granule staining with aldehyde fuchsin exhibited a markedly positive GBHA reaction whereas the exocrine tissue remained nearly unstained (Fig. 1). — The mice injected with insulin (Group 2) showed blood glucose values from 60 to 90 mg/100 ml. The islets were heavily stained with aldehydefuchsin and GBHA. — In the mice injected with anti-insulin serum (Group 3) the mean increase of the blood glucose was approximately 100 mg/100 ml after 90 minutes and approximately 150 mg/100 ml after 180 minutes. The islets appeared normal and were not involved in polymorphcellular immune insulitis. Some B cells in the islets were slightly degranulated. In general the GBHA reaction had normal intensities. Only single islets revealed varying staining patterns with sometimes increased intensities.

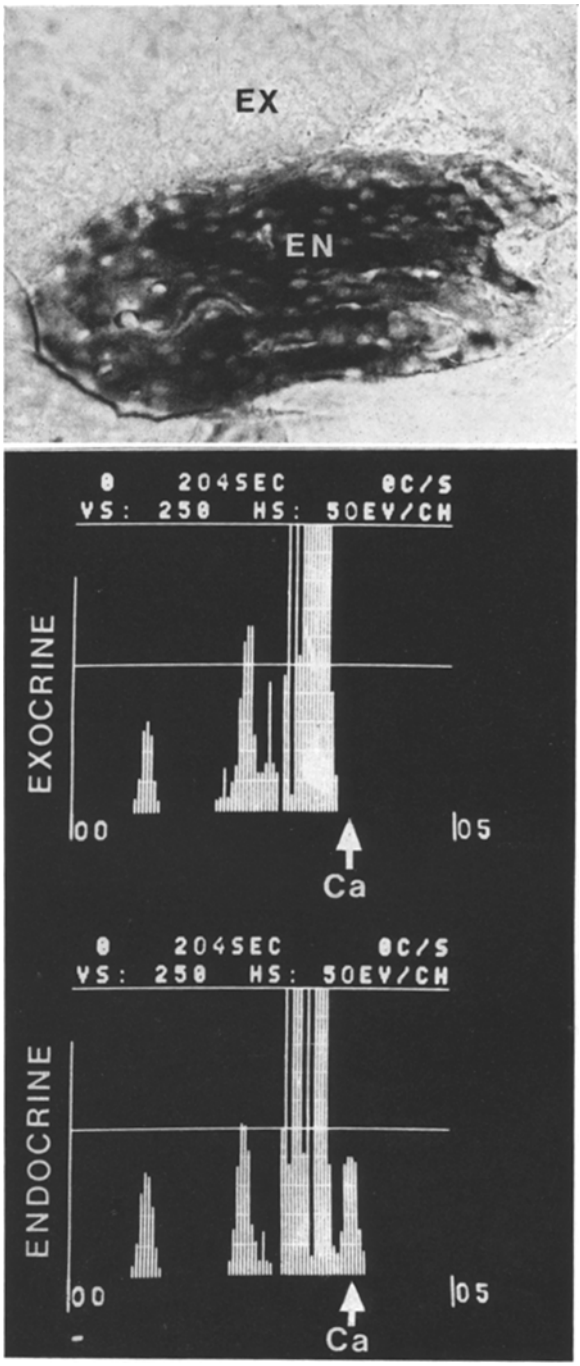

Fig. 1. Pancreas of untreated mouse: Elemental X-ray analysis confirming calcium specificity of the GBHA reaction. Upper part: islet with intense GBHA staining indicating a high calcium content in the endocrine tissue (*EN*). Negative reaction in the exocrine tissue (*EX*). $\times 300$. Lower parts: Elemental X-ray analysis of pancreatic tissue employing the EDAX system. Significant calcium content in the endocrine tissue. Absence of calcium in the exocrine tissue

Ultrastructural Ion Localization

The treatment of the pancreatic tissue with potassium pyroantimonate revealed many electron dense precipitates predominantly within and along the B cells. The round or oval shaped precipitates varied in size, and were sometimes arranged in small clusters. In general the precipitates were so small that an exact localization was possible. The ultrastructural preservation was good, i.e. secretory granules and membrane systems were well preserved. Clearest visualization of the dark precipitates was achieved in unstained sections, since the tissue structures could still be well recognized due to the osmification procedure. In stained and double stained sections differentiation between metallic material used for contrasting and pyroantimonate precipitates was sometimes difficult. In contrast to the abundant precipitates within B cells, less precipitates were observed within A or D cells. Dependent on their functional state the B cells showed characteristic distribution patterns of the precipitates.

In normoglycemia numerous small precipitates were found to be generally distributed in the cytoplasmic matrix and along the cytoplasmic membrane systems with modest impregnation of the endoplasmic reticulum. Some larger deposits were attached to the membranes of the secretory granules, few to the inner surface of the cell membrane.

Organelle bound deposits were mainly observed within some granule halos and sparsely within mitochondria. In the granule halos the dot-like precipitates are either in close contact to the granule itself or to the saccule membrane. Within the mitochondria the precipitates were fine and lay together in small clusters. Occasionally single precipitates were also found in association with the tubules and vesicles of the Golgi apparatus. The heterochromatin of the nucleus constantly exhibited some precipitates. Extracellular precipitation frequently occurred along the cell membrane with clear tracing of the cell boundaries.

In hypoglycemia (Group 2) the B cells revealed the signs of inactivation with increased density of secretory granulation and small Golgi complexes. The secretory granules were often separated from the cell membrane by a more or less distinct filamentous web. The precipitates showed an altered distribution pattern (Fig. 2). The main part of the deposits was localized in the cytoplasma between the numerous hormone granules. Close contact of the deposits to the granule membranes were only rarely observed, and halo deposits were almost absent. Moreover, very few precipitates were seen in association with the inner surface of the cell membranes. In contrast to the general disappearance of precipitates at these cell sites there was an increase of the deposits within the mitochondria. This increase corresponded to the increased cytoplasmic deposits, which seemed to be located as well in the cytoplasmic matrix as along the cytoplasmic membrane systems. Size and number of the precipitates in the nuclei remained unchanged.

In hyperglycemia (Group 3) the B cells showed the signs of acute activation with accentuation of the Golgi apparatus and the endoplasmic reticulum, as well as with a decreased number of granules. The secretory granules are predominantly located in the peripheral cell parts or within the filamentous web at the cell surface having close contact with the cell membrane (Fig. 3). All these alterations were most distinct 180 minutes after administration of the anti-insulin serum. The precipitation pattern of the pyroantimonate crystals show the most marked

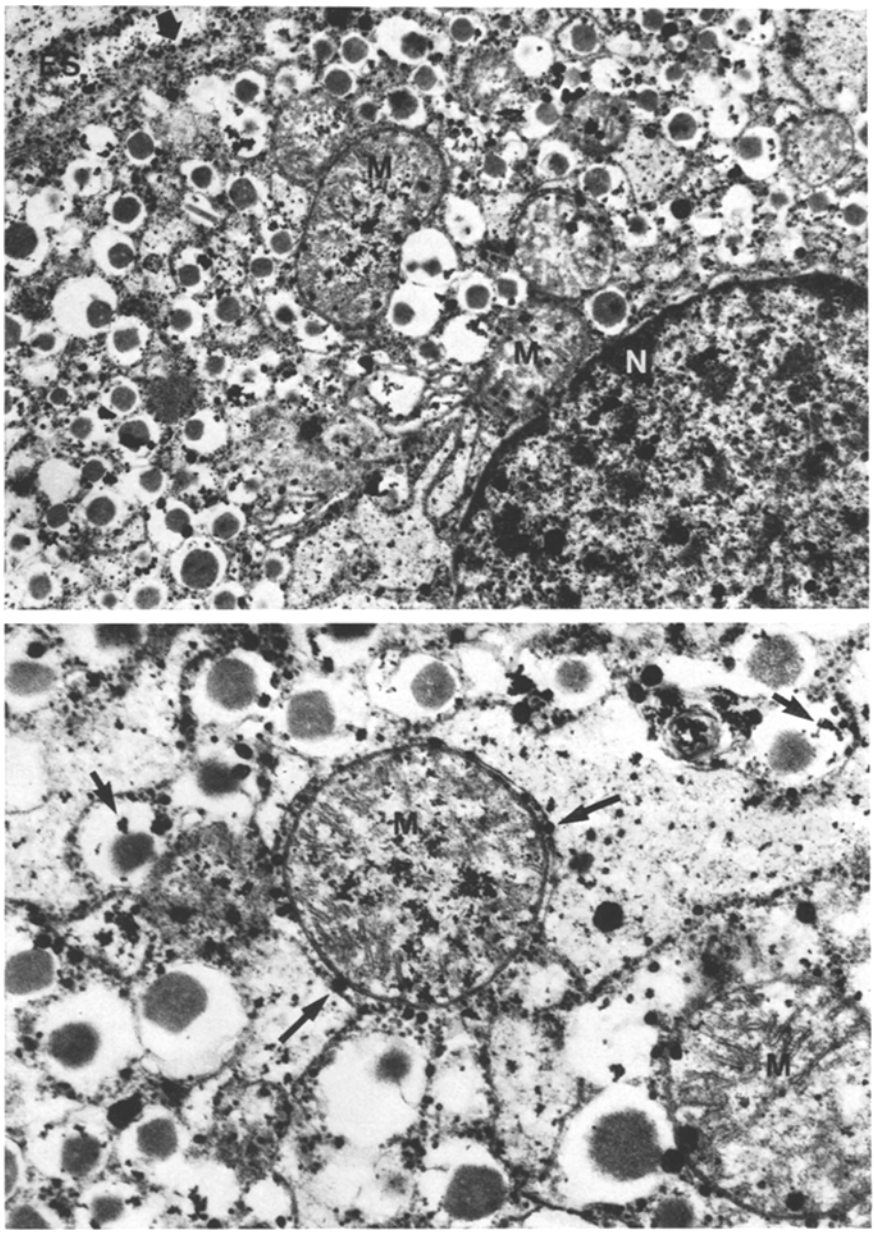

Fig. 2a and b. Inactivated B cell of mouse, 30 min after application of insulin: abundant granular precipitates within mitochondria (*M*) and at the endoplasmic reticulum (long thin arrow). Only few deposits within the halos of the secretory granules (short thin arrow) and at the cell membrane (thick arrow). Modest precipitation within the euchromatin of the nucleus (*N*) and in the extracellular space (*ES*). K-pyroantimonate reaction, uranylacetate and lead citrate staining. a) × 14000 b) × 28000

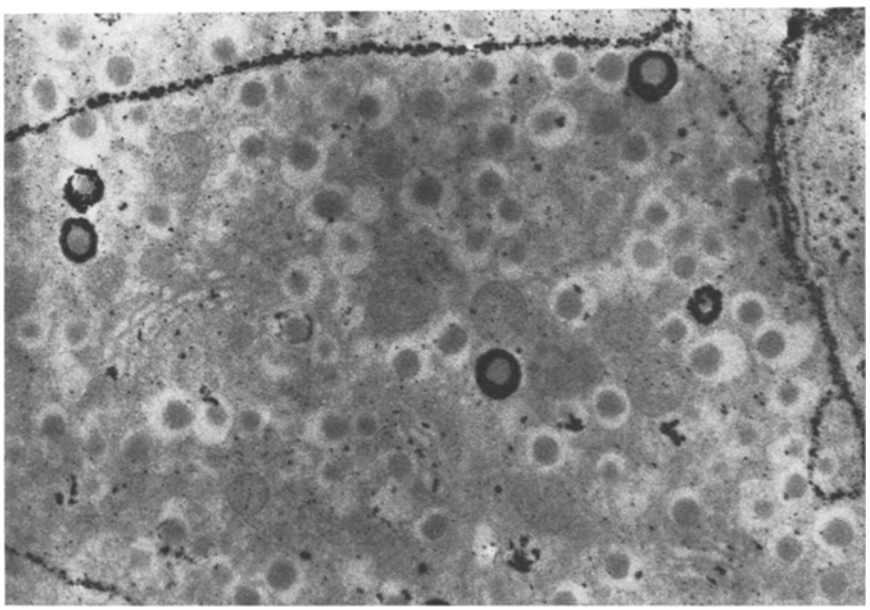

Fig. 3. Activated B cell of mouse, 180 min after application of anti-insulin serum: secretory granules in close contact with the cell membranes. Heavy precipitation within the halos of some granules and along the cell membranes. Decreased deposits in mitochondria and the cytoplasmic membrane systems. K-pyroantimonate reaction, unstained. × 14000

differences, when compared with the normal state. In particular there was an increase in frequency and size of the ion deposits within the halos. These deposits often surrounded the secretory granule filling out the whole halo (Fig. 3–6). They were attached to both the granules and the granule membranes. In some halos with less dense precipitates the pyroantimonate crystals appear to precipitate along a filamentous network within the halo, which encloses the granules (Fig. 6). In general, these heavy precipitates within granule saccules could only sporadically be observed in each B cell, but occurred regularly. They were distributed throughout the cytoplasma, although we occasionally got the impression that the granules were localized along a line leading straight to the cell surface (Fig. 4). Sometimes, especially near the cell membrane, we observed an increased number of precipitates attached to the outer surface of the granule saccule. In addition to the increased precipitation at the granule sites there was a marked increase in size and frequency of the ion deposits along the cell membranes. Both, the outer and particularly the inner surface of the cell membranes were bordered with dot-like precipitates. On the other hand decreased precipitation was found within the mitochondria and at the endoplasmic reticulum. The deposits within nuclei and Golgi apparatuses did not reveal significant alterations.

Elemental X-ray analysis was carried out for two reasons: (1) to confirm the calcium specificity of the highly positive GBHA reaction within the islet, (2) to ensure a significant amount of calcium within the pyroantimonate precipitates.

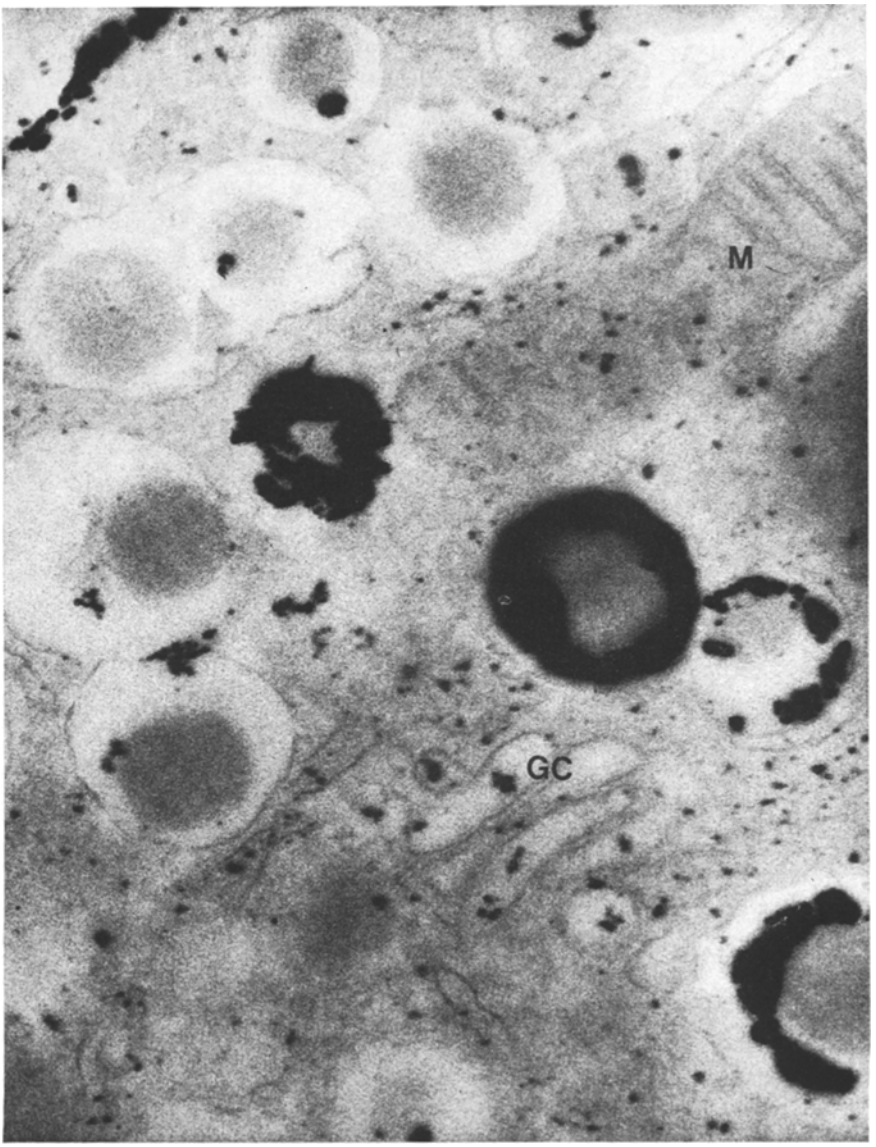

Fig. 4. Activated B cell of mouse, 180 min after application of anti-insulin serum: compact precipitates within the halos of some granules. The impregnated granules lie along a line straight to the cell membrane. Golgi complex (*GC*), mitochondrium (*M*). K-pyroantimonate reaction, unstained. × 60000

2 μ thick paraffin sections of the GBHA treated tissue were submitted to X-ray analysis with the EDAX system. For comparison the electron beam was directed to GBHA positive islets and GBHA negative exocrine pancreatic tissue. The obtained spectra were given in Fig. 1. Measurements within the islets revealed

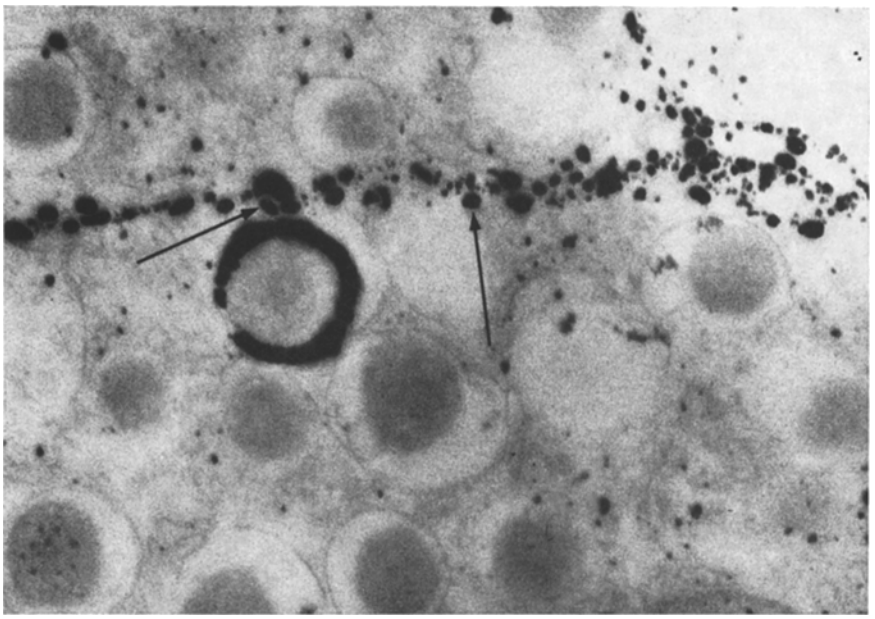

Fig. 5. Activated B cell of mouse, 180 min after application of anti-insulin serum: Impregnated granule in contact with the cell membrane. Precipitates at the outer and particularly at the inner surface of the cell membrane (↗). K-pyroantimonate reaction, unstained. × 50000

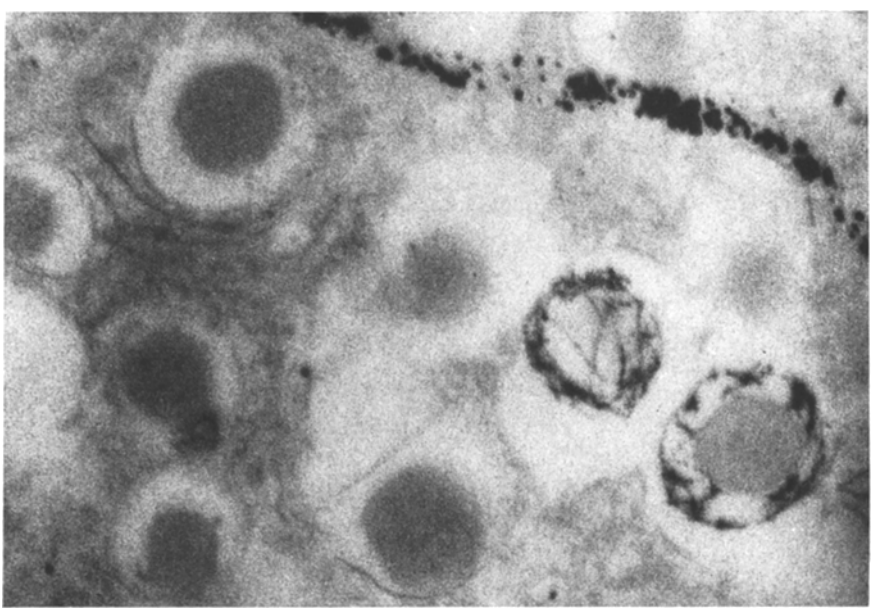

Fig. 6. Activated B cell of mouse, 180 min after application of anti-insulin serum: less intense precipitation often results in granular deposits within the halos. The precipitates seem to impregnate a network of filamentous material enclosing the hormone granules. K-pyroantimonate reaction, unstained. × 50000

a significant calcium peak, while this peak was completely absent in the surrounding exocrine tissue. The other elements, also detected by this method were constantly found in all areas of the specimen, even in those parts, which did not contain pancreatic tissue. This fact indicates that these latter elements originate from materials used to prepare and mount the tissue sections.

500 Å thick epoxy sections of pyroantimonate treated tissue did not reveal significant results when submitted to X-ray analysis employing the EDAX system. This failure might have been due to a specimen-detector distance, which was not optimal. Further explanation may result from the fact that the sections were too thin and that the energy resolution of the used system is limited. Applying the wavelength-dispersive system the limiting factor was a severe damage of the specimens, which rapidly occurred during examination. Therefore, we were only able to demonstrate a high calcium content of the precipitates particularly at the cell membrane and at the granule sites, but were unable to determine the exact quantitative relation to other ions in these precipitates.

Discussion

It is well established that the presence of calcium is essential for glucose induced insulin release both in animals (Grodsky and Bennett, 1966; Milner and Hales, 1967; Rasmussen, 1970; Malaisse, 1973) and in man (Heinze *et al.*, 1973). It was clearly shown by the work of Malaisse-Lagae, Malaisse and coworkers (for lit. see Malaisse, 1973) that an accumulation of calcium occurs in B cells following glucose stimulation. These authors conclude from their studies that intracellular calcium might trigger insulin release. Since these detailed concepts mainly derive from biochemical in vitro studies, it would be valuable to supplement them by morphological in vivo investigations. Thus using special light and electron microscopical techniques we studied the distribution and localization of calcium within the islet system of treated and untreated mice. Employing the GBHA technique (Kashiwa, 1966) for demonstrating calcium at the light microscopical level, we found very intense reactions of the islet tissue, suggesting a high calcium content of the B cells, while the exocrine tissue remained almost unstained (Schäfer and Klöppel, 1973).

The calcium-specificity of the GBHA reaction has been examined by Kashiwa (1966) employing chelating agents. Our studies on pancreatic tissue confirmed that the GBHA reaction really demonstrates mobile calcium. This was achieved by the observation that the GBHA stainable material was completely removed by aqueous fixation fluids, whereas it was retained by addition of oxalate to the fixation medium (Schäfer and Klöppel, 1973). Furthermore we were now able to prove the calcium specificity of the GBHA technique by elemental X-ray analysis using the EDAX system. From this it can be stated that the intensity of the GBHA reaction parallels the amount of cellular calcium.

Ultrastructurally calcium in addition to other cations and certain substances can be visualized, when it is precipitated by potassium pyroantimonate (Komnick and Komnick, 1963; Legato and Langer, 1969; Garfield *et al.*, 1972). The pyroantimonate can be used without any additional fixation compound (Tandler and Kierszenbaum, 1971) or in combination with OsO_4 or glutaraldehyde. Phosphate

buffers should be avoided, because they seem to interact in the precipitation reaction (Shiina *et al.*, 1970; Torack and Lavalle, 1970). Fixation by OsO_4 results in a bad preservation of secretory granules as noted by Herman *et al.* (1973). Therefore, we employed a glutaraldehyde fixation with subsequent OsO_4 fixation. In contrast to other authors we added the pyroantimonate not only to the OsO_4 but also to the glutaraldehyde (Gomba *et al.*, 1972) in order to avoid ion translocation by precipitating the ions already during their first contact with an aqueous fluid. The fixation fluids remained unbuffered and had only to be slightly adjusted to pH 7.3. An important preparation step seems to be an extensive rinsing in sucrose before dehydration, because ethanol precipitates the diluted potassium pyroantimonate in an unspecific way. Employing this procedure we achieved a good morphological preservation with characteristic and reproducable precipitation patterns.

In isolated islets of mice and rabbits Herman *et al.* (1973) observed numerous precipitates after pyroantimonate treatment of the tissue. These precipitates were predominantly localized at the cell membrane and the secretory granules of the B cells. They were found by X-ray analysis to contain particularly calcium. Studying the ion precipitation in pancreatic tissue of untreated normoglycemic mice we observed similar patterns of precipitates within the B cells. In addition to precipitates in the cytoplasmic matrix, at the microtubules, and at the outer surface of the secretory granule membrane, we found a modest deposition within the mitochondria, at the endoplasmic reticulum and sparsely within the Golgi apparatus. Modest deposition of precipitated material was also seen within the halos of the secretory granules. Ion impregnation of the cell membrane was predominantly at the extracellular site. In contrast to these marked and extensive precipitation pattern of the B cells the other islet cells and particularly the exocrine pancreatic cells hardly contained any deposits except a modest impregnation of the surrounding extracellular spaces. The most important disadvantage of the pyroantimonate procedure is its limited specificity. Additional X-ray analysis of the precipitates would therefore be very favourable (Mizuhira, 1973). In our study we were only able to identify calcium qualitatively within the precipitates along the secretory granules and the cell membrane, but not quantitatively because of the technical difficulties mentioned above. However, the portion of calcium within the deposits is thought to be high, for the number and the size of the precipitation products appear to correlate with the intensity of the calcium-specific GBHA reaction in the same functional state of the B cells. In the hypoglycemic mice GBHA reaction resulted in a marked staining of the islet tissue. Now the diffused precipitates in the cytoplasma, at the endoplasmic reticulum and within the mitochondria increased, while deposits within the halo of the abundant secretory granules and along the inner surface of the cell membrane only rarely occurred. This indicates that the inhibition of insulin release is obviously associated with a removal of cellular calcium from the secretory apparatus into the cytoplasmic membrane systems and the mitochondria.

Further support that the pattern of precipitates within the B cells somehow parallels the secretory process is particularly drawn from our findings in activated B cells. Herman *et al.* (1973) studied this functional state in isolated islets following glucose incubation. We investigated B cells in animals, which were

made hyperglycemic by a single injection of antiinsulin serum (Klöppel *et al.*, 1971). The results of both examinations generally correspond. The precipitates in mitochondria and the ergastoplasma decreased, while those within the granule saccules and at the inner surface of the cell membrane showed a marked increase. These results suggest that there may be a shift of calcium from the endoplasmic reticulum and the mitochondria into the secretory granules, and from outside of the cell membrane to its inner surface. Such an altered binding of calcium to cell membranes after certain hormonal influences is for example well established in liver cells (Shlatz and Marinetti, 1972). Glucagon and insulin seem to have there opposite effects.

The accumulation of deposits within the halo of some secretory granules was particularly striking. Often the granules were completely surrounded by the dense precipitates. Attention should be drawn to the finding that sometimes the precipitates seem to impregnate a fine filamentous matrix within the halos. The nature or even the existance of this matrix is not yet clear, for in the conventional electron microscopical pictures these halos are found as empty spaces. Further studies will have to elucidate this phenomenon. Sometimes the granules involved in precipitation reactions appeared to be localized along a line leading from central parts of the cell straight to the cell surface in a manner, as if granules, which will soon be discharged by emiocytosis, are transported to the cell border along certain ways. However, the microtubular system, held responsible for the translocation of the beta granule (Lacy *et al.*, 1968) and thought to be activated by intracellular calcium fluxes (Rasmussen, 1970), did not show any significant alteration in ion deposit contents during activation.

From our morphological data it may be possible to get some suggestive conclusions about the intracellular calcium fluxes during inactivation and activation of the B cells (Fig. 7). The increase and, moreover, the new appearance of deposits along the inner surface of the glucose-stimulated B cell, as well as the lack of such precipitates in B cells of hypoglycemic mice suggest that there is a calcium translocation across the membrane during stimulation of the glucoreceptor. These observations are in good correspondance with the biochemical data mentioned above (Malaisse, 1973). Once in the cell calcium can be stored in starage organelles. Well-known storage organelles of cellular calcium are the endoplasmic reticulum (Hasselbach, 1972; Fleckenstein, 1972) and the mitochondria. The mitochondria are the sites of a slowly exchangeable intracellular calcium pool (Borle, 1971). They contain energy-dependent calcium pumps within their membranes and can accumulate high amounts of calcium and store it in inactive form (Matthews *et al.*, 1971). They are thought to act as intracellular buffer systems against toxically high cytosolic calcium levels (Borle, 1971) and show characteristic alterations of their calcium-rich deposits as a response to altered cellular calcium metabolism (Matthews *et al.*, 1971; Sampson *et al.*, 1970; Schäfer, 1973).

Our findings provide evidence that these storage organelles accumulate calcium during inactivation of the B cell. In contrary acute stimulation of the B cells seems to release calcium from these organelles. This finding supports biochemical data given by the studies of Rasmussen. Furthermore, the release of calcium from the mitochondria was proposed by this author to be an effect of cyclic AMP (for lit. see Rasmussen, 1970; Rasmussen and Allen, 1973). An increase of cyto-

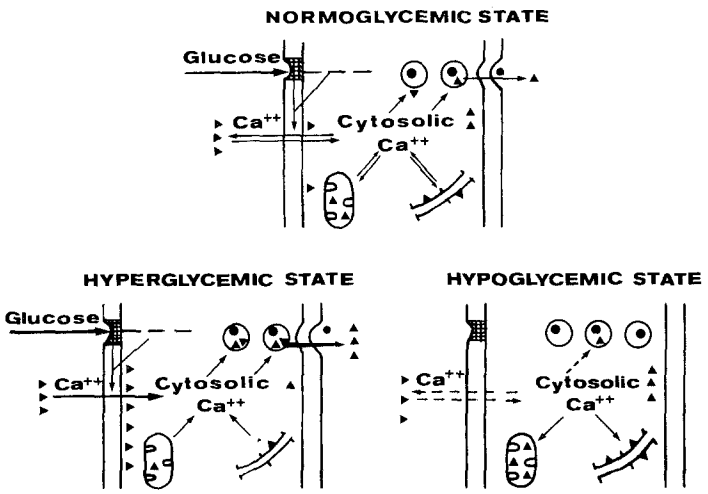

Fig. 7. Scheme of the proposed calcium shifts in activated and inactivated B cells on the basis of ultrastructural data. ▶ Precipitates

solic calcium may therefore result from two mechanisms: (1) The stimulation of the glucoreceptor mediates an increase of the calcium influx across the cell membrane. (2) The cyclic AMP mediates a calcium release from storage organelles of cellular calcium. According to the presented morphological findings during stimulation this increased cytosolic calcium seems to accumulate predominantly within the granule saccules. The effect and the function of the calcium accumulation in the hormone granules remain yet unknown. From the site of accumulation within the granule halos it may only be concluded that these calcium deposits are released together with the granule content by emiocytosis into the extracellular spaces. Evidence for this assumption is provided by recent biochemical data showing that a marked calcium efflux occurs, when glucose-induced insulin release starts (Malaisse *et al.*, 1973, in press). Prolonged stimulation might therefore lead to a loss of intracellular calcium. First investigations in mice (Schäfer and Klöppel, 1973; Klöppel and Schäfer, in preparation) submitted to prolonged hyperglycemia, indeed, show a striking calcium depletion of the hyperactive B cells.

In summarizing the morphological results on the localization of calcium in certain functional states of the B cell it can be stated that light microscopically marked alterations of cellular calcium content could only be observed with certainty after prolonged stimulation. Electron microscopic methods, however, demonstrated significant and marked intracellular calcium shifts also in the states of acute activation and inactivation of the B cells. Most striking findings during stimulation are the marked inward translocation of membrane associated calcium, the shift of the intracellular calcium from the storage organelles to the secretory granules and finally its outward transport along with the granules. The data, however, do not permit any conclusions with regard to the actual function and effect of calcium on the secretory structures of the B cell.

The authors wish to thank Miss Zeiger, Miss Fischer and Mrs. Baack for their skilful technical assistance. We are also indebted to Mr. and Mrs. Liebel in Kontron Application Laboratories, München, for effective cooperation in carrying out the X-ray analyses.

References

Borle, A.B.: Calcium transport in kidney cells and its regulation. In: Cellular mechanisms for calcium transfer and homeostasis, ed. by G. Nichols, and R.H. Wasserman, p. 151–174. New York-London: Academic Press 1971

Curry, D.L., Bennett, L.L., Grodsky, G.M.: Requirement for calcium ion in insulin secretion by the perfused rat pancreas. Amer. J. Physiol. **214**, 174–178 (1968)

Dean, P.M., Matthews, E.K.: Electrical activity in pancreatic islet cells: effect of ions. J. Physiol. (Lond.) **210**, 265–275 (1970)

Fleckenstein, A.: Physiologie und Pharmakologie der transmembranären Natrium-, Kalium- und Calcium-Bewegungen. Arzneimittel-Forsch. (Drug Res.) **22**, 2019–2028 (1972)

Garfield, R.E., Henderson, R.M., Daniel, E.E.: Evaluation of the pyroantimonate technique for localization of tissue sodium. Tissue & Cell **4**, 575–589 (1972)

Gomba, S., Szabo, J., Soltesz, B.M.: Electronhistochemical observations on the localization of sodium ions in glomerular basement membrane. Acta histochem. (Jena) **42**, 367–372 (1972)

Grodsky, G.M., Bennett, L.L.: Cation requirements for insulin secretion in the isolated perfused pancreas. Diabetes **15**, 910–913 (1966)

Hales, C.N., Milner, R.D.G.: The role of sodium and potassium in insulin secretion from rabbit pancreas. J. Physiol. (Lond.) **194**, 725–743 (1968)

Hasselbach, W.: Die sarkoplasmatische Calciumpumpe. Arzneimittel-Forsch. (Drug Res.) **22**, 2028–2036 (1972)

Heinze, E., Fussgänger, R., Teller, W.M.: Influence of calcium on insulin secretion in newborns. Pediat. Res. **7**, 100–102 (1973)

Herman, L., Sato, T., Hales, C.N.: The electron microscopic localization of cations to pancreatic islets of Langerhans and their possible role in insulin secretion. J. Ultrastruct. Res. **42**, 298–311 (1973)

Kashiwa, H.K.: Calcium in cells of fresh bone stained with glyoxal bis (2-hydroxyanil). Stain Technol. **41**, 49–55 (1966)

Klöppel, G., Altenähr, E., Freytag, G.: Elektronenmikroskopische Untersuchungen zur experimentellen Insulitis nach Injektion von Anti-Insulin-Serum. Virchows Arch. Abt. A Path. Anat. **354**, 324–335 (1971)

Klöppel, G., Schäfer, H.-J.: In preparation

Komnick, H., Komnick, U.: Elektronenmikroskopische Untersuchungen zur funktionellen Morphologie des Ionentransportes in der Salzdrüse von Larus argentatus. Z. Zellforsch. **60**, 163–203 (1963)

Lacy, P.E., Howell, S.L., Young, D.A., Fink, C.J.: New hypothesis of insulin secretion. Nature (Lond.) **219**, 1177–1179 (1968)

Legato, M.J., Langer, G.A.: The subcellular localization of calcium ion in mammalian myocardium. J. Cell Biol. **41**, 401–423 (1969)

Malaisse, W.J.: Insulin secretion: multifactorial regulation for a single process of release. Diabetologia **9**, 167–173 (1973)

Malaisse, W.J., Brisson, G.R., Baird, L.E.: The stimulus-secretion coupling of glucose-induced insulin release. X. Effect of glucose on 45 calcium efflux from perifused islets. Amer. J. Physiol., in press (1973)

Malaisse-Lagae, R., Malaisse, W.J.: Stimulus-secretion coupling of glucose-induced insulin release. III. Uptake of 45 calcium by isolated islets of Langerhans. Endocrinology **88**, 72–80 (1971)

Matthews, J.L., Martin, J.H., Arsenis, C., Eisenstein, R., Kuettner, K.: The role of mitochondria in intracellular calcium regulation. In: Cellular mechanisms for calcium transfer and homeostasis, ed. by G. Nichols, and R.H. Wasserman, p. 239–255. New York-London: Academic Press 1971

Milner, R.D.G., Hales, C.N.: The role of calcium and magnesium in insulin secretion from rabbit pancreas studied in vitro. Diabetologia **3**, 47–49 (1967)

Mizuhira, V.: Demonstration of the elemental distribution in biological tissues by means of the electron microscope and electron probe X-ray microanalyzer. Acta histochem. cytochem. **6**, 44–52 (1973)

Rasmussen, H.: Cell communication, calcium ion, and cyclic adenosine monophosphate. Science **170**, 404–412 (1970)

Rasmussen, H., Allen, J.E.: The calcium cyclic AMP relationship in cell activation. 9. Schwabinger Symposion der Forsch,gruppe Diabetes vom 21. 6. 73 in München

Sampson, H.W., Matthews, J.L., Martin, J.H., Kunin, A.S.: An electron microscopic localization of calcium in the small intestine of normal, rechitic and vitamin-D-treated rats. Calcif. Tiss. Res. **5**, 305–316 (1970)

Schäfer, H.-J.: Ultrastructure and ion distribution of the intestinal cell during experimental vitamin-D deficiency rickets in rats. Virchows Arch. Abt. A Path. Anat. **359**, 111–123 (1973)

Schäfer, H.-J., Klöppel, G.: Demonstration of calcium in pancreatic islets. Light microscopical observations in activated and inactivated B-cells of mice. Virchows Arch. Abt. A Path. Anat. in press

Schäfer, H.-J., Otto, H.F.: In preparation

Shiina, S., Mizuhira, V., Amakawa, T., Futaesaku, Y.: An analysis of the histochemical procedure for sodium ion detection. J. Histochem. Cytochem. **18**, 644–649 (1970)

Shlatz, L., Marinetti, G.V.: Hormone-calcium interactions with the plasma membrane of rat liver cells. Science **176**, 175–177 (1972)

Spicer, S.S., Swanson, A.A.: Elemental analysis of precipitate formed in nuclei by antimonate-osmium tetroxide fixation. J. Histochem. Cytochem. **20**, 518–526 (1972)

Tandler, C.J., Kierszenbaum, A.L.: Inorganic cations in rat kidney. Localization with potassium pyroantimonate-perfusion fixation. J. Cell Biol. **50**, 830–839 (1971)

Thureson-Klein, A., Klein, R.L.: Cation distribution and cardiac jelly in early embryonic heart: a histochemical and electron microscopic study. J. Molec. Cell. Cardiol. **2**, 31–40 (1971)

Torack, R.M., Lavalle, M.: The specificity of the pyroantimonate technique to demonstrate sodium. J. Histochem. Cytochem. **18**, 635–643 (1970)

Yarom, R., Meiri, U.: N lines in striated muscle: a site of intracellular Ca^{2+}. Nature (Lond.) New Biol. **234**, 254–256 (1971)

Dr. Hansjörg Schäfer
Dr. Günter Klöppel
Pathologisches Institut
der Universität
D-2000 Hamburg 20
Martinistraße 52
Federal Republic of Germany