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Preparation of Histochemical Sections for Quantitative Determination of Acid Phosphatase Activity by Means of Laser Microanalysis

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Summary. Based on former experiments dealing with a quantitative assay of acid phosphatase (acP) activity in histological sections by means of a laser microanalysis. The authors present results of investigations concerning optimal conditions needed for this purpose. The investigations were performed on rat liver sections. The realtion between incubation time and depth of acP reaction appearing in tissue blocks was investigated along with the dependence of photometric readings on thickness of histological sections and on incubation times. From results of experiments it appears that optimal conditions for quantitative determination of acP activity using the proposed laser method are provided by sections 30 to 50 μ in thickness which have been incubated in the substrate containing medium for 45 min. Sections thinner than 30 μ are not recommended for quantitative assay with this method because of low accuracy of readings caused by too low amounts of reaction product present there.

Introduction

In our previous investigations concerned with the applicability of laser microanalysis in histological and histochemical techniques—the possibility of detection of cations in tissue slices was raised (Kozik *et al.*, 1970a). Further investigations in this field indicated the possibility of quantitative evaluation of acid phosphatase (acP) activity in histological preparations (Kozik *et al.*, 1970b). From the results presented by Beatrice *et al.* (1969) and by Rosan *et al.* (1963) it appears that laser microanalysis may be applied provided the amount of element to be estimated is not smaller than 1×10^{-14} g.

The commonly known histoenzymatic assays in which heavy metal compounds are formed as reaction end products seem to be particularly suitable for quantification by means of laser microanalysis. However, the incubation conditions, such as thickness of tissue section and incubation times must be carefully chosen in order to ensure adequate amounts of reaction products to be formed, amounts which would enable photometric products to be formed, amounts which would

enable photometric evaluation of the emission band obtained from the tissue burnt out by laser rays. Furthermore, the conditions must be such, as to ensure proportionality, a straight line relationship between the amount of enzyme present in the incubation mixture and the measured activity, i.e. the amount of reaction product detected.

The aim of our present investigation was to establish optimal conditions of preparing and processing tissue specimens to make them suitable for quantitative assay of acP activity by means of laser microanalysis.

Material and Methods

Livers from 10 adult Wistar rats were used for experiments. The experimental material was treated in two different ways:

1. The livers were cut into blocks of approximately 0.3 cm long edges, which were then incubated in a substrate for acP assay according to Gomori (1956). The sections were incubated for five different time intervals (20, 30, 45, and 75 min) after which they were rinsed for 2–3 sec in distilled water and then fixed for 16 hrs in 10% neutral formaline at 4° C. The fixed and frozen sections were then cut into slices 15 μ thick, rinsed in distilled water and next transferred for 5 min into ammonium polysulphide solution. The rinsed sections were then placed on microscopic slides and mounted in glicero-gel on which measurements were taken of the widht of area on which the reaction was detectable with the aid of a micrometric occular. From one preparation 20 individual measurements were taken and means calculated.

2. The livers were fixed by perfusing with cold Baker's solution (1944). For acP assay, sections of 15, 30 and 50 μ in thickness were cut in a cryostat and then incubated for 20, 30, and 45 min respectively, in the Gomori substrate (1956). After incubation the sections were rinsed three times in distilled water for 2 sec each time and then transferred into ammonium polysulphide solution for 5 min. After repeated rinsing the sections were mounted on photographic plates (Kozik *et al.*, 1970b) and processed according to standard conditions applied in laser microanalysis (Rosan *et al.*, 1965). The preparations were then irradiated by a beam of laser rays using a Zeiss (type LMA-1) laser microanalysator. The radiation emitted during the action of laser rays was analyzed in a grating spectrograph (Zeiss, type PGS-2) and the density of the Pb-band (4057,8 Å) measured photometrically.

Results

As the result of incubation of whole tissue blocks for different time intervals it has been found that the depth of reaction for acP increased proportionally only during the first 30 min of incubation. Prolonged incubations did not affect significantly the extent of reaction in the tissue block. Table 1 demonstrates the effect of different incubation times on the extent of reaction for acP in tissue blocks.

Table 1. *Relation between incubation times and depth of reaction for acP in tissue blocks*

Incubation time (min)	Depth of reaction (mm)	Standard deviation
20	0.0525	0.006
30	0.074	0.005
45	0.080	0.006
60	0.081	0.004
75	0.082	0.004

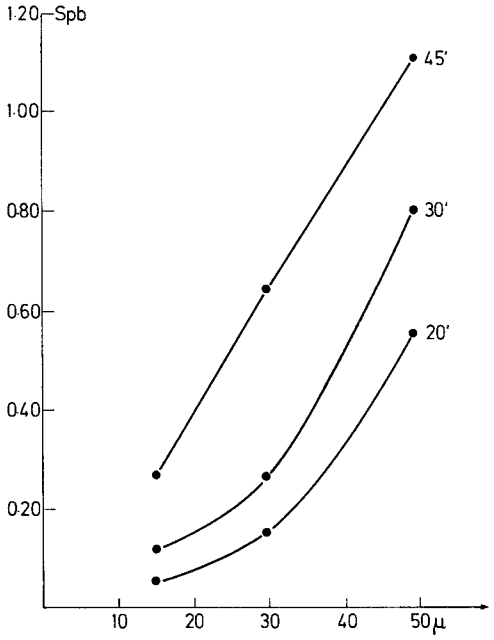

Fig. 1. Relation between photometric readings (enzyme activity) S_{Pb}, and thickness of the sections (amount of enzyme) in μ, for different incubation times

Table 2. *Photometric readings taken at 4057.8 Å, accounting for density of the Pb band, related to thickness of sections and to incubation times*

Thickness of sections (μ)	Incubation time (min)	S_{Pb}	Number of determinations	Dispersion of results
15	20	0.05	10	0.02
15	30	0.12	9	0.03
15	45	0.27	8	0.06
30	20	0.14	10	0.03
30	30	0.26	9	0.06
30	45	0.64	10	0.10
50	20	0.54	8	0.14
50	30	0.78	9	0.12
50	45	1.05	8	0.20

S_{Pb} = mean of individual photometric readings at 4057.8 Å.

Separate experiments aimed at establishing optimal incubation times and thickness of sections required for quantitative laser microanalysis. Sections differing in thickness were incubated in substrate for acP for different time intervals. Photometric readings at 4057.8 Å, taken from the obtained spectrographic Pb band are shown in Table 2. About 10 determinations were made for each group of

sections and for each incubation time. For calculation of results of laser analysis only such figures were considered, which based on the ratio of Ag band versus volume of the crater burnt out (S_{Ag}/Vol.) indicated similar excitation conditions.

Results presented in Table 2 indicate that the S_{Pb} values obtained from sections of equal thickness increase with increasing incubation time.

Relating the estimates (enzyme activity) to enzyme concentration (thickness of section), were obtained in the curves of Fig. 1. It appears that a straight line relationship between measured activity and amount of enzyme (enzyme concentration) has been obtained only with sections incubated for 45 min.

Discussion

Assuming the course of a histochemical reaction occuring in tissue sections to follow this scheme:

$$A \xrightarrow{K_1} A_1 \xrightarrow{K_2} B$$

where: A = substrate concentration in incubation medium,
A_1 = substrate concentration at the enzyme site,
B = concentration of reactiong product,
K_1 = diffusion velocity,
K_2 = reaction velocity,

one will realize that the amount of reaction product formed and thus the value of its representative, i.e. the density of the Pb-band (S_{Pb}), will be dependent on several factors, mainly on the diffusion rate of substrate and the enzyme activity itself. The diffusion rate in turn, depends on substrate concentration, thickness of tissue section and incubation temperature. In this situation following conditions are conceivable:

1. K_1 is lower than K_2 ($K_1 > K_2$)—the reaction follows kinetic rules.
2. K_1 is lower than K_2 ($K_1 < K_2$)—the reaction proceeds in the diffusion space.
3. K_1 and K_2 are similar ($K_1 \cong K_2$)—both the diffusion rate and reaction kinetics determine the final amount of product formed.

These possibilities appertain a situation, when only one surface is active and on this surface the histochemical process occurs. Such a situation is met in conditions where sections have been stuck to the slide prior to reaction, as in the case of unifixed tissues sectioned in a cryostat. When the reaction is performed on freely floating sections at least two surfaces must be taken into consideration.

From our experiments relating the extent of reaction for acP to incubation time it appears that the further from the surface the greater the influence of substrate diffusion rates on the amount of reaction product formed. It thus could be inferred that the effect of diffusion rates will increase with increasing thickness of sections. Hence it would appear that possibly thin sections should be used for determinations of acP activity by means of laser microanalysis. However, in order to obtain adequate amounts of reaction product which could be quantitatively measured—the incubation times would have to be considerably increased. From our results presented in Table 1 it appears that the extent of reaction increases proportionally only up to 30 min of incubation. Further extention of incubation times does not significantly increase the penetration of substrate into the tissue

(Fig. 1). According to our observations, the thickness of sections should not exceed 0.074 mm, independent of time of incubation. Having these considerations in mind, such incubation conditions should be chosen which would ensure proportionality between measured activity and amount of enzyme, as well as adequate amounts of reaction product suitable for quantitative detection with the laser technique (Rosan, 1965; Rosan *et al.*, 1965). The amount of product analyzed should yield readings between 0.30 and 1.4 ($0.30 < S < 1.4$).

From our experimental results shown in table 2 it emerges that in general requirements for laser microanalytical procedures are fulfilled by liver sections of different thickness and incubated for longer than 30 min. Excessive prolongation of incubation is however, according to common experience not to be recommended. From the point of view of amounts of reaction product available for quantitative laser microanalysis, sections ranging in thickness from 30 to 50 μ and incubated for 45 min should render reliable photometric readings. Thinner sections are not recommended because of yielding too low readings.

Anyhow, if quantification of enzyme activity is to be achieved and activities between different specimens have to be compared, the conditions must ensure proportionality between measured activity and amount of enzyme present in the incubated tissue.

In our experiments such a straight line relationship between activity and enzyme concentration has been established using increasing amounts of enzyme source (sections 15, 30, and 50 μ thick) but only when incubating for 45 min.

Conclusions

1. Using laser microanalysis, the acid phosphatase activity of rat liver can be measured quantitatively.

2. Under suitable conditions (45 min incubation time, sections 15–50 μ thick) acP activity is directly proportional to amount of enzyme.

3. Sections thinner than 30 μ are not recommended for quantification by means of laser microanalysis since the amount of reaction product formed is too low to be accurately measured.

4. The depth of reaction occuring in incubated tissues increases significantly during the first 30 min of incubation, then gradually levels down.

5. Penetration of substrate into the tissue is limited and does not exceed 0.08 mm even after prolonged incubation, thus creating a restriction as to the maximal thickness of tissue sections.

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