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THE USE OF A LINE SCAN RATEMETER FOR THE X-RAY MICROANALYTIC EVALUATION OF MEMBRANE-BOUND HISTOCHEMICAL ENDPRODUCTS

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Abstract

Although X-ray microanalysis represents a useful tool for identifying electron dense histochemical end-products, quantitative microanalytic measurements are seriously hampered in the case of the activities of certain membrane-bound enzymes. For example, the electron histochemical methods revealing K^+-dependent pNPPase activity result in a very fine, granular reaction product of lead phosphate. Therefore microanalytic, densitometric or similar evaluations of the reaction, even in semiquantitative terms are not practical by the usual procedures.

This paper describes a method of X-ray microanalysis of thick sections (0.5 µm) processed for K^+-pNPPase, where a sufficient amount of lead is present for X-ray microanalytic determination. The analysis is performed in the line scan mode on transversely cut membrane profiles by means of the line scan ratemeter of an EDAX System F. This yields quantitative data on the relative lead concentrations in the vicinity of the cell membrane. A method is proposed for calculation of relative enzyme activities based on the Pb-signal of the ratemeter curve and the average "noise"-level of the cytoplasm, containing also non-specifically bound lead.This method avoids the necessity of measuring the section thickness; it may be useful for a variety of purposes in the electron microscopic histochemistry of membrane-bound enzymes.

KEY WORDS: X-ray microanalysis, line scan ratemeter, electron histochemistry, membrane bound enzymes, Na^+-K^+-dependent ATPase, quantitative histochemistry, lead-phosphate reactions, perfusion technique, K^+-pNPPase.

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Introduction

Histochemistry represents a useful approach to a number of cell biological problems. It is especially interesting when we can perform reactions of well established mechanisms where the stoichiometry of a locally bound end-product formation is known, and suitable methods are at our disposal for quantitation of the reaction product. In most electron microscopic histochemical studies we are far from being able to fulfill these criteria. Yet histochemical studies of this type are obviously of great importance, since they can answer questions regarding the localization of certain enzymes, etc, which cannot be answered without using the high spatial resolution of this branch of histochemistry.

In electron microscopic histochemistry most of the methods are based on the formation of an electron-dense, heavy metal end-product. Therefore, one of the possibilities for quantitation would be a densitometric evaluation of the properly exposed and processed micrographs. Unfortunately, however, in many of the methods of electron microscopic histochemistry the specimens have to be osmium-fixed before or after the main reaction in order to enhance the electron contrast of the structural components of cells and tissues or the end-product of the histochemical reaction. This means that densitometry cannot be applied, since apart from the end-product of the reaction, the dense structures usually contain some osmium bound to the components independently from the specific histochemical reaction, too. It becomes,therefore,important to have also some information about the contribution of the osmium to the density of the reaction product.

The situation can be further complicated by peculiar enzyme localizations. For example, several membrane-bound enzymes can be revealed by electron microscopic histochemical reactions, like K^+-dependent pNPPase (indicating the "pump" enzyme) (Ernst, 1972a, 1972b, 1975; Ernst and Schreiber, 1981; Latham and Kashgarian, 1979; Mayahara et al., 1980, 1983; Ueno et al.,1983; Vorbrodt et al., 1982; Zaccone et al., 1984), guanylate cyclase (Kang et al., 1982) or nucleoside diphosphatase (Vorbrodt and Wisniewski, 1982). However, the reaction product is a very fine, granulated lead phosphate precipitate of

which the amount (or concentration) cannot be measured easily. Usually the amount of lead in the volume of an ultrathin section is too small for conventional X-ray microanalytic determination. Even if one is able to quantify the amount of lead in a given area of an ultrathin section, it is usually very difficult to obtain any realistic figure for its concentration related to the membrane surface area or any other morphological parameter due to the small size and uneven distribution of the granules. On the other hand, quantitative information about membrane-bound enzyme activities on special sites or in special cell types would be of great importance from various points of view of cell biology. The great need of quantitation is illustrated here with the example of guanylate cyclase activity in various cells of rat uterus subjected to different treatments (Kang et al., 1982). Due to the lack of any quantitative method, these authors had to be satisfied with only stating high (++), moderate (+), weak (+-) or missing (--) reaction levels of guanylate cyclase. It is unnecessary to emphasize how difficult it is to compare such an evaluations with results from other laboratories and how much bias can be involved in this type of evaluation.

X-ray microanalytic demonstration of the presence of a certain element in the end-products of some histochemical reactions may be a useful method to prove the nature of certain deposits, precipitates, etc., in qualitative terms. More important, however, is the quantitative determination of certain elements in the histochemical end-products. During recent years X-ray microanalysis has become a method of very general use in histochemistry (Beeuwkes and Rosen 1975; De Bruijn and Van Buitenen, 1980; De Bruijn et al., 1980; Berry et al., 1982; Davis et al., 1982; Jones et al., 1982; Mizuhira and Ueno, 1983; Ozawa and Yamamoto, 1983; Makita et al.,1983; Mitchell and Shepard, 1984; Appleton et al., 1985; Boekestein et al., 1985; Liposits et al., 1985; etc.). To the best of our knowledge, however, the application of the line scan ratemeter to the analysis of membrane-bound enzymes as described in the present paper has not been attempted.

In the present experiments we focussed our attention on Na^+-K^+-dependent ATPase revealed with the artificial substrate p-nitrophenylphosphate (pNPP) (Ernst, 1972a, 1972b; Mayahara et al., 1980). We are aware of the fact that questions about the electron microscopic histochemical methods for this enzyme are far from being completely resolved (see Mayahara et al., 1980). We felt it necessary, however, to make some efforts toward a possible quantification of this enzyme reaction. Especially since some new modifications of the method could be much better evaluated, if one has the ability to record measurements in addition to the very much biased subjective inspection of the specimens.

The present paper was aimed to show on a practical basis that the line scan ratemeter method of X-ray microanalysis can yield useful information about this "pump" enzyme activity in various types of cells. This methodology obviously will be applicable also for the activities of other enzymes which are localized in the cell membrane or in some other well distinguishable membranous structures.

Materials and Methods

All experiments were performed on the livers and kidneys of male CFY white rats, in the age range of 1 to 26 months. The main purpose of our study is to perform an investigation on the age-dependency of K^+-pNPPase, after having established a method for quantitation of its activity. All the reagents used were of analytical grade. For the quantitative electron microscopic demonstration of K^+-dependent pNPPase the following histochemical procedures were evaluated.

Histochemical procedures

1. Two-step method of Ernst (1972a, 1972b, 1975). In this method a Sr-phosphate deposit is formed initially when the enzyme splits the pNPP. This is then converted into lead-phosphate during the postincubation. The original method was modified as follows:

(i) Prefixation: Freshly excised small pieces of liver and kidney were sliced to about 0.050 mm thickness at room temperature, using a specially designed tool consisting of parallel razor blades, and then fixed in 1 % paraformaldehyde in 0.1 M cacodylate buffer (pH = 7.5) + 0.25 % glutaraldehyde for 10 min at 0^oC, i.e., we did not cut cryostat sections as Ernst (1972b) did. The fixation was followed by multiple washings in 0.1 M cacodylate buffer (pH = 7.5) for 3 min and two rinses in 0.1 M Tris-HCl buffer (pH = 7.5) for 3 min. (Both buffers contained appropriate concentrations of sucrose, i.e., 250 or 310 mOsm for liver and kidney, respectively).

(ii) Incubation: The tissue slices were incubated in the medium of Ernst (1975) at 37^oC for 30-40 min. The final concentrations in the incubation medium were: 20 mM pNPP-disodium salt; 20 mM $MgCl_2$; 20 mM KCl; 20 mM $SrCl_2$; and 250 mM Tris-HCl buffer, pH = 9.0). Substrate-free control samples were incubated in the absence of pNPP,and enzyme inhibitions were performed by using a preincubation in the presence of either 5 mM levamisole (Borgens, 1973) or 10 mM ouabain in appropriate buffers.

(iii) Washing: 0.1 M Tris-HCl (pH = 9.0) containing 250 mOsm sucrose, 2 x 5 min at room temperature.

(iv) Post-incubation: 2 x 5 min in 2 % $Pb(NO_3)_2$, containing 250 mOsm sucrose.

(v) Washing: in lead nitrate-free sucrose for 2 min, then a quick rinsing in Tris-HCl buffer (pH = 9.0) and subsequently in cacodylate buffer.

(vi) Postfixation: 1 % OsO_4 in 0.1 M cacodylate buffer, 40 min.

(vii) Dehydration through ethanol and propylene oxide, and embedding in Durcupan ACM as usual.

2. One-step method of Mayahara et al. (1980). The essential point of this method is that the lead-phosphate end-product of the reaction is formed directly from lead-citrate, when the pNPP is split by the enzyme, Furthermore, the

incubation medium also contains DMSO. This method has been applied by us exactly as described by Mayahara et al., (1980).

3. Experiments with perfusion technique: These experiments have been introduced in our laboratory in order to assure a better uniformity of the overall reaction intensity within the liver or kidney. The method consists of perfusion-fixation and incubation using the incubation medium of Mayahara et al. (1980). Since this method is still under development, a definitive technical description cannot be given. Details of this method and the results will be described elsewhere.

X-ray microanalysis

Specimens for X-ray microanalysis were 0.5 µm thick sections of resin-embedded tissue blocks. (The reasons for using such sections for this purpose will be described later). The sections were mounted on copper grids of 150 or 200 mesh, and studied in a scanning transmission mode of a JEOL JSM 35C scanning electron microscope equipped for energy-dispersive X-ray microanalysis with an EDAX System F instrument. The latter system is also equipped with a line scan ratemeter (LSR) type EDAX 352. The LSR must be properly calibrated before use in order to assure perfect linearity in the range of counts where one intends to perform the microanalysis. Preliminary experiments have shown that the most practical way of calibration is the following (Figure 1):

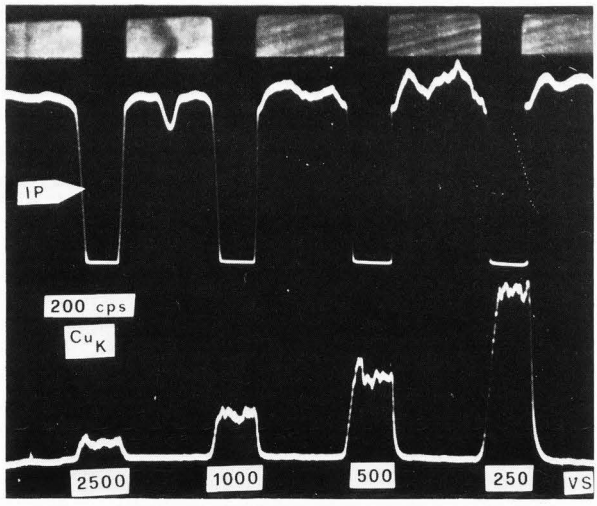


Figure 1. Demonstration of the calibration of the line scan ratemeter using a copper grid. In the upper part of the figure one can see details of the grid with four perpendicular copper columns in black. The line scan was run along the central line of one row of the holes. The upper curve (IP) is the image intensity profile, whereas the lower one shows the LSR profile modulated by the Cu_K intensity. VS is the vertical scale of the ratemeter changed when the beam was running in the hole of the grid. Numerical values of VS are indicated at each copper peak. The copper impulses arrived from the grid at an average rate of 200 cps.

A window of 220 eV width is created in the spectrum on the Cu_K line (at 8.04 keV) and a grid is analyzed at 25 kV accelerating voltage in the line scan mode so that the analysis line is running perpendicular to the vertical copper bars (Figure 1). The beam intensity is regulated so that 200 Cu_K impulses per second arrive to the analyzer when the beam is running on the grid. Using the variable functions of the ratemeter (smoothing, zero, threshold, gain) one has to reach a situation in which the constantly arriving Cu_K impulses give proportional signal heights when the vertical scale (i.e., the sensitivity of the LSR) is varied. Figure 1 demonstrates this situation in detail, showing in addition the line intensity profile (IP) of the scanning image together with the signal height of 200 cps of Cu_K impulses while the vertical scale of the LSR was varied from 250 to 2500 (the switch-over was made while the beam was running above the holes). This type of calibration can be performed also for lower intensities. As a matter of fact, for most of the specimens the vertical scale of 250 is quite satisfactory, but sometimes we used a vertical scale of 100.

The speed of the line scan should be rather slow (i.e., assuring a sufficiently long dwell time of the beam at a given site of the specimen) in order to allow the LSR to follow even the rather small changes in the frequency of the arriving X-ray impulses. In the case of Figure 1 the photographic speed of No.8 was chosen for the vertical scan which determines the horizontal run of the beam when the line scan mode is selected. At this position the line scan runs through the selected area representing on the screen 18 cm, in 250 sec. Knowing the linear magnification factor, one can calculate the actual scan speed on the surface of the analyzed specimen. When the intensity of the analyzed element is lower, one can perform the line scan at an even lower speed so that very small differences can also be detected by the LSR. (The JSM 35C microscope has another step for 500 sec scan speed, i.e. for longer dwell time).

The accelerating voltage was usually 25 kV which is quite sufficient for the scanning transmission investigation of the sections of 0.5 µm thickness, and at the same time, it was sufficient also for the excitation of Pb_M. (Higher voltages can also be applied. However, one has to consider also the eventual possibility of beam damage of the specimen at higher energies during the slow line scan). Further details will be discussed in the subsequent sections of this paper.

Results

First of all we want to demonstrate the histochemical appearance of K^+-pNPPase as an example for which the LSR method described in this paper can be applied. Figure 2 shows the detail of an epithelial cell of the thick ascending segment of Henle's loop from the kidney of a 24 months old rat, obtained by the 1-step method of Mayahara et al. (1980). The fine reaction product is localized on basal infoldings of plasma

membrane in this cell. In agreement with the observations of other authors (Ernst, 1975; Blitzer and Boyer, 1978) a reaction product of considerable intensity can be observed on the nuclear membrane of hepatocytes (Fig. 3) and also of kidney tubular cells. This latter activity, however, proved to be ouabain-insensitive (Fig. 3).

Figure 4 shows a cell from the pars recta of the descending tubule of the kidney obtained in the scanning transmission mode from a 0.5 µm thick section at 25 kV accelerating voltage. The microvillous area of several epithelial cells and the contacting membranes of two cells can be seen. The black deposits correspond to the lead-phosphate precipitate obtained by using the perfusion technique with the incubation medium of Mayahara et al. (1980) for the enzyme histochemical reaction. (As judged subjectively from the amount of lead precipitate, the enzyme activity in the liver was generally lower than in the kidney tubular epithelia. However, it was present both on the cell membrane and on the nuclear membrane). The scanning transmission images of thick sections are, of course, of poorer quality than the transmission electron micrographs of ultrathin sections. Nevertheless, the use of such sections offers the following advantages:

(i) Although the concentration of lead is the same in thin and thick sections, the larger total mass of the latter ones permits a more reliable X-ray microanalysis within a reasonably short time.

(ii) The relative influence of beam damage is much less significant for the thick sections.

(iii) In the thick section the lead-phosphate granules are not resolved individually as in the ultrathin sections, since they form a practically uniform layer on the cell membrane due to the superposition of about 10 ultrathin sections. Thus the analysis of the membrane by the LSR gives an acceptably homogenous peak of lead characterizing the average lead-content of a relatively large membrane area as shown below.

When running a line scan over the thick section and sending the Pb_M line (at 2.380 + 0.110 keV) to the LSR, results as shown in Figure 5 can be observed: A rather small but more or less constant number of Pb_M impulses arrives to the analyzer until the exciting beam runs in the cytoplasmic area of the cells. However, in complete coincidence with the reaction product of high density, localized on the cell membrane, a striking Pb-peak appears. This type of observation can repeatedly be obtained whenever the section contains transversally cut membranes. In the cells shown in Figure 5 the cell membrane is highly loaded with the lead-phosphate deposits. Therefore, the LSR detects a high Pb_M peak when the beam transverses the membranes. Since the plane of the membrane is oblique to the scanning line on the left side, the basis of the Pb_M peak is wider as compared to the right side where the membrane is running almost perpendicular to the analysis line. Nevertheless, no considerable difference can be seen in the heights of the 2 Pb_M lines. The line intensity is somewhat different in the right and left cytoplasmic regions which may be due to some local differences in section thickness.

Figure 2. Transmission electron micrograph of an ultrathin section of kidney medulla processed for the K^+-pNPPase activity by the 1-step method of Mayahara et al.(1980). Black deposits on the basal infoldings of an epithelial cell of the thick ascending segment of the Henle's loop are Pb-phosphate precipitate indicating the enzyme activity. The bar represents 1 µm.

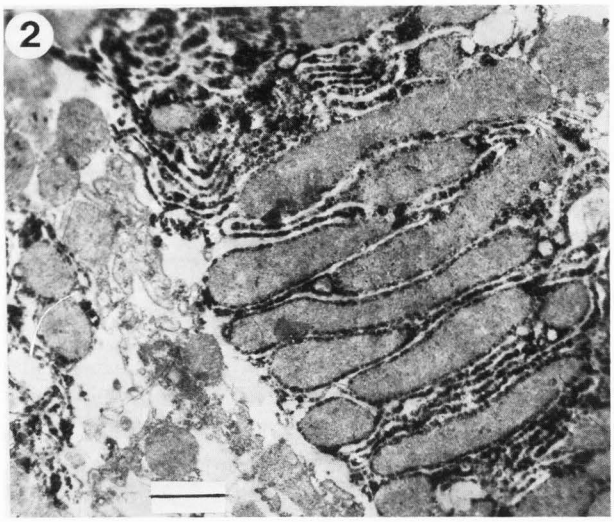
Figure 3. Transmission electron micrograph showing a detail of a hepatocyte from liver processed for K^+-pNPPase activity by the 2-step method of Ernst (1972b) after ouabain preincubation. Black deposits on the nuclear membrane (arrows) indicate non-specific alkaline phosphatase activity. The bar represents 1 µm.

Figure 4. Detail of pars recta of a descending kidney tubule processed by the perfusion technique for K^+-pNPPase activity, as seen in the scanning transmission mode in a 0.5 µm thick section at 25 kV. The right side of the tubular epithelium shows heavy black deposits corresponding to the microvillous internal surface, which contains mainly alkaline phosphatase. On the left side, the contact of two cells can clearly be seen, where the specific enzyme activity is present on the plasmalemma. The bar represents 10 µm.

Figure 5. The upper part of this figure demonstrates the scanning transmission image of two cell membranes from a kidney processed by the perfusion technique for K^+-pNPPase activity. The horizontal white line indicates the position of the line scan run from left to right. In the lower part of the figure one can see the zero level (at the very bottom), the Pb_M line profile (in the middle) and the image intensity profile (upper curve). Vertical scale of the LSR was 250. Note the slight differences in width of the bases of the two Pb_M peaks due to the different position of the analyzed membrane in relation to the analysis line. The bar represents 10 µm.

Figure 6. Energy dispersive X-ray spectrum collected during a complete line scan from the kidney cells shown in Figure 5. The Pb_M line is highlighted at 2380 + 110 eV. On the left side of this peak one can observe the overlapping peaks of P_K and Os_M, and still further to the left an Al_K peak. Small peaks of Ca, K and Cl are also present.

Figure 6 demonstrates the X-ray spectrum obtained during the line scan run. It is evident from the spectrum that apart from Pb, a considerable amount of P and Os is also present. Unfortunately, however, the Os_M line (1.96 keV) closely overlaps with the P_K line (2.00 keV). Therefore, it is not possible to investigate a possible correlation between the Pb and P elemental intensities. (It should be noted that the spectrum contains an Al-peak, too, derived from the specimen holder, and also some traces of copper from the supporting grid. Ca, K, and Cl are also present in minor quantities. These contributions, however, do not disturb the evaluation of the LSR curves as described below).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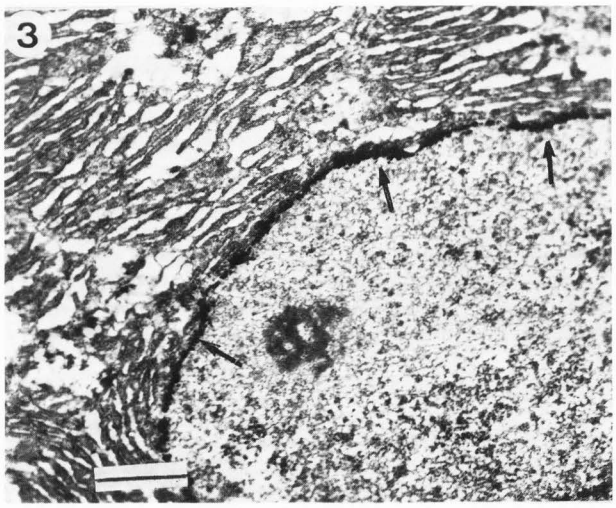
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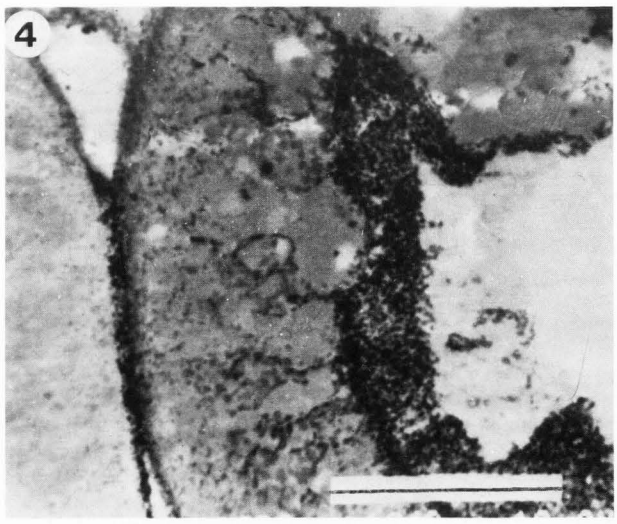
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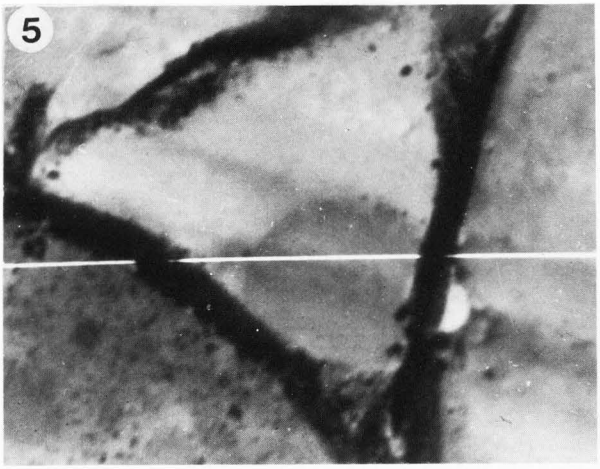
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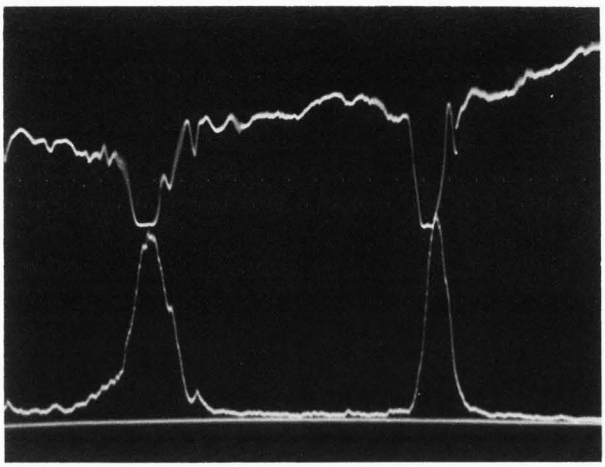

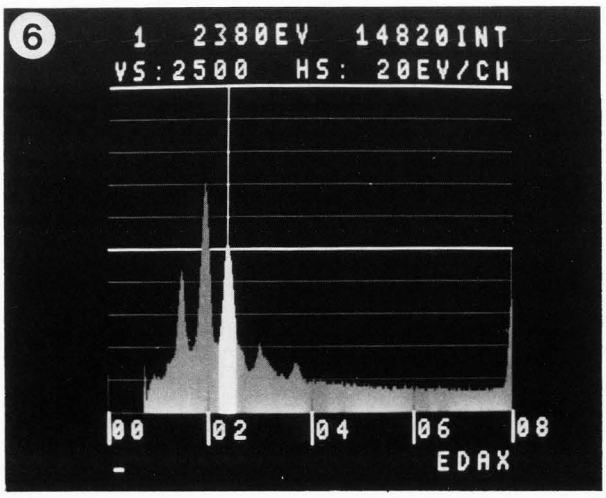
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Figure 7 shows a kidney cell where the line scan run was repeated twice on the same place: first the Pb_M impulses, and secondly the Os+P-impulses were sent to the LSR. It is quite evident that a considerable part of the electron density derives from the presence of Os. However, in this particular case, Pb is by far the dominant contribution.

With regard to the mathematical evaluation of the LSR curves, several considerations should be made. It is obvious that the counts collected in the energy range of 2.380 ± 0.110 keV (sent to the LSR) contain not only the Pb_M X-rays but also the background intensity (Bremsstrahlung) which is proportional to the actually analyzed mass. The absolute lead concentrations can obviously be calculated if these two X-ray counts are separated. This might be resolved in principle by a special computer program being able to perform the background subtraction on the actually collected spectrum at various time intervals (for example several times per second) and send only the net Pb_M counts to the ratemeter. However, this method would require an extremely fast computer, and in addition, also the ratemeter itself should be redesigned properly. Since the application of such highly powerful programs is very expensive, we had to find a more realistic compromise.

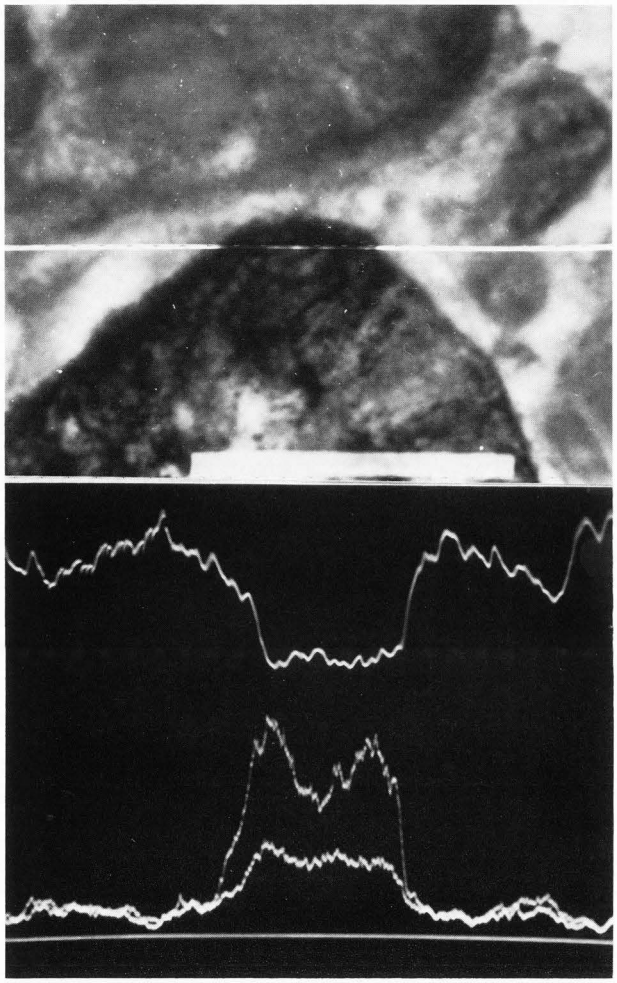

Figure 7. Scanning transmission electron micrograph from a 0.5 µm thick section of an epithelial cell from the medullary ascending thick limb of the kidney processed for K^+-pNPPase activity by the 2-step method of Ernst (1972a, 1972b), after levamisole preincubation (upper picture). The bar indicates 10 µm, the horizontal white line corresponds to the site of line scan analysis. In the lower part we recorded 4 curves: at the very bottom the zero line of the LSR is shown; above this the $Os_M + P_K$ line; then the Pb_M line can be found. In addition the figure shows the intensity profile (upper curve). The vertical scale of the LSR was 500 in this case for both elements.

One has to start from the facts outlined in Figure 8. The LSR curve displays a triangular peak with a basis m and a net height of x, respectively. This peak will be called signal; the size of the signal is characterized by its integral (S). S is superposed on a background which will be designated noise. The average height of this noise is y, its width m, and its integral will be symbolized by N. This terminology is used in order to distinguish S and N from the terms "peak" and "background" of the X-ray spectra. With respect to the situation in the cell, S represents the perimembranous area, whereas N corresponds to the cytoplasmic (or extracellular) regions.

However, as we mentioned before, the LSR curve contains the Pb_M X-rays and the continuum radiation. These two components will be called p and c in N, as well as P and C in S,

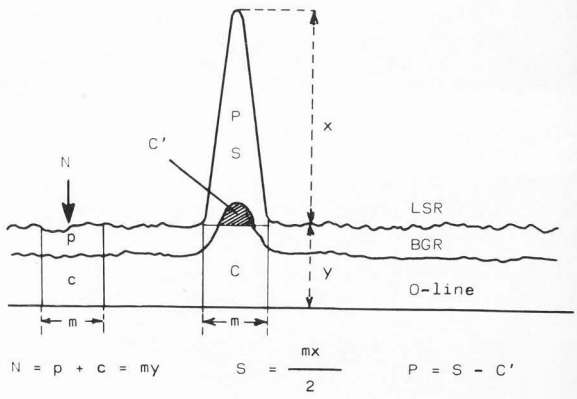

Figure 8. A schematic outline of the method suggested for the mathematical evaluation of the LSR curves obtained on the transversal membrane sections. S is the area of the signal, and N represents the cytoplasmic "noise" area belonging to the same width m. The width of the signal basis depends on the magnification used. Further explanations in the text.

respectively. The presence of Pb in the cytoplasmic area, i.e., in N, is shown by the fact that a Pb peak is obtained even if the LSR analysis is performed only in cytoplasmic regions. This lead is bound most probably in a non-specific way by the cytoplasmic components. LSR analyses performed only in the cytoplasmic region have shown that the fraction of the lead impulses in N, i.e., the ratio r = p/N is fairly constant, at least in the same type of tissue. Therefore, the specific lead precipitations due to the enzyme activities in the membranous region can be related to the non-specifically bound lead.

A further problem is, however, that the perimembranous area has a somewhat larger specific mass than the cytoplasm, just because of the higher concentration of lead being present in those places. It means that a fraction of C (C') contributes to the value of S, i.e., P < S. Figure 8 demonstrates schematically the situation. Nevertheless, it is quite obvious that the ratio C'/S should be again fairly constant, since C' is directly correlated with the lead content of the perimembranous region. In other words, the equation P = Sq holds true, where q = (1 - C'/S), i.e., the fraction of Pb_M X-rays in S.

If we want to characterize the enzyme activity (EA) by the ratio (R) of the true lead contents in the perimembranous (P) and cytoplasmic (p) regions, it can be written:

$$R = P/p = Sq/Nr \qquad (1)$$

If two activities (R_1 and R_2) in the same type of tissue have to be compared in relative terms:

$$\frac{R_1}{R_2} = \frac{S_1 q N_2 r}{S_2 q N_1 r} = \frac{S_1 N_2}{S_2 N_1} \qquad (2)$$

i.e., the factors q and r cancel out. Since S = mx/2 and N = my, equation (2) does not require any other parameter than x and y, if m is equal in both cases (Figure 8). In possession of these two parameters, one can obtain a relative measure for EA without being forced to measure the section thickness, or to perform the background subtraction during the line scan run. In other words, LSR curves can be evaluated by a rather simple graphic method. One can also obtain digitalized printouts from the ratemeter, if the necessary development is performed.

This method of calculation is obviously valid only, if the Pb-peak can be considered as a triangle which is always the case, if single membrane profiles are involved in the measurement. The situation is not very different, if we have two cell membranes in a close contact with each other. Therefore, it is obviously important to record with each LSR curve whether the analyzed membrane was single or double in various measurements. In the case if the shape of the signal is not triangular, other mathematical formulas can be used as well, however, based always on the same principle, i.e., calculation of R = S/N.

The approach of quantitative evaluation described above has several advantages as follows:

(i) The magnification used for the line scan is not critical for this type of study, provided that the line scan speed chosen is sufficiently low for the detection of differences in the lead concentration outside and inside the membrane.

(ii) As has been mentioned, one can avoid the necessity of measuring the section thickness which is unfeasible if one has to analyze a great number of membrane profiles. (Nominal section thicknesses taken just from the microtome knobs may be very misleading). The non-specific Pb-binding of the organic matrix is relatively constant, therefore, the EA parameter as described above is certainly more reliable than only a subjective judgment of the density.

Although the principal purpose of the present paper was merely to show that the LSR method can yield useful quantitative information about the K^+-pNPPase activity of the membranes, several additional results, although incomplete, can be mentioned to illustrate the applicability of this method. The results obtained so far using this method of evaluation, allow us to draw the following general conclusions:

(i) The 2-step and 1-step methods can be compared quantitatively, and it is evident that the latter one gives considerably higher values for EA than the former when identical tissues are compared. This observation is in agreement with the known inhibitory effect of Sr^{2+} on the enzyme (Ernst, 1972a, 1972b).

(ii) The liver cell membranes generally contain much lower K^+-pNPPase activity than most of the tubular epithelium of the kidney, whereas the ouabain-insensitive (most probably alkaline phosphatase) activity localized on the nuclear membrane behaves in an opposite way.

(iii) Using specific enzyme inhibitors like ouabain and levamisole (Borgens, 1973), one can discriminate the proportions of the various enzyme activities (e.g. alkaline phosphatase) in the overall lead-deposition on the membranes.

(iv) The efficiency of new methodical approaches can be tested by using this method of evaluation.

Further studies are underway in our laboratory, aimed especially to reveal some age-dependent differences in the enzyme activities in various parts of the body. One of the principal problems to be initially resolved is the determination of the statistical scatter of the obtainable data, in order to see how large the data pool must be for a safe comparison. This, however, can be resolved only after the highest possible level of standardization of the histochemical procedure itself has been established.

Discussion

Obviously, one of the first criteria one has to check is the stoichiometry of the reaction involved. According to Ernst (1972a), the reaction of the Na^+-K^+-dependent ATPase can be described as follows:

$$ATP + E \xrightarrow{Mg^{2+} + Na^+} ADP + EP \qquad (3)$$

$$EP \xrightarrow{K^+} E + P_i \qquad (4)$$

where E means the enzyme, EP is the enzyme-phosphate and P_i is the inorganic phosphate liberated from EP.

The reaction (2) can take place also as follows:

$$RP + E \xrightarrow{K^+} R + P_i + E \quad (5)$$

where RP means some artificial substrate containing a phosphate group (e.g., pNPP). The essential point of the histochemical method is to capture this P_i in form of an insoluble precipitate of high density. It has been shown that in reaction (5) the initial product is a mixture of $KMgPO_4$ and $Mg_3(PO_4)_2$ which is then converted according to a linear correlation into the final, visible product (Beeuwkes and Rosen, 1975). These authors used X-ray microanalysis for the demonstration of the linearity of this conversion for cobalt as the capturing ion, and showed also a strict linearity between cobalt and sulfur when the end-product of the reaction was converted by $(NH_4)_2S$ into a cobalt-sulfur complex. These findings indicate the existence of a fixed stoichiometry between the original enzyme product and the final visible product. Obviously there is no theoretical reason for assuming that Sr^{2+} or Pb^{2+} ions would behave differently. Therefore, it seems to be legitimate to measure the lead-content of the cell membrane as a parameter proportional to the activity of K^+-pNPPase.

The second point to be discussed is the evaluation of the LSR curves. One cannot be sure that the cell membrane is always running perfectly perpendicular to the section surface, i.e., it may have some inclination within the section. However, as shown in Figure 8, this will not seriously influence the relative value of EA as calculated from $\underline{x}$ and $\underline{y}$, since in this case the amount of Pb found in the area of membrane cross-section will be proportionally larger, i.e., the LSR peak becomes wider. Since the width of the membrane cross-section is not involved in the final calculation, we can neglect the inclination of the membrane plane within the depth of the section. Of course this statement is valid only within certain limits of inclination, namely, as long as the membrane profile is well distinguishable, i.e., the signal of LSR remains triangular. Above this level of inclination we are not going to analyze anyway, because the membrane profile becomes blurred. The evaluation of the LSR curves can also be computerized, although such a program has not yet been initiated in our laboratory.

Some comments should be made on the sensitivity of this type of X-ray microanalysis. Provided that the LSR is properly calibrated, very minute differences of S/N can be distinguished. For example, the usual average count rate in the applied energy range is about 10 per sec in the cytoplasm, whereas it may reach values greater than 100 in the perimembranous space. Accordingly, the relative values for x/y obtainable fall in the range from 3 to 15. However, even if the count rate is only double in the membrane as compared to the cytoplasm, the LSR curve still reveals a well defined peak, i.e., $x/y = 1$ can still be accurately measured. Such a sensitivity seems to be sufficient for most histochemical purposes.

Another point to be mentioned concerns the quality of the histochemical specimens. Obviously, for any type of quantitation it is desirable to have a uniform reaction everywhere in the tissue, since local differences in the intensities due to diffusion idiosyncrasies of the components of the incubation media, etc may seriously compromise the validity of the results. It is well known that the basic methods of two-step or one-step pNPPase histochemistry are not ideal in this sense, since one rather frequently encounters areas of extremely variable reaction intensities even when using only 50 µm thick sections. The only way to achieve a more uniform reaction seems to be the development of some perfusion techniques where each component of the incubation medium reaches each cell with equal probability. Our laboratory is actually dealing with such a methodological development and some preliminary results have already been obtained and shown in the figures of this paper.

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Discussion with Reviewers

J.S.Hanker and B.L.Giammara: Does the use of thick sections enable the measurement of histochemical reaction end-products containing less electron-opaque metals which ordinarily could not be quantified by X-ray microanalysis on ultrathin sections?

Authors: Most probably it does, however, the sensitivity of the method must be checked very carefully for each type of histochemical reaction to be measured.

H. Mayahara: The authors used their own "perfusion cytochemistry" to overcome the unevenness of the cytochemical reaction when the routine technique was used. However, the validity of their technique has not yet been confirmed. Judging from their figures, this "perfusion cytochemistry" seems to be accompanied by a new type of artifact. For instance, the authors state in the explanation of Figure 4 that "... whereas on the left side the contact of two cells can clearly be seen, where the specific enzyme activity is present." However, this lead deposition is not the K^+-pNPPase activity, but it is a non-specific deposition of lead on the basal lamina. The reason for this opinion is as follows: first, the distribution of K^+-pNPPase activity in the proximal cells is not like this (Ernst 1972, 1975; Mayahara et al. 1980). Second, the lead deposit was too thick for a membrane enzyme activity and as thick as the basal lamina. The nonspecific lead deposition was shown to occur when tissue blocks were used for cytochemical

incubation (see Mayahara and Ogawa, J. Histochem. Cytochem. 16, 721-724, 1968). Perhaps, the use of tissue blocks in the authors' "perfusion cytochemistry" caused a similar type of artifact, lead deposition on the basal lamina. Please comment.

Authors: The critical comments with regard to our perfusion technique are noted. The aim of this paper was not to establish this technique and it is still subject to development. We only mentioned it to indicate the strategy we are following in this field. However, regardless of the nature of the precipitate, the problem of quantitation of lead remains the same.

W.C. De Bruijn: Isn't the poor separation of the Os and Pb M lines a problem?

Authors: The difference in energy is about 400 eV which is sufficient for a complete peak separation.