Histol Histopathol (1995) 10: 463-479

Histology and Histopathology

Invited Review

The kinetics of enzymes *in situ*, with special reference to lactate and succinate dehydrogenases

Y. Nakae[1] and P.J. Stoward[2]

[1]Department of Oral Anatomy 1/, School of Dentistry, Tokushima University, Tokushima, Japan and [2]Department of Anatomy and Physiology, University of Dundee, Dundee, Scotland, UK

Summary. The kinetics of two enzyme systems *in situ* that have been studied with real-time image analysis systems are reviewed in detail. The enzymes are a structurally-bound mitochondrial enzyme, succinate dehydrogenase (SDH) and a soluble cytoplasmic enzyme, lactate dehydrogenase (LDH). The image analysis system is used to capture successive images of a tissue section at constant time intervals whilst it is being incubated on a substrate-containing gel film. The increasing absorbances of the final reaction products in each cell are measured in the successive images as a function of incubation time. The absorbances of the formazan reaction products formed by SDH, for example, in sections of liver determined by such means increase linearly during the first minute of incubation, but non-linearly afterwards. The initial velocities of SDH in single hepatocytes in sections incubated on gel substrate films are calculated from the activities during the first 20 s of incubation. In contrast, the activities of LDH measured in various cell types, including hepatocytes, with the gel film technique increase non-linearly during the first minute of incubation, but linearly for incubation times between 1 and 3 min. The initial velocities (v_i) of LDH in single cells can be, calculated, however from the activities during the first interval, 10 s, of the image capturing sequence. Unfortunately, the experimental errors of the initial velocities of LDH determined in this way are relatively high. To overcome this problem, we have found empirically that the equations $v_i = a_1{}^oA$ and $v_i = \underline{v}+a_2{}^oA$ enable reliable initial velocities (v_i) of the LDH reaction in single cells of various types to be calculated using the data of the linear activities for incubation times between 1 and 3 min. Dependence of the initial velocities of the SDH and LDH reactions on substrate concentrations gave the Michaelis constants (K_m) and maximum velocities (V_{max}). The K_m values determined *in situ* for SDH in hepatocytes and for LDH in various cell types with the gel film technique are in the same order of magnitude as the corresponding values determined biochemically. The constants a_1, a_2 and K_m of LDH are characteristic for each cell type and seem to be related to the intracellular localization of the enzyme and to its ligand-binding rather than to the different isozyme compositions in various cell types.

Key words: Enzymes, Kinetics, Image analysis system, Quantitative histochemistry, Gel film technique

Offprint requests to: Dr. Yoshiko Nakae, Department of Oral Anatomy 1, School of Dentistry, Tokushima University, 3 Kuramoto-cho, Tokushima 770, Japan

Introduction

Many studies on enzyme kinetics in solution have been undertaken using tissue homogenates or purified enzymes to elucidate the reaction mechanisms of enzymes. The findings of such studies have greatly contributed to predicting their physiological functions in living cells. However, enzymes in living cells do not exist in isolation. They are not 'pure'. Instead they function in a complex microenvironment in close association with other proteins. As a consequence, their kinetics in the real life situations of cells may differ considerably from those in solutions. The purpose of this review is to examine some of these differences as revealed by modern quantitative histochemistry.

In general, enzymes are classified into two groups, soluble and structurally-bound (Wilson, 1978). There is some evidence for the partition of several soluble enzymes between soluble and particulate fractions of tissue homogenates being influenced by specific metabolites such as substrates, products and allosteric effectors of the enzymes (Wilson, 1978; Masters, 1981, 1992). The binding of soluble enzymes to subcellular structures modifies their kinetic parameters (Wilson, 1968; Knull et al., 1974; Nitisewojo and Hultin, 1976; Walsh et al., 1977; Swezey and Epel, 1986) and allosteric properties (Karadsheh and Uyeda, 1977). Thus, it has been suggested that the interactions between soluble enzymes and cellular structures function to regulate enzyme activities in response to various

physiological states of the cell such as development (Reid and Masters, 1985a,b; Swezey and Epel, 1986), disease processes (Knull et al., 1973, 1974; Bachelard, 1976), and muscle contraction (Walsh et al., 1977; Pagliaro and Taylor, 1988; Masters, 1981, 1984).

Validated quantitative histochemical techniques (Stoward, 1980; Stoward et al., 1991; Van Noorden and Butcher, 1991), in which the morphological integrities of cells are preserved in sections and smears, permit investigation of the diversity of the interactions and the microenvironmental factors *in situ* affecting the equilibrium between soluble enzymes and cellular structures. There have been some reports that the kinetic parameters of enzymes determined *in situ* agree with those determined *in vitro* (Gutschmidt et al., 1979; Kugler, 1981; Stoward and Al-Sarraj, 1981; Nakae and Shono, 1984; Gordon and Robertson, 1986; Blanco et al., 1988; Old and Johnson, 1989; Jonges et al., 1990, 1992; Nakae and Stoward, 1992, 1993b, 1994b). There are, not unexpectedly, also contrary reports. Thus, for example, Michaelis constants (K_m) of several enzymes determined *in situ* are higher than those determined *in vitro* (Butcher, 1970; Robertson et al., 1982; Sinowatz et al., 1983; Van Noorden and Butcher, 1986; Van Noorden and Jonges, 1987; Van Noorden, 1988; Jonges and Van Noorden, 1989; Lawrence et al., 1990; Patel et al., 1991; Nakae and Stoward, 1994b). The differences has been attributed to the interactions *in situ* between enzymes and cellular structures (Van Noorden and Vogels, 1989a; Lawrence et al., 1990) or specific metabolites (Nakae and Stoward, 1994b). Hence, it is extremely important in understanding the physiological functions of enzymes in cells to determine their activities and kinetic parameters *in situ* and compare them to biochemical data.

In this review, the details of how *in situ* activities are determined with a computer-assisted image analysis system are described. Two examples are discussed. The first is lactate dehydrogenase (LDH), a soluble enzyme that catalyses the last step of glycolysis. The second is succinate dehydrogenase (SDH), a mitochondrial membrane-bound enzyme involved in the Krebs citric acid cycle. The initial velocities of the SDH reaction *in situ* can be determined from its initial linear kinetics during the first minute of incubation (Pette, 1981; Nakae and Stoward, 1992). In contrast, it is much more difficult to determine the activities of a soluble enzyme *in situ* because more than half the enzyme originally present rapidly diffuses out of unfixed sections during its histochemical assay (Fahimi and Amarasingham, 1964; Kalina and Gahan, 1965; Meijer, 1980; Nakae and Stoward, 1993a). However, a new approach has been developed (Nakae and Stoward, 1993a,b,c, 1994a,b) for determining the initial reaction velocities of the soluble enzyme *in situ* with the agarose gel film technique originally introduced by Pette and his colleagues (Pette and Brandau, 1962; Nolte and Pette, 1972a,b; Pette and Wimmer, 1979). This approach is reviewed here as an example of studies that have overcome this problem. The diverse kinetic parameters of LDH *in situ* determined by this means in various cell types are discussed in terms of its isozyme composition and microenvironment in each cell type.

Real-time image analysis system

Modern image analysis system are based on computer and television technologies. Fig. 1 shows a typical system fitted to a light microscope. The principal steps in image analysis are image capture, image processing, and measurement. The analysis can involve measurements of optical densities, areas, diameters, perimeters, lengths, and distances. Several image analysis systems available commercially digitize images at constant intervals in real time. Examples are the Seescan Solitaire Plus image analysis system (Seescan Analytical Services Ltd, Cambridge, UK), the ARGUS-100 image analysis system (Hamamatsu Photonics K.K., Hamamatsu, Japan) and the MCID image analysis system (Imaging Research Ltd, Ontario, Canada). There are several advantages in using image analysis systems with this function in the field of quantitative enzyme histochemistry. Images based on the absorption of monochromatic light by the final reaction product (FRP) produced by an enzyme in a tissue section are captured at constant intervals during its histochemical assay and stored on mini-floppy disks. The stored images can then be analysed at leisure. Optical density measurements of successive images enable the enzyme activities in a large number of single cells in a section to be monitored continuously. Topographical data for a number of cells can also be obtained quickly in the same time. Fewer samples and less time and labour are required for this kind of work compared with using a scanning microdensitometer of the type available in the 1970s and 1980s.

A scanning spot in a scanning microdensitomer

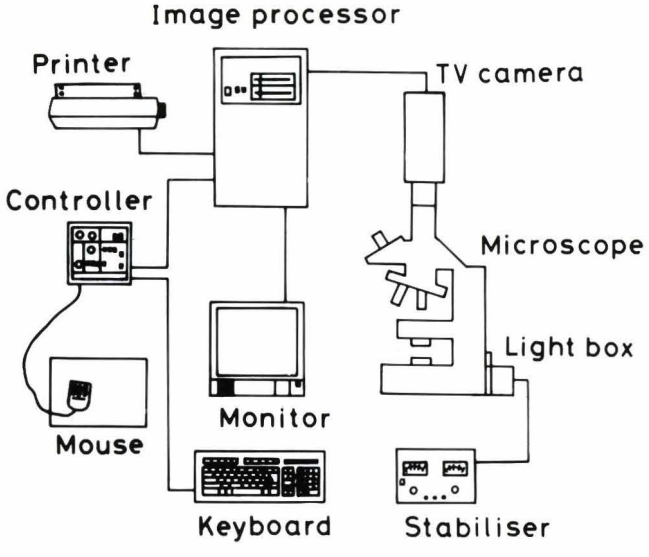


Fig. 1. Schematic diagram of an ARGUS-100 image analysis system.

Table 1. Transmission (%) of a background image captured using an ARGUS-100 image analysis system. A diode-gun Plumbicon camera was used as the scanner. A substrate gel film for SDH was sandwiched between two cover slips and placed on the stage of a microscope fitted with a x40 objective and irradiated by monochromatic light at 584 nm. The image (512 x 483 pixels) was divided into 64 rectangles (lengthwise A-H and crosswise 1-8). The mean transmission and SD for each rectangle image was calculated regarding the transmission of the centre pixel in the whole image as 100%. The coefficient of variation (CV) for the mean transmission among the 64 measurements is 1.8% (see also Blanco et al., 1988).

	1	2	3	4	5	6	7	8
A Mean	91.2	95.9	98.6	99.9	100.0	99.9	98.9	96.5
SD	1.78	0.976	0.831	0.215	1.78	0.976	0.831	0.215
B Mean	92.1	97.4	99.7	100.0	100.0	100.0	99.8	98.0
SD	2.03	1.21	0.462	0.00	2.03	1.21	0.462	0.00
C Mean	94.3	99.1	100.0	100.0	100.0	100.0	100.0	99.6
SD	2.22	0.926	0.013	0.00	2.22	0.926	0.013	0.00
D Mean	96.9	99.9	100.0	100.0	100.0	100.0	100.0	100.0
SD	1.53	0.29	0.00	0.00	1.53	0.29	0.00	0.00
E Mean	98.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
SD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
F Mean	98.6	100.0	100.0	100.0	100.0	100.0	100.0	100.0
SD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
G Mean	98.3	100.0	100.0	100.0	100.0	100.0	100.0	100.0
SD	0.00	0.00	0.00	0.070	0.00	0.00	0.00	0.070
H Mean	98.2	100.0	100.0	100.0	100.0	100.0	100.0	99.9
SD	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.20

corresponds to a pixel, a picture element, in image analysis. Each pixel is related to 64 or 256 grey levels depending on a 6 or 8 bit number respectively in digital image processing (Joyce Loebl, 1985; Russ, 1990). The grey level 0 corresponds to 100% transmission (T) of monochromatic light and the grey level 255 to the dark current of a scanner (T= 0%). The transmission of each pixel is converted to optical density (OD) using the equation $OD = 2 - \log_{10} T$. The scanner is usually a high quality commercial TV camera. Several TV cameras with appropriate and characteristic spectral response, noise and sensitivity are available for image analysis systems (Joyce Loebl, 1985; Inoué, 1986). For microdensitometry, a Plumbicon, Newvicon or, best of all, a CCD camera (Donovan and Goldstein, 1985; Chieco et al., 1994) is preferred because of the proportionality between input and output intensities of light (gamma= 1), making calibration simple and more reliable than other cameras (Joyce Loebl, 1985).

Calibration for microdensitometric measurements

For microdensitometric measurements, objects (sections or smears) are best illuminated with monochromatic light obtained by placing a narrow-band interference filter in the light path of the microscope (Blanco et al., 1988; Lawrence et al., 1989; Nakae and Stoward, 1992). This permits the valid application of Lambert-Beer's law. Interference filters (e.g. from Vacuum Optics Co. of Japan, Tokyo) combined with a filter which eliminates near infra-red light are preferable to either an interference wedge or a prism monochrometer because the band width is much narrower. The light source should preferably be stabilised by a regulated DC power supply. A thermoregulated stage should be attached to the light microscope of the image analysis system (Nakae and Stoward, 1992, 1993a, 1994a,b) because enzyme activity depends on the temperature of the incubation medium. The optical system of the light microscope and the image analysis system should be adjusted for Köhler illumination. The variations of transmission within a background image without specimen (shading effects) should be less 10% (Inoué, 1986) as shown in Table 1 (see also Blanco et al., 1988). To correct for the inevitable uneven illumination, this residual image is subtracted from each test image (shading correction). The light intensity of the microscope is adjusted to just below the saturation point for all pixels within the array of the light detector. However, Lawrence et al. (1989) has reported that after shading correction there is no significant effect on the absorbance readings in a Seescan Solitaire Plus image analysis system even if the relative light intensity is reduced from 114 to 35 grey scale. The same microscope objective should be used in a series of measurements because objective magnification affects absorbance readings (Blanco et al., 1988; Lawrence et al., 1989). The focus of images should be adjusted precisely during capturing. Though the out-of-focus error on absorbance reading is about ±10% with a conventional microdensitometer, thinner sections of tissue are preferred (Bitensky, 1980).

Lawrence et al. (1989) has reported the calibration of a Seescan Solitarie Plus image analysis system using neutral density filters. Fig. 2 shows the calibration of an ARGUS-100 image analysis system using neutral-density filters of known absorbances measured with a Vickers M85 scanning microdensitometer (Nakae and Stoward, 1993a). The absorbances at 584 nm of various neutral-density filters measured in an ARGUS image analysis system agree within 10% for absorbances to at least 1.2. Absorbances also increase lienarly with section thicknesses, thus confirming that Lambert-Beer's law holds true for such objects (Martin et al., 1985; Blanco et al., 1988; Chalmers and Edgerton, 1989b; Lawrence et al., 1989; Nakae and Stoward, 1992).

Distributional error due to the heterogeneous distribution of absorbing material in cells can be assessed by measuring their absorbances with various frame sizes in the same images. An example is shown in Fig. 3 in which the observed initial velocities of the LDH reaction measured in various areas (measuring frames) in single fibres of skeletal muscle are plotted against the frame sizes (Nakae and Stoward, 1994a). The wavelength of monochromatic light used for these measurements is 584 nm, which corresponds to the isobestic point of the principal FRPs, a red monoformazan and a blue diformazan of Nitro BT (Butcher, 1978). The initial velocities were calculated from the absorbance changes of two images captured at incubation times 0 and 10 s. Using x40 objective

(1 pixel= 0.25 μm^2) the distributional error of formazans deposited in skeletal muscle cells is within about 4% using the ARGUS-100 image analysis system.

Continuous monitoring of enzyme activity

To measure enzyme activities *in situ*, validated quantitative histochemical techniques (Stoward, 1980; Stoward et al., 1991; Van Noorden and Butcher, 1991) are applied to either cell smears or tissue sections cut at constant speed at a constant temperature between -20 and -30 °C in a cryostat. Usually cells and sections are used without fixation for histochemical assays in order to avoid enzyme denaturation. However, this leads to another problem, namely that enzymes, especially soluble ones, originally present in unfixed cells and tissue sections diffuse into aqueous media during their histochemical assay (Fahimi and Amarasingham, 1964; Meijer, 1980; Wachsmuth, 1980; Nakae and Stoward, 1988, 1993a). Three methods have been developed to overcome or control this diffusion: agarose gel films (Pette and Brandau, 1962; Fahimi and Amarasingham, 1964; Nolte and Pette, 1972a,b), polyvinyl alcohol (PVA)-containing media (Altman and Chayen, 1965; Altman, 1980; Van Noorden and Vogels, 1989a), and semipermeable membranes (McMillan, 1967; Meijer, 1972, 1980). However, with the first two of these methods, enzymes still diffuse out of sections during incubation and subsequent rinses in distilled water (Butcher et al., 1980; Frederiks et al., 1988; Stoward and Nakae, 1988; Nakae and Stoward, 1988, 1993a). Therefore, ideally enzyme activities *in situ* should be determined in the initial phase of incubation by continuous monitoring of the formation of enzyme FRP during incubation rather than from end-point measurements. The continous monitoring of enzyme reactions *in situ* using real-time image analysis systems has been reported by Marković et al. (1983), Sieck and Sacks (1986), Sieck et al. (1987), Blanco et al. (1988), Chalmers and Edgerton (1989a,b), Lawrence et al. (1989, 1990), and Nakae and Stoward (1992, 1993a, 1994a,b) and others.

The monitoring is carried out as follows. First, an image of an incubation medium (the 'background image') (Table 1) is captured and stored in the image processor. The enzyme reaction is then started by placing a tissue section in contact with an assay medium. Monochromatic images of the section are captured at constant time-intervals in real time continuously during the incubation, and stored after subtraction of the background image (shading correction). The background and test images displayed on the monitor are sharply focused during the capturing. Each image requires 1/30 or 1/25 s for digitisation for 525 or 625 scanning lines respectively (Joyce Loebl, 1985; Inoué, 1986; Russ, 1990). The images are integrated an appropriate number of times (usually 16) before storage.

Fig 4a shows an image of an unfixed section of liver captured in the ARGUS-100 image analysis system 100 s after starting an incubation of a section placed on a substrate (succinate)-containing agarose gel film for SDH (Nakae and Stoward, 1992). The principal FRPs are a mixture of Nitro BT mono- and diformazans. The grey levels (activities) in Fig. 4a are related to a colour scale. The pseudo-coloured image is shown in Fig. 4b. From this image it can be seen that the SDH activity is

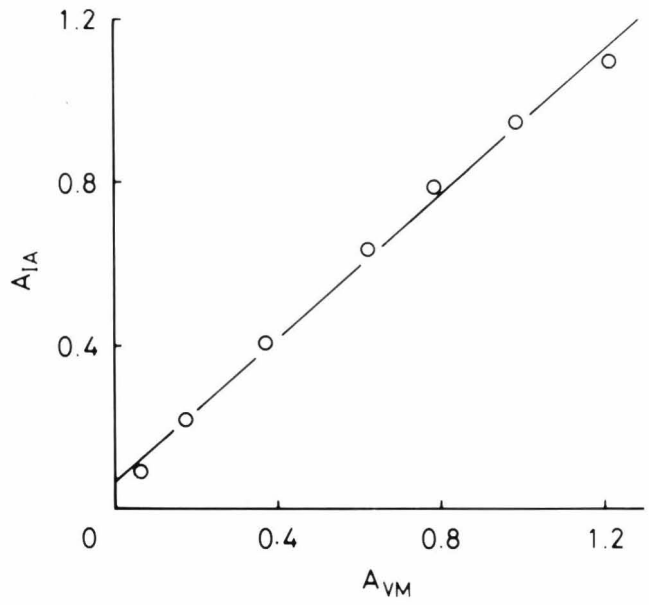


Fig. 2. Absorbance (A_{IA}) at 584 nm of neutral density filters measured with an ARGUS-100 image analysis system as a function of their calibrated absorbance (A_{vm}) at 584 nm with a Vickers M85 scanning microdensitometer. The regression of A_{IA} on A_{VM} fits the equation A_{IA}= 0.879A_{VM} + 0.0639 (r= 0.997, $p< 0.001$, n= 7). (From Nakae and Stoward, 1993a).

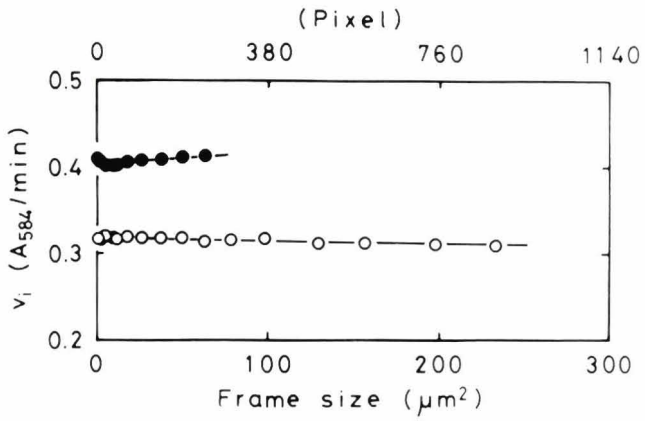


Fig. 3. Observed initial velocities (A_{584}/min) calculated using various frame sizes in the images for uncorrected LDH in single muscle fibres of mouse gastrocnemius. The sections, 4 μm thick, were incubated on substrate gel films containing 0.8% lowgelling temperature (LGT) agarose, 70 mM L-lactate and other ingredients. pH 7.5, 37 °C. ARGUS-100 image analysis system was used. ○= a large fibre; ●= a small fibre. (From Nakae and Stoward, 1994a).

lower in the pericentral region than in the periportal region.

Kinetic analysis

Here we review, first, how kinetic data of enzymes *in situ* are obtained from images captured at predetermined intervals of tissue sections during their incubation on substrate-containing media and, second, how the data are analysed in order to determine kinetic constants.

Time courses of enzyme reactions

The absorbances of the FRP of an enzyme deposited in a delimited area of single cells are determined as a function of incubation time from images captured continuously during incubation using an image analysis system. Typical time courses of the SDH and LDH reactions in the cytoplasm of single pericentral and periportal hepatocytes are shown in Figs. 5a,b (Nakae and Stoward, 1992, 1993a). They were obtained using an ARGUS-100 image analysis system, from 28 sequential images of liver sections incubated on agarose gel films containing the appropriate enzyme substrate (Nakae and Stoward, 1992, 1993a). The time courses of their control reactions without substrates (the 'nothing dehydrogenase' reactions) are also shown in these Figures. The absorbances of the SDH FRPs (i.e. activities) formed from Nitro BT increase linearly during the first minute of incubation, but non-linearly afterwards. In contrast, the activities of LDH and 'nothing dehydrogenase' increase non-linearly during the first minute of incubation, but linearly for incubation times between 1 and 3 min. A similar non-linearity and linearity is observed at various concentrations of substrate (L-lactate) for LDH reactions in mouse cardiac and skeletal muscle fibres, gastric parietal cells, duct epithelial and acinar cells of the parotid gland, oocytes, and human hepatocytes (Nakae and Stoward, 1994a,b). The corrected (intrinsic) activity curves for SDH and LDH obtained by subtracting the 'nothing dehydrogenase' activity at each time point of incubation from the corresponding SDH and LDH activities are similar to their uncorrected activity curves (Figs. 5a,b).

A number of quantitative histochemical studies on single cells have revealed that, for several enzymes, including SDH and LDH, the enzyme reaction rate is initially not linear with time (Nolte and Pette, 1972a,b; Altman, 1978; Pette and Wimmer, 1979; Kugler, 1981; Pette, 1981; Robertson et al., 1982, 1984; Butcher and Van Noorden, 1985; Van Noorden et al., 1985; Van Noorden and Butcher, 1986, 1987; Van Noorden and Jonges, 1987; Stoward and Nakae, 1988; Chalmers and Edgerton, 1989b; Frederiks et al., 1989; Jonges and Van Noorden, 1989; Lawrence et al., 1989; Lomax et al., 1989; Ruhnke and Gossrau, 1989; Van Noorden and Vogels, 1989b; Frederiks et al., 1991; Nakae and Stoward, 1992, 1993a,b, 1994a,b). However, Van Noorden's group (Van Noorden et al., 1985; Van Noorden and Butcher, 1986; Van Noorden and Jonges, 1987; Van Noorden, 1988; Jonges and Van Noorden, 1989; Van Noorden and Vogels, 1989b; Frederiks et al., 1991), among others, have shown that some enzymes do exhibit linear reaction rates when the kinetics of the 'nothing dehydrogenase' reaction is subtracted, particularly when sections are incubated in media containing PVA as a tissue protectant. In contrast, these reactions are often not linear when sections are incubated on gel substrate films (Nakae and Stoward, 1993a,b, 1994a,b).

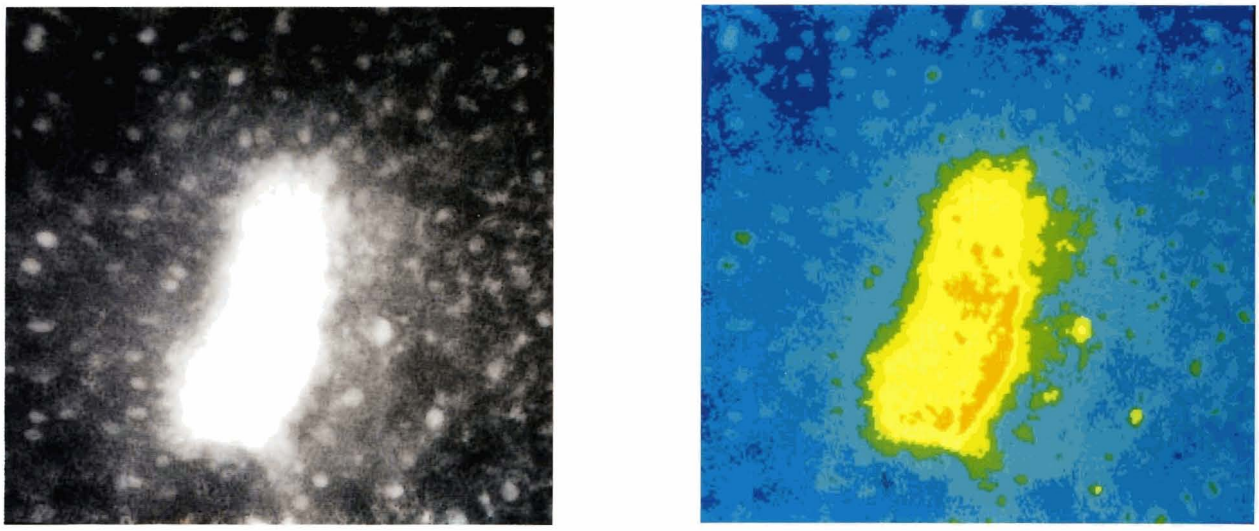


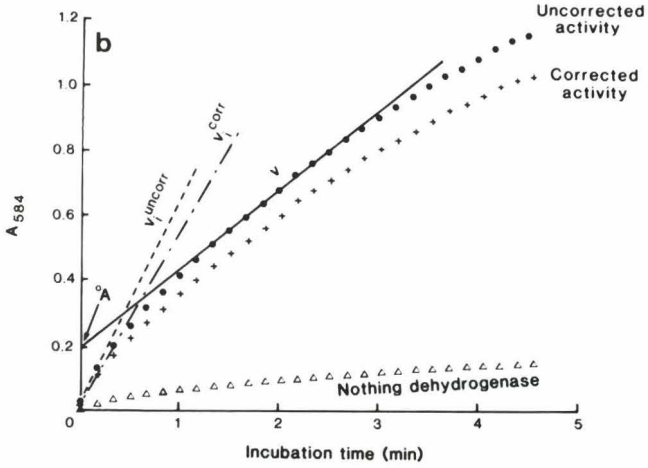
Fig. 4. Display on the monitor of an image analysis system of the image of the pericentral region in a liver section incubated for 100 s on a gel substrate film for SDH. 8 µm section, 20mM succinate, 1.6% LGT agarose gel film, pH 7.5, 37 °C. A x40 objective was used. **(a)** Grey scale image (left) (512 x 483 pixels) irradiated with monochromatic light at 584 nm. Each pixel was related to 256 grey levels. The white dots are the nuclei of hepatocytes. **(b)** Pseudo-coloured image (right) of (a). The images were corrected for shading effects.

The activities of LDH in liver homogenates (Van Noorden and Vogels, 1989b) and purified glucose-6-phosphate dehydrogenase (Pette and Wimmer, 1979) incorporated in polyacrylamide gel films have been measured in aqueous incubation media. They were reported to be linear with incubation time. Therefore, the non-linearity observed in the initial phase of the enzyme reactions *in situ* appears to be characteristic of enzyme reactions in tissue sections. Van Noorden and Vogels (1989b) claimed that product inhibition of LDH by pyruvate does not occur in the polyacrylamide gel films because pyruvate diffuses more easily from the gels when immersed in aqueous media than from tissue section incubated in PVA-containing media. There is another explanation for the linear reactions of the enzymes incorporated in the polyacrylamide gels: diffusion of the enzymes from the gels into the incubation media is prevented by the gel matrices.

Initial velocity

The reasons why *in situ* enzyme activities are initially non-linear with time are possibly either inhibition of the enzymes by their products (Van Noorden et al., 1985; Van Noorden and Vogels, 1989b) or FRPs (Kugler, 1981; Pette, 1981) or diffusion of the enzymes (Nakae and Stoward, 1988, 1993a) or their endogenous substrates (Van Noorden and Butcher, 1987) or reduced coenzymes and exogenous electron carriers (McMillan, 1967; Andersen and Høyer, 1974) out of tissue sections during incubation, depending on the intracellular localization and molecular properties of the enzymes. Whatever the reason, the initial velocities of enzyme reactions should be measured to avoid these effects.

The initial velocities of an enzyme can be calculated from the gradients of the linear initial phases of the reaction. For example, the observed initial velocities of the SDH and LDH reactions in single hepatocytes in liver sections incubated on gel substrate films can be determined from the linear portions of the activity-time plots between 0 and 20 s, and 0 and 10 s, respectively (Figs. 5a,b) (Nakae and Stoward, 1992, 1993a). However, the initial velocities of the LDH reaction determined in this way are subject to considerable errors because the initial phase of the reaction is very short. Therefore, the initial velocity of the LDH reaction is better determined from the linear steady-state phase for incubation times between 1 and 3 min rather than from the initial phase (Fig. 5b) (Nakae and Stoward, 1993a,b, 1994a,b). This can be achieved as follows. The steady-state velocity v is the gradient of the linear portion for incubation times between 1 and 3 min. When this linear portion extrapolates, it intercepts the absorbance axis at ^{o}A (Fig. 5b). The observed initial velocities v_i and $(v_i - v)$ of the uncorrected LDH and 'nothing dehydrogenase' reactions in single hepatocytes in the tissue sections of various thicknesses are then plotted against ^{o}A (Figs. 6a,b) (Nakae and Stoward, 1993a,c, 1994a). The intercepts ^{o}A correlate highly significantly with the observed v_i and $(v_i - v)$ for both the LDH and 'nothing dehydrogenase' reactions. The regression best-fit lines for both reactions pass near the origin. It is reasonable that the points for the 'nothing dehydrogenase' reaction in Figs. 6a,b are approximated by the same lines as those for LDH because the 'nothing dehydrogenase' activities are mainly due to LDH oxidising endogenous lactate in the cells (Van Noorden et al., 1985; Van Noorden and



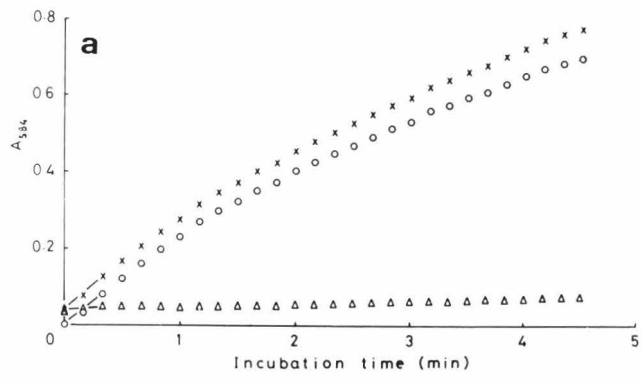


Fig. 5. Typical plots of absorbance at 584 nm *(A_{584})* of FRP formed by (a) SDH in single pericentral hepatocytes and (b) LDH in single periportal hepatocytes as a function of incubation time. Absorbances were determined at 10 s intervals using an ARGUS-100 image analysis system. x, ●= uncorrected activities; Δ= 'nothing dehydrogenase' activity; ○, += activities corrected for 'nothing dehydrogenase' by subtracting Δ from x and ● at each time point. pH 7.5, 37 °C. **(a)** 8 μm-thick sections, 20 mM succinate, 1.6% LGT agarose gel films. Regression lines for SDH are drawn for incubation times (t) between 0 and 20 s and correspond to A_{584}= 0.256*t* + 0.0383 (*r*= 0.998, *p*< 0.05, *n*= 3) for uncorrected SDH, *A584*= 0.0222*t* + *0.0392* (*r*= 0.999, *p*< 0.05, *n*= *3*) for «nothing dehydrogenase» and *A584*= 0.234*t* - 0.000918 *(r= 0.998*, *p*< 0.05, *n*= 3) for corrected SDH. Their initial velocities v_i correspond to the gradients of these equations. (From Nakae and Stoward, 1992). **(b)** 4 μm sections, 70 mM L-lactate, 0.8% agarose gel films. The straight line drawn for incubation times between 1 and 3 min is the linear regression of A_{584} on time. Above 3 min, the reaction becomes non-linear. The gradients of the plots for incubation times betwen 0 and 10 s (dotted lines) were assumed to be the observed initial velocities. (From Nakae and Stoward, 1993a).

Vogels, 1989b). Similar kinds of plots have been obtained for the LDH reaction in mouse cardiac and skeletal muscle fibres, gastric parietal cells, oocytes, duct epithelial and acinar cells of the parotid gland, and human hepatocytes (Nakae and Stoward, 1994a,b). Hence the initial velocity of the LDH reaction is approximated by the following equations (Nakae and Stoward, 1993a,b, 1994a,b)

$$v_i = a_1 {}^{o}A \qquad (1)$$

$$v_i - v = a_2 {}^{o}A \qquad (2)$$

where a_1 and a_2 are constants characteristic for each cell type and are respectively in the range 2.2 - 3.0 and 1.2-2.2 (Table 2) (Nakae and Stoward, 1994a). The mean differences (%), irrespective of sign, d_1 and d_2, between the observed v_i and the v_i calculated from equation 1 and equation 2 respectively (v_{i1} and v_{i2}) for various cell types are also shown in Table 2. The means of d_1 and d_2 for eight different cell types are 16 and 11% respectively. The close agreement between the observed v_i and the v_i calculated from equations 1 and 2 for mouse hepatocytes are shown in Figs. 7a,b.

The calculated initial velocities v_{i1} and v_{i2} of the LDH reaction *in situ*, but not the steady-state velocity v, are proportional to the thickness of a tissue section, that is the total amount of the enzyme in the section (Fig. 8) (Nakae and Stoward, 1993a,c). This emphasizes the importance of determining initial velocities in quantitative histochemical assays of enzyme activities (Nakae and Stoward, 1993c).

Michaelis constant and maximum velocity

Fig. 9 shows the dependence of the calculated initial velocity v_{i2} of the intrinsic LDH reaction corrected for 'nothing dehydrogenase' in single cardiac muscle fibres of mouse on substrate (L-lactate) concentration (S) (Nakae and Stoward, 1994b). The v_{i2} was calculated using equation 2 from the data obtained with the gel film technique and an ARGUS-100 image analysis system. The initial velocity increases as the substrate concentration rises and approaches its maximum value at lactate concentrations higher than 50 mM. Inhibition of LDH by the substrate does not occur at lactate

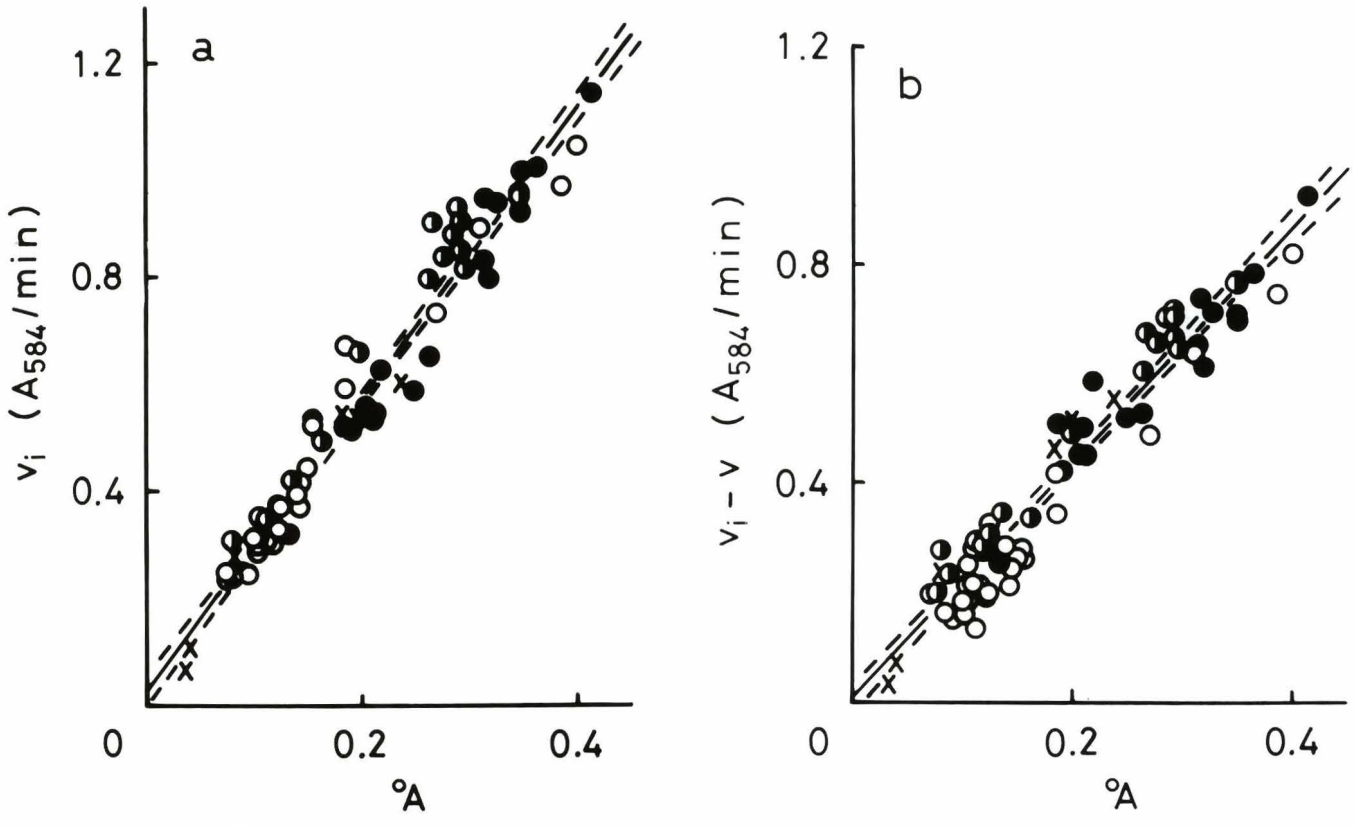


Fig. 6. Regression of **(a)** observed initial velocities (v_i) and **(b)** (v_i- v) on intercepts ^{o}A of LDH absorbance-incubation time plots for mouse periportal and pericentral hepatocytes. x= no substrate (i.e. 'nothing dehydrogenase'). 0.8% LTG agarose gel films, pH 7.5, 37 °C. ○= 4 μm sections (L-lactate concentrations= 0.07-70 mM). Semi-filled circles= 7 μm sections (L-lactate concentrations= 0.0175 - 14 mM). ●= 14 μm sections (substrate concentrations as for 7 μm sections). Each point is the mean for 10 hepatocytes around the same blood vessel. The solid regression lines in (a) and (b) correspond to the equations $v_i = 2.71\ ^{o}A + 0.0230$ (r= 0.980, p< 0.001, n = 71) and $v_i - v = 2.16\ ^{o}A + 0.00127$ (r= 0.967, p< 0.001, n = 71) respectively. The dotted lines show 95% confidence limits to the regression estimates. (From Nakae and Stoward, 1993c).

Table 2. Constants a_1 and a_2 ±SEM determined from linear regression plots of v_i on ^oA and those of (v_i - v) on ^oA respectively, and mean differences (%), irrespective of sign, d_1 and d_2, between observed v_i and v_i calculated from eq. 1 ($v_i = a_1{}^oA$) and eq. 2 ($v_i = v + a_2{}^oA$) respectively. The correlation coefficients (r_1 and r_2) of the two plots are highly significant (p<0.001) for all cell types. n=number of sections. (From Nakae and Stoward, 1994a).

SPECIES	ORGAN	CELL	a_1	r_1	d_1	a_2	r_2	d_2	n
Mouse	Heart	Muscle fibre	2.8±0.2	0.955	22	1.5±0.1	0.936	12	34
	Gastrocnemius	Muscle fibres	2.6±0.1	0.935	20	1.2±0.1	0.902	11	66
	Stomach	Parietal cell	3.0±0.2	0.961	17	1.7±0.1	0.946	12	21
	Parotid gland	Striated ductal cell	3.0±0.3	0.930	15	2.2±0.2	0.933	10	19
		Acinar cell	2.5±0.4	0.826	17	1.4±0.2	0.878	10	24
	Ovary	Oocyte	2.2±0.2	0.869	19	1.6±0.2	0.865	19	28
	Liver	Periportal and pericentral hepatocytes	2.7±0.1	0.980	11	2.2±0.1	0.967	11	71
Human	Liver	Periportal hepatocyte	3.0±0.2	0.964	8	1.9±0.2	0.929	6	19

Table 3. Mean kinetic constants ± SEM of LDH (against L-lactate) calculated from Hanes plots. The correlation coefficients (r) of the Hanes plots were all highly significant (p<0.001) except for *(p<0.005) and **(p<0.02), n=number of experimental points. Observed initial velocities (v_i^{obs}) in the presence of 70 mM lactate are included for comparison with V_{max}. (From Nakae and Stoward, 1994b).

SPECIES	ORGAN	CELL	VARIABLE	K_m (mM)	V_{max} (µmoles/cm³/min)	v_i^{obs} (µmoles/cm³/min)	r	n
Mouse	Heart	Muscle fibre	v	9.69±1.17	34.2±1.6		0.972	
			v_{i1}	23.5±3.1	66.4±6.2	61±4	0.897	30
			v_{i2}	13.4±1.5	67.5±3.7		0.961	
	Gastrocnemius	Small fibre	v	10.1±0.5	43.6±1.1		0.995	
			v_{i1}	27.2±9.9	62.5±19.2	59±3	0.618*	19
			v_{i2}	10.9±1.0	67.3±3.4		0.979	
		Intermediate fibre	v	11.2±0.5	43.6±0.9		0.997	
			v_{i1}	22.1±7.8	53.6±12.5	52±5	0.743	17
			v_{i2}	12.5±1.5	67.8±3.7		0.978	
		Large fibre	v	10.3±0.6	41.4±0.9		0.996	
			v_{i1}	20.4±11.0	38.3±13.0	45±6	0.604**	17
			v_{i2}	10.4±1.4	59.2±3.1		0.980	
	Stomach	Parietal cell	v	9.61±1.33	32.8±1.4		0.985	
			v_{i1}	9.67±4.07	50.2±6.5	59±5	0.878	20
			v_{i2}	9.66±2.33	62.2±4.6		0.954	
	Parotid	Striated ductal cell	v	8.59±1.77	19.1±1.2		0.972	
			v_{i1}	11.3±4.7	40.6±6.4	46±4	0.845	18
			v_{i2}	7.75±2.24	47.8±3.7		0.955	
		Acinar cell	v	9.79±2.57	19.4±1.5		0.944	
			v_{i1}	6.57±4.27	17.2±2.2	24±3	0.860	23
			v_{i2}	7.64±2.27	29.2±2.0		0.954	
	Ovary	Oocyte	v	10.7±1.1	37.5±1.5		0.984	
			v_{i1}	4.28±3.62	34.2±4.6	55±11	0.846	24
			v_{i2}	7.98±2.27	65.0±5.3		0.934	
	Liver	Periportal hepatocyte	v	15.3±2.0	35.5±1.8		0.994	
			v_{i1}	12.9±4.3	68.8±7.6	105±11	0.971	95
			v_{i2}	14.3±2.1	102±5		0.993	
		Pericentral hepatocyte	v	19.8±1.6	38.1±1.5		0.996	
			v_{i1}	10.6±3.1	77.2±6.1	105±11	0.985	42
			v_{i2}	16.7±2.0	110±5		0.994	
Human	Liver	Periportal hepatocyte	v	14.8±1.7	28.0±1.4		0.980	
			v_{i1}	14.0±3.2	48.1±4.6	46±4	0.941	16
			v_{i2}	14.2±1.9	58.9±3.4		0.978	

concentrations up to at least 70 mM (Fig. 9). The steady-state velocity v and the calculated initial velocity v_{i1} show the same dependence on substrate concentration as v_{i2} (Nakae and Stoward, 1994b). Hanes plots (1932) of S/v_{i2} against S for the LDH reaction in cardiac muscle fibres are shown in Fig. 10. The negative intercept of the linear regression line on the abcissa in this Figure corresponds to the apparent Michaelis constant, K_m

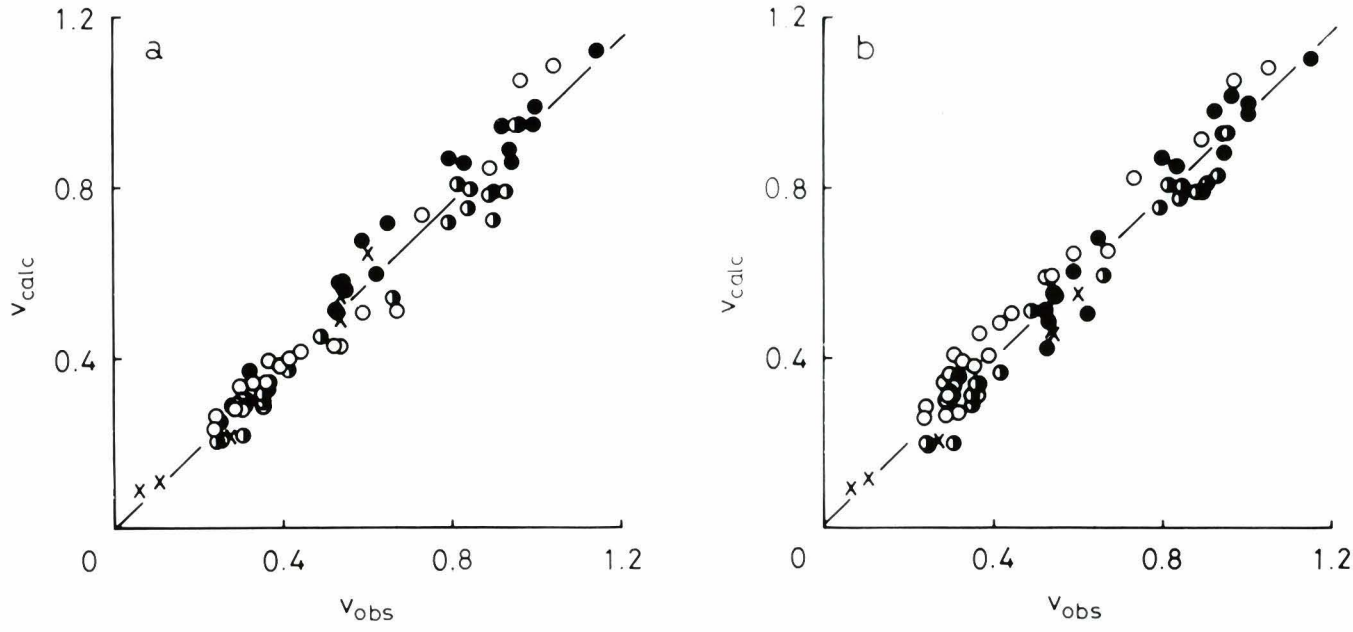


Fig. 7. Relationships between observed v_i and v_i calculated from **(a)** equation 1 ($v_i = 2.71°A$) and **(b)** equation 2 ($v_i = v+2.16$ °A) for LDH in mouse periportal and pericentral hepatocytes. Experimental conditions and symbols as in Fig. 6. The regression (solid lines) fit the equations $v_{calc} = 0.968v_{obs} - 0.00533$ ($r = 0.980$, $p < 0.001$, $n = 71$) and $v_{calc} = 0.978v_{obs} + 0.00999$ ($r = 0.978$, $p < 0.001$, $n = 71$) for (a) and (b) respectively, where v_{calc} and v_{obs} are the calculated and observed v_i respectively.

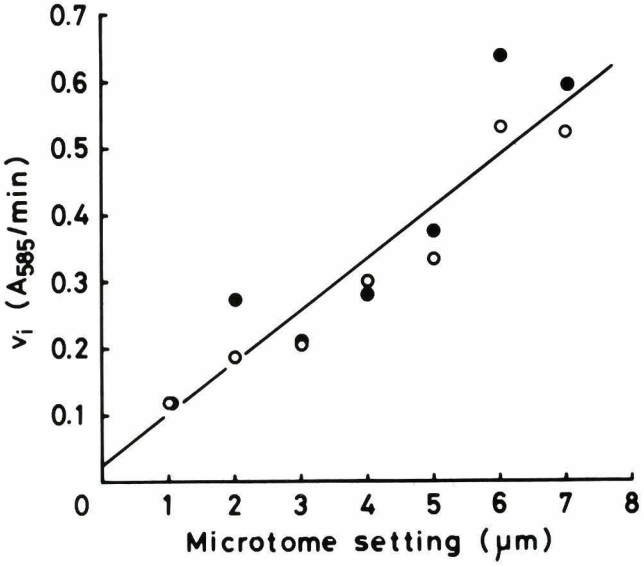


Fig. 8. Highly significant correlation between calculated v_i for LDH in periportal hepatocytes and section thickness, d (microtome setting). 0.8% LTG agarose gel films, 70 mM L-lactate. pH 7.5, 37 °C. Each point is the mean for 3-10 hepatocytes. The regression of v_i on d fits the equation $v_i = 0.0781d + 0.0230$ ($r = 0.931$, $p < 0.001$, $n = 14$). ○ = v_i calculated from $v_i = 2.71$ °A; ● = v_i calculated from $v_i = v + 2.16$ °A. (From Nakae and Stoward, 1993a, c).

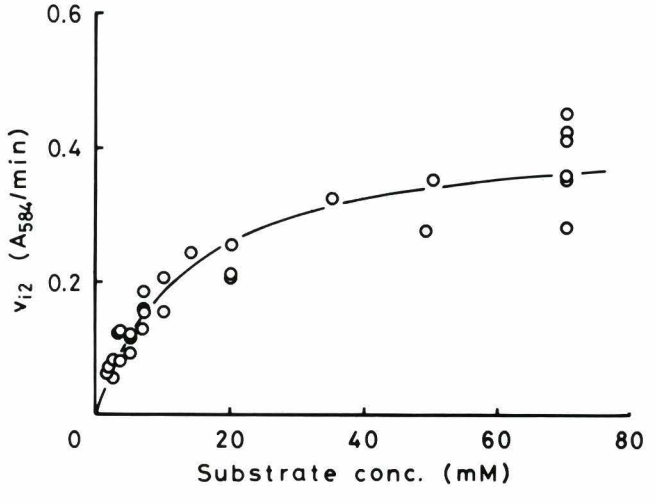


Fig. 9. Dependence of the initial velocity v_{i2} on the intrinsic (corrected) LDH reaction in mouse cardiac muscle fibres on substrate concentration. 4 μm sections, 0.8% LGT agarose films. pH 7.5, 37 °C. The curve is the best-fit calculated using K_m and V_{max} values 13.4 mM and 0.432 absorbance units/min respectively and the Michaelis-Menten equation. (From Nakae and Stoward, 1994b).

(Michaelis and Menten, 1913). The gradient of the regression line is the reciprocal of the maximum initial velocity (V_{max}) corrected for 'nothing dehydrogenase'. The apparent K_m and V_{max} values of LDH in various cell types, determined from linear regression analysis of Hanes plots of S/v, S/v_{i1} and S/v_{i2} against S, are summarised in Table 3 (Nakae and Stoward, 1994b). The higher correlation coefficients of the Hanes plots for all cell types using v_{i2} compared to v_{i1}, the significantly lower SEM of K_m and V_{max} using v_{i2} compared to v_{i1} (Table 3) and the higher d_1 compared to d_2 (Table 2) indicate that the observed initial velocities of the LDH reaction fit equation 2 better than equation 1 (Nakae and Stoward, 1994b).

The K_m values of the intrinsic LDH in all cell types except hepatocytes are similar when determined from the apparent steady-state velocities but diverse when determined from v_{i2}. The significantly different K_m values determined from v and v_{i2} suggest that K_m values should be determined from initial reaction velocities and not from steady-state velocities.

Interpretation of kinetic constants

a_1 and a_2

The constants a_1 and a_2 (Table 2) determined from the linear regression plots of the observed v_i on ^oA and those of $(v_i - v)$ on ^oA respectively for the LDH reaction in a particular cell do not seem to be related to the LDH isozyme composition of the cell. On the contrary, they appear to depend more on the extent to which the enzyme diffuses out of cells during its assay on gel substrate films, or conversely on the properties of the activity of insoluble LDH bound to mitochondria and other intracellular components (Nakae and Stoward, 1994a). Evidence for the lack of correspondence of a_1 and a_2 with isozyme composition is the similar low values of a_1 and a_2 in skeletal and cardiac muscle fibres. Skeletal muscle contain principally M-type LDH (Markert and Ursprung, 1962; Costello and Kaplan, 1963; Ohashi, 1966; Battellino and Blanco, 1970; Lluis, 1985; Reid and Masters, 1985b) but cardiac muscle H-type LDH (Markert and Ursprung, 1962; Costello and Kaplan, 1963; Battellino and Blanco, 1970; Hori et al., 1970) and yet their a_2 values are both low. Conversely, mouse and human hepatocytes which have high a_2 values also contain mostly M-type LDH like skeletal muscle fibres (Wróblewski and Gregory, 1961; Markert and Ursprung, 1962; Costello and Kaplan, 1963; Ohashi, 1966; Shaw, 1969; Battellino and Blanco, 1970; Hori et al., 1970; Reid and Masters, 1985b).

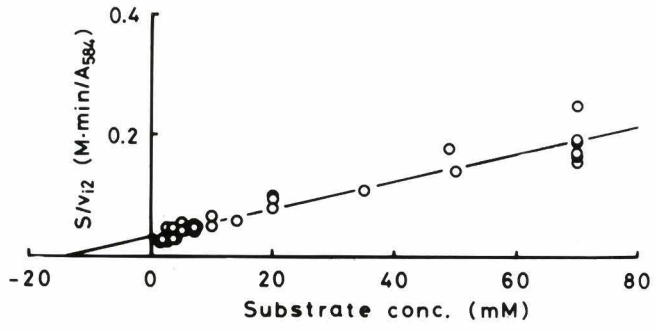


Fig. 10. Hanes plot of the data in Fig. 9. S= substrate concentration. The drawn line is a linear regression best-fit and gives K_m and V_{max} values of 13.4 mM and 0.432 absorbance units/min respectively.

On the other hand, cells with low values of a_2 appear to contain a significant proportion of their LDH bound to mitochondria, as has been demonstrated by both histochemical (Van Wijhe et al., 1964; Fahimi and Karnovsky, 1966; Baba and Sharma, 1971) and biochemical (Lluis, 1984, 1985; Pruñonosa et al., 1989; Sagristá et al., 1989; Szczęsna-Kaczmarek, 1990) techniques. Similarly, mouse cardiac muscle cells with a mitochondrial volume density in the range 35-48% (Herbener, 1976; Kainulainen et al., 1979; Else and Hulbert, 1981) are also likely to have much of their LDH activity associated with mitochondria (Baba and Sharma, 1971). Some of the LDH in both skeletal and cardiac muscle fibres may actually be cytosolic, but it is believed that it interacts with mitochondria (Van Wijhe et al., 1964; Fahimi and Karnovsky, 1966; Baba and Sharma, 1971; Lluis, 1984, 1985; Pruñonosa et al., 1989), sarcoplasmic reticulum (Fahimi and Amarasingham, 1964; Fahimi and Karnovsky, 1966; Baba and Sharma, 1971), isotropic bands (Pette and Brandau, 1962; Fahimi and Amarasingham, 1964; Dölken et al., 1975), Z-band regions (Van Wijhe et al., 1964; Fahimi and Karnovsky, 1966; Dölken et al., 1975), troponin (Yasykova et al., 1990) and reconstituted thin filaments (Masters, 1981, 1992). These interactions may lessen or protect the enzyme from diffusion out of cells during its histochemical assay. Likewise, the low values of a_1 and a_2 for oocytes and parietal cells, the cytoplasm of which displays strong LDH activities similar to those reported by Pupkin et al. (1966) and Asoo (1969), may be attributed to LDH present in, or bound to, mitochondrial clusters (Zamboni, 1970; Wassarman and Josefowicz, 1978; Helander et al., 1986; Van Blerkom and Bell, 1986).

In contrast, the relatively high values of a_1 and a_2 for hepatocytes can be attrributed to the higher cytosolic LDH and lower mitochondrial LDH activity (Elduque et al., 1982; Reid and Masters, 1985b; Kline et al., 1986; Brandt et al., 1987; Sagristá and Bozal, 1987; Sanz and Lluis, 1988; Sagristá et al., 1989; Sanz et al., 1990). Parotid gland duct epithelial cells also have high a_2 values. These cells have parallel arrays of mitochondria in their basal infoldings (Parks, 1961), but the uniformly strong LDH activity observed in their basal and apical cytoplasm suggests the activity is equally distributed in the cytosol and mitochondria.

Biochemcial studies indicate that inhibition of the LDH reaction by pyruvate is much greater in crude extracts and purified H_4 isozyme from cardiac muscle than for crude extracts and purified M_4 isozyme from

Table 4. Apparent Michaelis constants (against succinate) of soluble and membrane-bound succinate dehydrogenase from mammalian livers. (From Nakae and Stoward, 1992, with slight modification).

SPECIES	pH	TEMPERATURE (°C)	ELECTRON ACCEPTOR	K_m (mM)		ASSAY METHOD	REFERENCE
				Soluble	Membrane-bound		
Rat	7.4	38	0.0133mM cytochrome *c*		1[a]	Biochemical	Ackermann and Potter,1949
	8.0	37	0.3% NT		1.54	Histochemical	Butcher, 1970
	8.0	37	0.66 mM PMS		6.5	Histochemical	Butcher, 1970
	7.6	25	1.09 mM PMS		0.42-0.81	Biochemical	Susheela et al., 1977
	7.4	25	0.1mM cytochrome *c*	0.65	1.01	Biochemical	Wei et al., 1984
	7.4	?	1.62 mM PMS	0.095-0.226		Biochemical	Velez et al., 1985
Sheep	6.5	?			2.5	Biochemical	Rao and Swami, 1969
Human	7.5	38	1mM PMS	0.3	0.9	Biochemical	Shaw et al., 1981
Mouse	7.5	37	0.26mM PMS		1.2[b], 1.4[c]	Histochemical	Nakae and Stoward, 1992

NT: neotetrazolium salt (without PMS as an intermediate acceptor); PMS: phenazine methosulphate; [a]: K_s value; [b]: for periportal hepatocytes; [c]: for pericentral hepatocytes.

skeletal muscle (Latner et al., 1966; Stambaugh and Post, 1966). If the non-linearity of the activity curves of LDH during the first minute of incubation is due to product inhibition by pyruvate, then the a_1 and a_2 values for cardiac muscle fibres should be higher than those for skeletal muscle fibres and hepatocytes. However, the a_1 and a_2 values for cardiac and skeletal muscle fibres are actually lower than those for hepatocytes (Table 2). Thus, the non-linearity of the LDH activity does not seem to be attributable to product inhibition.

K_m and V_{max}

The following mean kinetic constants have been determined for SDH from Hanes plots of the initial velocities measured using the gel film method and an ARGUS-100 image analysis system (Nakae and Stoward, 1992). For periportal hepatocytes, the apparent K_m is 1.2±0.8 mM and V_{max} 29±2 µmoles hydrogen equivalents formed/cm^3 hepatocyte cytoplasm/min. For pericentral hepatocytes, K_m is 1.4±1.0 mM and V_{max} 21±2 µmoles hydrogen equivalents/cm^3/min. There is no significant difference between the K_m values for periportal and pericentral hepatocytes. These K_m values are very similar to those determined previously from biochemically assays, as shown in Table 4 (Nakae and Stoward, 1992). The K_m values determined biochemically for solubilized SDH (0.095-0.65 mM) are lower than those for membrane-bound SDH (0.42-2.5 mM). K_m values determined *in situ* by Butcher (1970) and Nakae and Stoward (1992) are 1.2, 1.4 and 1.54 mM and are in the latter range. The agreement is fairly close. However, the K_m value (6.5 mM) determined histochemically in PVA-containing medium in the presence of phenazine methosulphate (Butcher, 1970) is considerably higher. K_m values for SDH in skeletal muscle fibres (0.31 - 1.35 mM; Nakae and Shono, 1984; Blanco et al., 1988; Old and Johnson, 1989) and hippocampus (0.56 mM; Kugler, 1988) determined histochemically in aqueous media are also similar to those determined biochemically. Weiss and Deichsel (1978) and Kugler (1981) found the K_m for SDH in kidney cells was 1.05 and 1.75 mM with a semi-permeable membrane technique and a gel sandwich technique respectively.

The V_{max} values that we have found are higher than the V_{max} values reported previously for rat liver (Nakae and Stoward, 1992): 1.5 times higher than Butcher's (1970) value, about 10-fold more than that reported by Nolte and Pette (1972a), and 18 times higher than that determined by Van Noorden and Vogels (1989b).

The ratio of the mean V_{max} of SDH in periportal hepatocytes to that in pericentral hepatocytes is 1.4 (Nakae and Stoward, 1992). This value agrees with the corresponding ratio (1.58) for the activity reported for the adult male rat by Nolte and Pette (1972a), but differs from the activity ratio (1.91) found by Wimmer and Pette (1979) for liver of male rats of a different strain. The two lower values (1.4, 1.58) correlate with the ratio of 1.5 for the mitochondrial volume density in mouse periportal hepatocytes to that in the pericentral hepatocytes (Asada-Kubota et al., 1982). Hence, it is conceivable that the same kind of SDH molecule is distributed to the same density in the mitochondrial inner membranes of the two kinds of hepatocytes (Nakae and Stoward, 1992).

In situ K_m values of LDH in various cell types (Table 3) have been discussed in detail by us elsewhere (Nakae and Stoward, 1994b). The K_m value (13.4 mM) of the intrinsic LDH reaction in cardiac muscle cells determined from v_{i2} (see p. 469) is significantly higher than the K_m for small and large muscle fibres of

gastrocnemius, gastric parietal cells, oocytes, and ductal and acinar cells of the parotid gland, but similar to those for hepatocytes and intermediate fibres of gastrocnemius (Table 3). The K_m we found (14.0-14.2 mM) for LDH in human periportal hepatocytes is in good agreement with the values (14.3-15.5 mM) determined previously with a biochemical assay technique (Nisselbaum and Bodansky, 1963; Nisselbaum et al., 1964) for human M_4 isozyme, which is the main component of liver LDH (Wróblewski and Gregory, 1961; Markert and Ursprung, 1962; Costello and Kaplan, 1963; Battellino and Blanco, 1970). The range of K_m determined from v_{i2} measurements for mouse periportal and pericentral hepatocytes, 14.2-14.3 mM and 12.1-16.7 mM respectively (Nakae and Stoward, 1993b, 1994b), are remarkably similar to the human K_m values, but are considerably higher than the value of 5.5 mM found previously for the mouse M_4 isozyme *in vitro* (Battellino and Blanco, 1970). The three-fold discrepancy between the *in situ* and *in vitro* values could be due in part to the different assay conditions used (e.g. pH, temperature, ionic strength) but it is unlikely that any such differences account for the magnitude of the discrepancy.

The mean K_m value of LDH derived from v_{i2} in three types of skeletal muscle fibre in mouse gastrocnemius is 11.3 mM (Table 3). This is similar to the K_m value (12.5 mM) of the M_4 isozyme from bovine skeletal muscle (Pesce et al., 1964, 1967) but disagrees with the K_m values of 3.9 and 5 mM for M_4 isozymes isolated from skeletal muscles of the mouse (Hawtrey et al., 1975) and pig (Bennett and Gutfreund, 1973) respectively and with the K_m values (20 and 30 mM) determined previously with a histochemical method for LDH in two fibre types of human skeletal muscle (Wachsmuth, 1980). Nitisewojo and Hultin (1976) reported that the K_m value of chicken LDH M_4 isozyme for pyruvate is double when it is bound to the cellular particulate fraction of homogenized skeletal muscle. Therefore, it is reasonable that the K_m value determined biochemically for the soluble LDH purified from mouse skeletal muscle is roughly half compared to the corresponding value determined *in situ*.

The K_m value determined from v_{i2} for mouse cardiac muscle fibres (13.4 mM) is close to the K_m for hepatocytes (14.3-16.7 mM), but is significantly higher than the K_m for small and large muscle fibres of gastrocnemius (10.4-10.9 mM), gastric parietal cells (9.7 mM), ductal and acinar cells of the parotid gland (7.7 mM) and oocytes (8.0 mM) (Table 3). Previous biochemical studies have reported that the K_m values of LDH H_4 isozymes isolated from hearts of mammalian species are in the range 2-9 mM (Nisselbaum and Bodansky, 1963; Nisselbaum et al., 1964; Pesce et al., 1964, 1967; Battellino et al., 1968; Battellino and Blanco, 1970; Südi, 1974; Hawtrey et al., 1975). The K_m value we determined *in situ* for cardiac muscle fibres disagrees with these values. Therefore, it seems that the kinetic behaviour of LDH *in situ* in cardiac muscle fibres is different from that of isolated LDH from these cells. Everse and Kaplan (1973) reported that a significant proportion of LDH in fresh heart extracts is present in an inactive form, presumably the abortive enzyme-NAD^+-pyruvate complex. These results suggest that ideally enzyme activity should be measured both *in situ* and *in vitro* in order to determine its actual physiological function in the cell. There is also the possibility that some LDH in cardiac muscle fibres interacts with mitochondria *in situ* (Baba and Sharma, 1971) and that the K_m value of the bound form is higher than that of the soluble form (Nitisewojo and Hultin, 1976).

The K_m values cited here for LDH in mouse gastric parietal cells, oocytes, and ductal and acinar cells (in the range, 7.6-9.7 mM, Table 3) are slightly higher than the K_m values of H_4 isozymes from mammalian species determined biochemically (Nisselbaum and Bodansky, 1963; Nisselbaum et al., 1964; Pesce et al., 1964, 1967; Battellino et al., 1968; Battellino and Blanco, 1970; Südi, 1974; Hawtrey et al., 1975). Since the LDH isozymes in mouse stomach, ovary and parotid gland are principally the H-type, M-type and M-type respectively (Markert and Ursprung, 1962; Battellino and Blanco, 1970; Masters, 1981), it is unlikely that the wide variation of K_m in other cell types can be attributed solely to differences of isozyme composition. Instead, it is more probable that the variations are due to intrinsically different kinetics, substrate binding and intracellular localization of the enzyme in a variety of cell types.

The wide range of V_{max} determined from Hanes plots of v_{i2} also reflects the diverse kinetics of LDH in different cell tyeps (Table 3). They range from 29 μmoles hydrogen equivalents formed/cm^3 cell cytoplasm/min units in mouse parotid gland acinar cells to 59-68 units in skeletal and cardiac muscle fibres, 62-65 units in gastric parietal cells and oocytes, and 102-110 units in hepatocytes. With the exception of skeletal muscle fibres, the mean V_{max} values for LDH in each cell type are, on average, about 9% higher than the corresponding mean observed initial velocities (v_i^{obs}). The differences between the two values are not significant. In contrast, V_{max} in skeletal muscle fibres are significantly higher than the observed initial velocities by, on average, 25%.

The steady-state activities of LDH *in situ* in mouse skeletal muscle (Altman, 1978), rat cardiac muscle (Altman, 1978; Butcher, 1983; Van Noorden and Vogels, 1989b) and rat liver (Altman, 1978; Van Noorden and Vogels, 1989b) determined with assay media containing PVA are 4 - 41% of the observed initial activity (v_i^{obs}) we obtained with a gel film technique (Table 3) (Nakae and Stoward, 1994a). The difference is explained in part by the fact that we determined initial reaction velocities, which are 2-3 times higher than the steady-state velocities generally reported. However, the lower rates obtained using the PVA media may also be due to either a reduction of the diffusion constants of substrate and other reactants in the media (Ogston et al., 1973), or to inhibitions of the activities by PVA itself (Van Noorden

and Vogels, 1989a).

The similarity of the V_{max} of LDH in male mouse periportal and pericentral hepatocytes as shown in Table 3 accords with the data, also obtained with a gel film method, reported for rat liver by Nolte and Pette (1972a) and Bengtsson et al. (1981) but not with other studies published previously. Wimmer and Pette (1979), for example, claimed that the mean LDH activity in rat periportal hepatocytes was 32% higher than that in pericentral hepatocytes; Morrison et al. (1965) claimed that it was 58% higher, Shank et al. (1959) 80% higher, and Katz (1989) 30-80% higher.

In general, K_m values determined histochemically in media containing PVA as a tissue protectant are up to 10 times higher than those determined biochemically in media without PVA (Butcher, 1970; Robertson et al., 1982; Sinowatz et al., 1983; Van Noorden and Butcher, 1986; Van Noorden and Jonges, 1987; Van Noorden, 1988; Jonges and Van Noorden, 1989). Van Noorden and Vogels (1989a) claimed that the higher K_m values *in situ* arise either from the cellular microenvironment of the enzymes or from reductions of the diffusion constants of the substrates and other reactants in the PVA media (Ogston et al., 1973). On the other hand, there are reports where K_m values determined histochemically and biochemically agree (Gutschmidt et al., 1979; Kugler, 1981; Stoward and Al-Sarraj, 1981; Nakae and Shono, 1984; Gordon and Robertson, 1986; Blano et al., 1988; Frederiks and Marx, 1988; Old and Johnson, 1989; Jonges et al., 1990, 1992; Nakae and Stoward, 1992, 1993b, 1994b). In most of these studies, the K_m values were determined *in situ* in the absence of PVA. There is thus a need to determine the kinetic parameters of the same enzymes *in situ* with different histochemical techniques and compare the results.

Conclusions

The coincidence of the orders of magnitude of K_m values determined histochemically with the gel film technique and biochemically in aqueous media confirms that the Michaelis-Menten kinetics (1913) established from studies in solution can be applied in the same way to enzyme reactions *in situ*. The diversity of the values of a_1, a_2, K_m and V_{max} in different cell types described in this review confirms the unique contribution of quantitative histochemistry for indicating that the metabolism of the same enzyme system in different cells *in situ* is heterogeneous, even in cells of the same type within the same organ (e.g. periportal and pericentral hepatocytes). Although several biochemical and histochemical studies have recognised that the kinetics of an enzyme *in situ* may differ from those in a dilute homogenate (e.g. Reid and Masters, 1985b; Swezey and Epel, 1986; Pagliaro and Taylor, 1988; Lawrence et al., 1990; Jonges et al., 1992), others have not appreciated that this may be responsible for the cellular heterogeneity or zonation that exists in tissues such as the liver (Katz, 1989; Jungermann and Katz, 1989; Gebhardt, 1992). The data and approach referred to this review are a step toward bridging this gap.

References

Ackermann W.W. and Potter V.R. (1949). Enzyme inhibition in relation to chemotherapy. Proc. Soc. Exp. Biol. Med. 72, 1-9.

Altman F.P. (1978). The use of a heated stage for following histochemical reactions under a microdensitometer. Histochem. J. 10, 611-614.

Altman F.P. (1980). Tissue stabilizer methods in histochemistry. In: Trends in enzyme histochemistry and cytochemistry. Ciba Foundation Symposium. Vol. 73 (new ser.). Evered D. and O'Connor M. (eds). Excerpta Medica. Amsterdam, Oxford. pp 81-93.

Altman F.P. and Chayen J. (1965). Retention of nitrogenous material in unfixed sections during incubation for histochemical demonstration of enzymes. Nature 1205-1206.

Andersen H. and Høyer P.E. (1974). Simplified control experiments in the histochemical study of coenzyme-linked dehydrogenases. Histochemistry 38, 71-83.

Asada-Kubota M., Kanai K. and Kanamura S. (1982). Development of ultrastructural heterogeneity among hepatocytes in the mouse. Anat. Rec. 202, 395-405.

Asoo K. (1969). Cytological and histochemical study on the human stomach cancer cells. J. Kumamoto Med. Soc. 43, 540-562 (in Japanese).

Baba N. and Sharma H.M. (1971). Histochemistry of lactic dehydrogenase in heart and pectoralis muscles of rat. J. Cell Biol. 51, 621-635.

Bachelard H.S. (1976). Biochemistry of coma. In: Biochemistry and neurological disease. Davison A.N. (ed). Blackwell. London. pp 228-277.

Battellino L.J. and Blanco A. (1970). Catalytic properties of the lactate dehydrogenase isozyme 'X' from mouse testis. J. Exp. Zool. 174, 173-186.

Battellino L.J., Jaime F.R. and Blanco A. (1968). Kinetic properties of rabbit testicular lactate dehydrogenase isozyme. J. Biol. Chem. 243, 5185-5192.

Bengtsson G., Kiessling K.-H., Smith-Kielland A. and Mørland J. (1981). Partial separation and biochemical characteristics of periportal and perivenous hepatocytes from rat liver. Eur. J. Biochem. 118, 591-597.

Bennett N.G. and Gutfreund H. (1973). The kinetics of the interconversion of intermediates of the reaction of pig muscle lactate dehydrogenase with oxidized nicotinamide-adenine dinucleotide and lactate. Biochem. J. 135, 81-85.

Bitensky L. (1980). Microdensitometry. In: Trends in enzyme histochemistry and cytochemistry. Ciba Foundation Symposium. Vol. 73 (new ser.) Evered D. and O'Connor M. (eds). Excerpta Medica. Amsterdam, Oxford. pp 181-199.

Blanco C.E., Sieck G.C. and Edgerton V.R. (1988). Quantitative histochemcial determination of succinic dehydrogenase activity in skeletal muscle fibres. Histochem. J. 20, 230-243.

Brandt R.B., Laux J.E., Spainhour S.E. and Kline E.S. (1987). Lactate dehydrogenase in rat mitochondria. Arch. Biochem. Biophys. 259, 412-422.

Butcher R.G. (1970). Studies on succinate oxidation. I. The use of intact tissue sections. Exp. Cell Res. 60, 54-60.

Butcher R.G. (1978). The measurement in tissue sections of the two

formazans derived from Nitroblue Tetrazolium in dehydrogenase reactions. Histochem. J. 10, 739-744.

Butcher R.G. (1983). Enzyme histochemistry of the myocardium. In: Cardiac metabolism. Drake-Holland A.J. and Noble M.I.M. (eds). John Wiley & Sons. Chichester, New York. pp 445-469.

Butcher R.G. and Van Noorden C.J.F. (1985). Reaction rate studies of glucose-6-phosphate dehydrogenase activity in sections of rat liver using four tetrazolium salts. Histochem. J. 17, 993-1008.

Butcher R.G., Dawson A.L., Knaab S.A. and Gahan P.B. (1980). Dehydrogenase activity and loss of formazan from tissue sections. Histochem. J. 12, 591-598.

Chalmers G.R. and Edgerton V.R. (1989a). Marked and variable inhibition by chemical fixation of cytochrome oxidase and succinate dehydrogenase in single motoneurons. J. Histochem. Cytochem. 37, 899-901.

Chalmers G.R. and Edgerton V.R. (1989b). Single motoneuron succinate dehydrogenase activity. J. Histochem. Cytochem. 37, 1107-1114.

Chieco P., Jonker A., Melchiorri C., Vanni G. and Van Noorden C.J.F. (1994). A user's guide for avoiding errors in absorbance image cytometry: a review with original experimental observations. Histochem. J. 26, 1-19.

Costello L.A. and Kaplan N.O. (1963). Evidence for two forms of M-type lactate dehydrogenase in the mouse. Biochim. Biophys. Acta 73, 658-660.

Dölken G., Leisner E. and Pette D. (1975). Immunofluorescent localization of glycogenolytic and glycolytic enzyme proteins and of malate dehydrogenase isozymes on cross-striated skeletal muscle and heart of the rabbit. Histochemistry 43, 113-121.

Donovan R.M. and Goldstein E. (1985). A charge coupled device-based image cytophotometry system for quantitative histochemistry and cytochemistry. J. Histochem. Cytochem. 33, 551-556.

Elduque A., Casadó F., Cortés A. and Bozal J. (1982). Intramitochondrial location of the molecular forms of chicken liver mitochondrial malate dehydrogenase. Int. J. Biochem. 14, 221-229.

Else P.L. and Hulbert A.J. (1981). Comparison of the "mammal machine" and the "reptile machine": energy production. Am. J. Physiol. 240, R3-R9.

Everse J. and Kaplan N.O. (1973). Lactate dehydrogenases: structure and function. In: Advances in enzymology. Vol. 37. Meister A. (ed). John Wiley & Sons. New York, London. pp 61-133.

Fahimi H.D. and Amarasingham C.R. (1964). Cytochemical localization of lactic dehydrogenase in white skeletal muscle. J. Cell Biol. 22, 29-48.

Fahimi H.D. and Karnovsky M.J. (1966). Cytochemical localization of two glycolytic dehydrogenases in white skeletal muscle. J. Cell Biol. 29, 113-128.

Frederiks W.M. and Marx F. (1988). A quantitative histochemical study of 5'-nucleotidase activity in rat liver using the lead salt method and polyvinyl alcohol. Histochem. J. 20, 207-214.

Frederiks W.M., Marx F. and Van Noorden C.J.F. (1988). Quantitative histochemistry of creatine kinase in rat myocardium and skeletal muscle. Histochem. J. 20, 624-628.

Frederiks W.M., Marx F. and Myagkaya G.L. (1989). The 'nothing dehydrogenase' reaction and the detection of ischaemic damage. Histochem. J. 21, 565-573.

Frederiks W.M., Marx F. and Van Noorden C.J.F. (1991). Homogeneous distribution of phosphofructokinase in the rat liver acinus: a quantitative histochemical study. Hepatology 14, 634-639.

Gebhardt R. (1992). Metabolic zonation of the liver: regulation and implications for liver function. Pharm. Ther. 53, 275-354.

Gordon M. and Robertson W.R. (1986). The application of continuous monitoring microdensitometry to an analysis of NAD^+ binding and 3ß-hydroxy-Δ^5-steroid dehydrogenase activity in the regressing corpus luteum of the pro-oestrous rat ovary. Histochem. J. 18, 41-44.

Gutschmidt S., Kaul W. and Riecken E.O. (1979). A quantitative histochemical technique for the characterization of α-glucosidases in the brush-border membrane of rat jejunum. Histochemistry 63, 81-101.

Hanes C.S. (1932). Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. Biochem. J. 26, 1406-1421.

Hawtrey C.O., Naidu A., Nelson C. and Wolfe D. (1975). The physiological significance of LDH-X. In: Isozymes II: physiological function. Markert C.L. (ed). Academic Press. New York, London. pp 171-180.

Helander H.F., Leth R. and Olbe L. (1986). Stereological investigations on human gastric mucosa. I. Normal oxyntic mucosa. Anat. Rec. 216, 373-380.

Herbener G.H. (1976). A morphometric study of age-dependent changes in mitochondiral populations of mouse liver and heart. J. Gerontol. 31, 8-12.

Hori Y., Takamori Y. and Nishio K. (1970). The effects of X-irradiation on lactate dehydrogenase isozymes in plasma and in various organs of mice. Radiat. Res. 43, 143-151.

Inoué S. (1986). Video microscopy. Plenum Press. New York, London.

Jonges G.N. and Van Noorden C.J.F. (1989). *In situ* kinetic parameters of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase in different areas of the rat liver acinus. Histochem. J. 21, 585-594.

Jonges G.N., Van Noorden C.J.F. and Gossrau R. (1990). Quantitative histochemical analysis of glucose-6-phosphatase activity in rat liver using an optimized cerium-diaminobenzidine method. J. Histochem. Cytochem. 38, 1413-1419.

Jonges G.N., Van Noorden C.J.F. and Lamers W.H. (1992). *In situ* kinetic parameters of glucose-6-phosphatase in the rat liver lobulus. J. Biol. Chem. 267, 4878-4881.

Joyce Loebl (1985). Image analysis: Principles and Practice. Short Run Press. Exeter.

Jungermann K. and Katz N. (1989). Functional specialization of different hepatocyte populations. Physiol. Rev. 69, 708-764.

Kainulainen H., Pilström L. and Vihko V. (1979). Morphometry of myocardial apex in endurance-trained mice of different ages. Acta Physiol. Scand. 107, 109-114.

Kalina M. and Gahan P.B. (1965). A quantitative study of the validity of histochemical demonstration for pyridine nucleotide-linked dehydrogenases. Histochemie 5, 430-436.

Karadsheh N.S. and Uyeda K. (1977). Changes in allosteric properties of phosphofructokinase bound to erythrocyte membranes. J. Biol. Chem. 252, 7418-7420.

Katz N.R. (1989). Methods for the study of liver cell heterogeneity. Histochem. J. 21, 517-529.

Kline E.S., Brandt R.B., Laux J.E., Spainhour S.E., Higgins E.S., Rogers K.S., Tinsley S.B. and Waters M.G. (1986). Localization of L-lactate dehydrogenase in mitochondria. Arch. Biochem. Biophys. 246, 673-680.

Knull H.R., Taylor W.F. and Wells W.W. (1973). Effects of energy

metabolism on *in vitro* distribution of hexokinase in brain. J. Biol. Chem. 248, 5414-5418.

Knull H.R., Taylor W.F. and Wells W.W. (1974). Insulin effects on brain energy metabolism and the related hexokinase distribution. J. Biol. Chem. 249, 6930-6935.

Kugler P. (1981). Kinetic and morphometric measurements of enzyme reactions in tissue sections with a new instrumental setup. Histochemistry 71, 433-449.

Kugler P. (1988). Quantitative succinate dehydrogenase histochemistry in the hippocampus of aged rats. Histochemistry 88, 299-307.

Latner A.L., Siddiqui S.A. and Skillen A.W. (1966). Pyruvate inhibition of lactate dehydrogenase activity in human tissue extracts. Science 154, 527-529.

Lawrence G.M., Beesley A.C.H. and Matthews J.B. (1989). The use of continuous monitoring and computer-assisted image analysis for the histochemical quantification of hexokinase activity. Histochem. J. 21, 557-564.

Lawrence G.M., Beesley A.C.H., Mason G.I., Thompson M., Walker D.G. and Matthews J.B. (1990). A comparison of histochemically and biochemically determined kinetic parameters for brain hexokinase type I. Inst. Phys. Conf. Ser. 98, 667-670.

Lluis C. (1984). Lactate dehydrogenase associated with the mitochondrial fraction and with a mitochondrial inhibitor - I. Enzyme binding to the mitochondrial fraction. Int. J. Biochem. 16, 997-1004.

Lluis C. (1985). Lactate dehydrogenase binding to the mitochondrial fraction and to a mitochondrial inhibitor as a function of the isoenzymatic composition. Int. J. Biochem. 17, 1219-1226.

Lomax R.B., Daglish A., Taylor R.J., Gordon M.T. and Robertson W.R. (1989). Rapid continuous monitoring of enzyme activity in tissue sections: experience with the M85A and Zeiss UMSP-30 systems. Histochem. J. 21, 595-599.

Markert C.L. and Ursprung H. (1962). The ontogeny of isozyme patterns of lactate dehydrogenase in the mouse. Dev. Biol. 5, 363-381.

Marković N.S., Marković O.T. and Young D.S. (1983). Simplified methods for measuring enzyme kinetics in a single cell. Clin. Chem. 29, 1578-1581.

Martin T.P., Vailas A.C., Durivage J.B., Edgerton V.R. and Castleman K.R. (1985). Quantitative histochemical determination of muscle enzymes: biochemical verification. J. Histochem. Cytochem. 33, 1053-1059.

Masters C.J. (1981). Interactions between soluble enzymes and subcellular structure. CRC Crit. Rev. Biochem. 11, 105-143.

Masters C.J. (1984). Interactions between glycolytic enzymes and components of the cytomatrix. J. Cell Biol. 99, 222s-225s.

Masters C.J. (1992). Microenvironmental factors and the binding of glycolytic enzymes to contractile filaments. Int. J. Biochem. 24, 405-410.

McMillan P.J. (1967). Differential demonstration of muscle and heart type lactic dehydrogenase of rat muscle and kidney. J. Histochem. Cytochem. 15, 21-31.

Meijer A.E.F.H. (1972). Semipermeable membranes for improving the histochemical demonstration of enzyme activities in tissue sections. I. Acid phosphatase. Histochemistry 30, 31-39.

Meijer A.E.F.H. (1980). Semipermeable membrane techniques in quantitative enzyme histochemistry. In: Trends in enzyme histochemistry and cytochemistry. Ciba Foundation Symposium. Vol. 73 (new ser.). Evered D. and O'Connor M. (eds). Excerpta Medica. Amsterdam, Oxford. pp 103-116.

Michaelis L. and Menten M.L. (1913). Die Kinetik der Invertinwirkung. Biochem. Z. 49, 333-369.

Morrison G.R., Brock F.E., Karl I.E. and Shank R.E. (1965). Quantitative analysis of regenerating and degenerating areas within the lobule of the carbon tetrachloride-injured liver. Arch. Biochem. Biophys. 111, 448-460.

Nakae Y. and Shono M. (1984). Kinetic behaviour of succinate dehydrogenase of three fibre types in skeletal muscle. I. Effects of temperature and a competitive inhibitor. Histochem. J. 16, 1207-1217.

Nakae Y. and Stoward P.J. (1988). Loss of succinate dehydrogenase from tissue sections during its histochemical assays. Proc. R. Microsc. Soc. 23, 45.

Nakae Y. and Stoward P.J. (1992). Initial reaction kinetics of succinate dehydrogenase in mouse liver studied with a real-time image analyser system. Histochemistry 98, 7-12.

Nakae Y. and Stoward P.J. (1993a). Estimating the initial reaction velocity of a soluble dehydrogenase *in situ*. Histochem. J. 25, 199-205.

Nakae Y. and Stoward P.J. (1993b). Kinetic analysis of lactate dehydrogenase *in situ* in mouse liver determined with a quantitative histochemical technique. Histochem. J. 25, 206-212.

Nakae Y. and Stoward P.J. (1993c). Kinetic analysis of enzymes *in situ* determined with a real-time image analysis system. Biophysics 33, 263-268 (in Japanese).

Nakae Y. and Stoward P.J. (1994a). The initial reaction velocities of lactate dehydrogenase in various cell types. Histochem. J. 26, 283-291.

Nakae Y. and Stoward P.J. (1994b). The diverse Michaelis constants and maximum velocities of lactate dehydrogenase *in situ* in various types of cell. Histochem. J. 26, 292-297.

Nisselbaum J.S. and Bodansky O. (1963). Purification, kinetic, and immunochemical studies of the major variants of lactic dehydrogenase from human liver, hepatoma, and erythrocytes; comparison with the major variant of human heart lactic dehydrogenase. J. Biol. Chem. 238, 969-974.

Nisselbaum J.S., Packer D.E. and Bodansky O. (1964). Comparison of the actions of human brain, liver, and heart lactic dehydrogenase variants on nucleotide analogues and on substrate analogues in the absence and in the presence of oxalate and oxamate. J. Biol. Chem. 239, 2830-2834.

Nitisewojo P. and Hultin H.O. (1976). A comparison of some kinetic properties of soluble and bound lactate dehydrogenase isoenzymes at different temperatures. Eur. J. Biochem. 67, 87-94.

Nolte J. and Pette D. (1972a). Microphotometric determination of enzyme activity in single cells in cryostat sections. I. Application of the gel film technique to microphotometry and studies on the intralobular distribution of succinate dehydrogenase and lactate dehydrogenase activities in rat liver. J. Histochem. Cytochem. 20, 567-576.

Nolte J. and Pette D. (1972b). Microphotometric determination of enzyme activity in single cells in cryostat sections. II. Succinate dehydrogenase, lactate dehydrogenase and triosephosphate dehydrogenase activities in red, intermediate and white fibers of soleus and rectus femoris muscles of rat. J. Histochem. Cytochem. 20, 577-582.

Ogston A.G., Preston B.N. and Wells J.D. (1973). On the transport of compact particles through solutions of chain-polymers. Proc. R. Soc. Lond. A. 333, 297-316.

Ohashi M. (1966). The control factors on the isozyme expression of transplantable tumors. Jpn. J. Clin. Med. 24, 365-370 (in Japanese).

Old S.L. and Johnson M.A. (1989). Methods of microphotometric assay of succinate dehydrogenase and cytochrome *c* oxidase activities for use on human skeletal muscle. Histochem. J. 21, 545-555.

Pagliaro L. and Taylor D.L. (1988). Aldolase exists in both the fluid and solid phases of cytoplasm. J. Cell Biol. 107, 981-991.

Parks H.F. (1961). On the fine structure of the parotid gland of mouse and rat. Am. J. Anat. 108, 303-329.

Patel H.R.H., Frederiks W.M., Marx F., Best A.J. and Van Noorden C.J.F. (1991). A quantitative histochemical study of D-amino acid oxidase activity in rat liver in relationship with feeding conditions. J. Histochem. Cytochem. 39, 81-86.

Pesce A., McKay R.H., Stolzenbach F., Cahn R.D. and Kaplan N.O. (1964). The comparative enzymology of lactic dehydrogenases. I. Properties of the crystalline beef and chicken enzymes. J. Biol. Chem. 239, 1753-1761.

Pesce A., Fondy T.P., Stolzenbach F., Castillo F. and Kaplan N.O. (1967). The comparative enzymology of lactic dehydrogenases. III. Properties of the H_4 and M_4 enzymes from a number of vertebrates. J. Biol. Chem. 242, 2151-2167.

Pette D. (1981). Microphotometric measurement of initial maximum reaction rates in quantitative enzyme histochemistry *in situ*. Histochem. J. 13, 319-327.

Pette D. and Brandau H. (1962). Intracellular localization of glycolytic enzymes in cross-striated muscles of *Locusta migratoria*. Biochem. Biophys. Res. Commun. 9, 367-370.

Pette D. and Wimmer M. (1979). Kinetic microphotometric activity determination in enzyme containing gels and model studies with tissue sections. Histochemistry 64, 11-22.

Pruñonosa J., Sagristá M.L. and Bozal J. (1989). Comparative binding of lactate dehydrogenase to mitochondrial fractions. Ital. J. Biochem. 38, 311-323.

Pupkin M., Bratt H., Weisz J., Lloyd C.W. and Balogh K. (1966). Dehydrogenases in the rat ovary. I. A histochemical study of Δ^5-3ß- and 20α-hydroxysteroid dehydrogenases and enzymes of carbohydrate oxidation during the estrous cycle. Endocrinology 79, 316-327.

Rao K. and Swami K.S. (1969). Kinetic characteristics of selected oxidases in sheep liver homogenate. Arch. Int. Physiol. Biochim. 77, 812-818.

Reid S. and Masters C. (1985a). On the ontogeny of aldolase isozymes and their interactions with cellular structure. Mech. Ageing Dev. 30, 299-317.

Reid S. and Masters C. (1985b). Ontogenic variations in the interactions of lactate dehydrogenase isozymes with cellular structure. Mech. Ageing Dev. 31, 69-87.

Robertson W.R., Earnshaw R.J. and Lambert A. (1982). The determination by microdensitometry of the initial maximum velocity rate of rat ovarian 3ß hydroxysteroid dehydrogenase activity. Histochemistry 74, 435-442.

Robertson W.R., Davison B., Gandy M., Burdge R. and Lambert A. (1984). Computerized continuous monitoring of cytochemical enzyme reaction product formation by the Vickers M85A microdensitometer. Histochem. J. 16, 651-656.

Ruhnke M. and Gossrau R. (1989), Reaction rate measurements of proteases and glycosidases with chromogenic methods. Histochem. J. 21, 535-544.

Russ J.C. (1990). Computer-assisted microscopy: the measurement and analysis of images. Plenum Press. New York, London.

Sagristá M.L. and Bozal J. (1987). Lactate dehydrogenase activity in the mitochondrial fraction of chicken liver: enzyme binding and kinetic behaviour of soluble and bound enzyme. Biochimie 69, 205-214.

Sagristá M.L., Pruñonosa J. and Lluis C. (1989). Modulation of lactate dehydrogenase activity by enzyme-protein interaction. J. Enzym. Inhib. 3, 57-66.

Sanz M.C. and Lluis C. (1988). Ambiquitous behavior of rabbit liver lactate dehydrogenase. Experientia 44, 203-208.

Sanz M.C., Sagristá M.L. and Lluis C. (1990). Kinetic behaviour of soluble and mitochondrial bound lactate dehydrogenase. Ital. J. Biochem. 39, 21-29.

Shank R.E., Morrison G., Cheng C.H., Karl I. and Schwartz R. (1959). Cell heterogeneity within the hepatic lobule (quantitative histochemistry). J. Histochem. Cytochem. 7, 237-239.

Shaw C.R. (1969). Isozymes: classification, frequency, and significance. Int. Rev. Cytol. 25, 297-332.

Shaw M.-A., Edwards Y. and Hopkinson D.A. (1981). Human succinate dehydrogenase: biochemical and genetic characterization. Biochem. Genet. 19, 741-756.

Sieck G.C. and Sacks R.D. (1986). SDH activity and cross-sectional area of muscle fibers in cat diaphragm. J. Appl. Physiol. 60, 1284-1292.

Sieck G.C., Sacks R.D. and Blanco C.E. (1987). Absence of regional differences in the size and oxidative capacity of diaphragm muscle fibers. J. Appl. Physiol. 63, 1076-1082.

Sinowatz F., Scheubeck M., Wrobel K.-H. and Zwack M. (1983). Histochemical localization and quantification of glucose-6-phosphate dehydrogenase in bovine Leydig cells. Histochem. J. 15, 831-844.

Stambaugh R. and Post D. (1966). Effects of tissue extracts and temperature on lactate dehydrogenase isozymes. Biochim. Biophys. Acta 122, 541-543.

Stoward P.J. (1980). Criteria for the validation of quantitative histochemical enzyme techniques. In: Trends in enzyme histochemistry and cytochemistry. Ciba Foundation Symposium. Vol. 73 (new ser.). Evered D. and O'Connor M. (eds). Excerpta Medica. Amsterdam, Oxford. pp 11-27.

Stoward P.J. and Al-Sarraj B. (1981). Quantitative histochemical investigations of semipermeable membrane techniques for the assay of acid phosphatase in skeletal muscle. I. Meijer's method. Histochemistry 71, 405-431.

Stoward P.J. and Nakae Y. (1988). Simultaneous histochemical assay of two dehydrogenases in the small cell. Histochem. J. 20, 610-616.

Stoward P.J., Meijer A.E.F.H., Seidler E. and Wohlrab F. (1991). Dehydrogenases. In: Histochemistry. Theoretical and applied. Vol. 3. 4th ed. Stoward P.J. and Pearse A.G.E. (eds). Churchill Livingstone. Edinburgh. pp 27-71.

Südi J. (1974). Macroscopic rate constants involved in the formation and interconversion of the two central enzyme - substrate complexes of the lactate dehydrogenase turnover. Biochem. J. 139, 261-271.

Susheela L., Venkatesan K. and Ramasarma T. (1977). Structural and kinetic studies on the activators of succinate dehydrogenase. Biochim. Biophys. Acta 480, 47-55.

Swezey R.R. and Epel D. (1986). Regulation of glucose-6-phosphate dehydrogenase activity in sea urchin eggs by reversible association with cell structure elements. J. Cell Biol. 103, 1509-1515.

Szcesna-Kaczmarek A. (1990). L-lactate oxidation by skeletal muscle mitochondria. Int. J. Biochem. 22, 617-620.

Van Blerkom J. and Bell H. (1986). Regulation of depelopment in the fully grown mouse oocyte: chromosome-mediated temporal and spatial differentiation of the cytoplasm and plasma membrane. J.

Embryol. Exp. Morphol. 93, 213-238.

Van Noorden C.J.F. (1988). Enzyme reaction rate studies in tissue sections. Proc. R. Microsc. Soc. 23, 93-97.

Van Noorden C.J.F. and Butcher R.G. (1986). A quantitative histochemical study of NADPH-ferrihemoprotein reductase activity. Histochem. J. 18, 364-370.

Van Noorden C.J.F. and Butcher R.G. (1987). Linearity in dehydrogenase reaction rate studies in tissue sections is affected by loss of endogenous substrates during the reaction. J. Histochem. Cytochem. 35, 1401-1404.

Van Noorden C.J.F. and Butcher R.G. (1991). Quantitative enzyme histochemistry. In: Histochemistry. Theoretical and applied. Vol. 3. 4th ed. Stoward P.J. and Pearse A.G.E. (eds). Churchill Livingstone. Edinburgh. pp 355-432.

Van Noorden C.J.F. and Jonges G.N. (1987). Quantification of the histochemical reaction for alkaline phosphatase activity using the indoxyl-tetranitro BT method. Histochem. J. 19, 94-102.

Van Noorden C.J.F. and Vogels I.M.C. (1989a). Polyvinyl alcohol and other tissue protectans in enzyme histochemistry: a consumer's guide. Histochem. J. 21, 373-379.

Van Noorden C.J.F. and Vogels I.M.C. (1989b). Cytophotometric analysis of reaction rates of succinate and lactate dehydrogenase activity in rat liver, heart muscle and tracheal epithelium. Histochem. J. 21, 575-583.

Van Noorden C.J.F., Kooij A., Vogels I.M.C. and Frederiks W.M. (1985). On the nature of the 'nothing dehydrogenase' reaction. Histochem. J. 17, 1111-1118.

Van Wijhe M., Blanchaer M.C. and George-Stubbs S.St. (1964). The distribution of lactate dehydrogenase isozymes in human skeletal muscle fibers. J. Histochem. Cytochem. 12, 608-614.

Velez M., Machado A. and Satrústegui J. (1985). Age-dependent modifications of rat heart succinate dehydrogenase. Mech. Ageing Dev. 32, 131-140.

Wachsmuth E.D. (1980). Assessment of immunocytochemical techniques with particular reference to the mixed-aggregation immunocytochemical technique. In: Trends in enzyme histochemistry and cytochemistry. Ciba Foundation Symposium. Vol. 73. (new ser.). Evered D. and O'Connor M. (eds). Excerpta Medica. Amsterdam, Oxford. pp 135-153.

Walsh T.P., Clarke F.M. and Masters C.J. (1977). Modification of the kinetic parameters of aldolase on binding to the actin-containing filaments of skeletal muscle. Biochem. J. 165, 165-167.

Wassarman P.M. and Josefowicz W.J. (1978). Oocyte development in the mouse: an ultrastructural comparison of oocytes isolated at various stages of growth and meiotic competence. J. Morphol. 156, 209-236.

Wei Y.-H., Ding W.-H. and Wei R.-D. (1984). Biochemical effects of PR toxin on rat liver mitochondrial respiration and oxidative phosphorylation. Arch. Biochem. Biophys. 230, 400-411.

Weiss von G. and Deichsel G. (1978). Methodenkritische Studien zur Enzymkinetik in der Histochemie am Beispiel der Glucose-6-Phosphat-Dehydrogenase und der Succinat-Dehydrogenase. Untersuchungen mit der Membrantechnik an der Rattenniere. Acta Histochem. Suppl. 20, 261-265.

Wilson J.E. (1968). Brain hexokinase. A proposed relation between soluble-particulate distribution and activity *in vivo*. J. Biol. Chem. 243, 3640-3647.

Wilson J.E. (1978). Ambiquitous enzymes: variation in intracellular distribution as a regulatory mechanism. Trends Biochem. Sci. 3, 124-125.

Wimmer M. and Pette D. (1979). Microphotometric studies on intraacinar enzyme distribution in rat liver. Histochemistry 64, 23-33.

Wróblewski F. and Gregory K.F. (1961). Lactic dehydrogenase isozymes and their distribution in normal tissues and plasma and in disease states. Ann. N.Y. Acad. Sci. 94, 912-932.

Yasykova M., Yu., Gusev N.B., Muronetz V.I. and Nagradova N.K. (1990). Interaction of lactate dehydrogenase with skeletal muscle troponin. Biochim. Biophys. Acta 1036, 85-87.

Zamboni L. (1970). Ultrastructure of mammalian oocytes and ova. Biol. Reprod. Suppl. 2, 44-63.