# Trends in Enzyme Histochemistry and Cytochemistry

Ciba Foundation Symposium 73 (new series)


1980

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# Trends in Enzyme Histochemistry and Cytochemistry

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# Preface

T.F. SLATER

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This symposium, planned by the Ciba Foundation at the suggestion of Dr Peter Stoward, has ensured that a number of specialists took a hard look at the current state of the art of enzyme histochemistry and cytochemistry. Of course, a rather severe process of selection of topics for discussion was necessary, as a limited time was available; nonetheless, several major aspects have been covered in depth.

The discipline of histochemistry and cytochemistry is concerned with the chemical composition and behaviour of biological materials in relation to their original structure, be it at the tissue (i.e. histological), cellular (i.e. cytological), or cell organelle (i.e. submicroscopic morphological) level. To some extent controlled fragmentation of tissues and cells, followed by separation of the component cells or organelles by centrifugation, can be used for such studies. The separated fractions can be analysed by conventional biochemical techniques since these separation procedures generally provide enough material for such analysis. With modern developments in centrifuging techniques (e.g. zonal centrifuging with small rotors) it is possible to obtain very good separations of the component structures in tissue biopsy samples weighing only a few milligrams. Moreover, biochemical studies of isolated cell populations (e.g. isolated hepatocytes) are now producing much valuable biochemical data with respect to cell to cell variations in enzymic and other biological activities. The chemical data obtained in these ways, however, although revealing much about the molecular composition of certain types of cells and organelles, have not contributed much to our knowledge of the variations in biological activity and chemical composition between individual cells (or cell parts) within organized


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tissue structures. The problem of identifying and quantifying biological and chemical differences between cells in organized tissues remains a problem for microscopic techniques. One approach that has not been extensively used is microdissection of small pieces of tissue or individual cells from their surroundings, followed by microchemical estimation of the isolated samples. Such techniques (e.g. as practised particularly by the work of Glick, Lowry and Morrison) give quantitative information about the distribution of enzyme activity or biochemical substrates within different regions of a tissue that is heterogeneous in cellular type and in the distribution of biological activities. A second approach, which was the subject of this symposium, is microscopic histochemistry and cytochemistry.

In the latter approach, microscopic methods are used to provide data on individual cells or cell components preserved in an organized tissue framework or matrix. Histochemistry and cytochemistry are related closely in this way to histology, cytology and pathology, and, in fact, form a disciplinary bridge between these subjects and biochemistry.

The major experimental objective in histochemistry and cytochemistry is to develop 'staining' (or other visualization) methods for tissues and cells such that observation and measurement of the visualized object under the microscope (using optical absorption, fluorescence or electron-scatter techniques, for example) reveal an aspect of its molecular composition in relation to associated structures. This approach dates back in principle to Raspail's 'Essai de Chimie Microscopique Appliqué à la Physiologie' which appeared in 1830. Although over the subsequent 100 years some (very limited) success was achieved in combining microscopic and chemical approaches to cellular studies, the lack of relevant theory and of appropriate techniques inhibited most approaches. In consequence, morphology and biochemistry developed along largely independent lines.

Around 1930 Raspail's approach was taken up more successfully not only by qualitative studies of the microscopic images of stained cells, but also by quantitative studies of cellular components *in situ*. In particular, the groups of Caspersson and Pollister began to use the microscope as a quantitative measuring instrument. Since their early work there has been a progressive refinement in technology, with the recent introduction of microprocessing being a major event. Automated and computer-directed microscopes and flow instruments are now operating that can apply pattern recognition techniques, morphometry and cytophotometry to naturally present (or chemically produced) variables in cells and tissues.

Such rapid technological developments over the last few years emphasize the need to define precisely the chemical mechanisms of the histochemical and

cytochemical methods being used, and to ensure that the staining or visualization methods should localize the component in question at its original site within the cell or tissue. It is also desirable that the staining intensity (or other visualization procedure) should reflect in a reproducible manner the amount or activity of the components under study. It is probable, however, that compromise will have to be made on these aims *in toto* since, in these objectives, the histochemist/cytochemist is virtually aiming at the impossible: to study chemical components accurately within a complex intracellular matrix without significantly altering the structural relationships and interrelated biological activities. This makes such quantitative studies liable to criticism from the points of view of specificity, topology or quantitative reliability, or all three. In fact, histochemistry has for many years been a largely qualitative discipline. This is in marked contrast to biochemistry, where the emphasis has been mostly on measurement. There were at least two reasons for this. Firstly, the chemistry behind the often complex chromogenic histochemical reactions was poorly understood, and secondly, equipment for measuring histochemical end-products inside tissue sections was not readily available. In recent years, much of this has changed. More is known about the intricacies of histochemical reactions, and suitable measuring equipment can now be obtained commercially. Thus, one can now place a stained section in such equipment and obtain precise values for the intensity of staining in any desired region of the section. However, it is important not to confuse the concepts of precision and accuracy. Precise results obtained from a sample lie closely together, with only a small spread of values. They can, however, be far from the 'true result' in terms of biological activity and relevance. Although modern instrumentation can give us precise results, are they accurate? They can only be so if the histochemical methods used are valid.

It was this concern over validity that in part led to the present symposium.

Among the early objectives discussed for the symposium were, first, to attempt to obtain agreement on the minimum number of criteria that quantitative histochemical and cytochemical techniques should meet when applied to the localization and quantitative measurement of enzymes and other components in tissues and cells; and, secondly to assess how far existing techniques meet such 'agreed criteria'.

It became clear during the symposium, however, that some participants felt that histochemical systems are so complex and, at present, so little understood in chemical terms that it would be premature to draw up hard and fast recommendations. Another view expressed was that even if firm criteria of rather general applicability could be stated, they would be so complicated and hedged with qualification and traps for the unwary, that they would be no advantage for most workers entering the field.

In the event, the participants at the symposium spent little time talking of 'agreed criteria' and devoted most time to discussing the authoritative presentations of the new and varied methodologies currently used in quantitative histochemistry and cytochemistry. In addition, some considerable time was devoted to the relationships of quantitative cytochemical techniques and concepts to other relevant disciplines.

Of course, in an evaluation of the appropriate methodology for tackling a biological problem involving the association of structure and activity, histochemistry is only one type of approach and no more. It is not exclusive of other approaches and may not even be appropriate in the particular circumstances under study. In many cases, histochemical and cytochemical techniques may be better used in conjunction with other approaches, such as traditional biochemical techniques, to obtain a clearer understanding of the functions of cells and tissues, and the mechanisms operating in disease states.

During the symposium there were repeated references to the chemical complexity of many of the staining reactions currently used, and to the importance of increasing our understanding of the underlying mechanisms of such reactions. I have no doubt that this is one of the important and more immediate goals for work in this discipline, and one which should be coupled to a more general use and availability of chemically purified reagents. At present, there are obvious deficiencies in studies of certain reactions: modern technology has allowed very precise measurements to be made but the accuracy (or biological significance) of such measurements is clouded by lack of understanding of the basic reactions and, very often, by the use of uncharacterized initial reactants.

At present histochemical and cytochemical techniques fall into two main classes, qualitative and quantitative. The qualitative staining methods are useful where the stained product can be related empirically to some cellular or physiological function. A much-used example has been the reaction for succinate dehydrogenase as a morphological marker for mitochondria. Where such a reaction is used as a qualitative pointer to mitochondrial location it has, in general, some considerable value, and for such observations it is not necessary to understand the detailed chemical kinetics of the reaction. However, when such a reaction is used to reflect mitochondrial respiratory activity the reflection obtained may be grossly distorted. As pointed out clearly in the symposium, for such quantitative data on enzyme sequences to be obtained, the enzyme to be measured must be carefully chosen to reflect metabolic flux through the sequence. Given such a choice of enzyme it is further necessary, for quantitative studies, to validate the method so that changes in enzyme activity arising from alterations in the physiology of the cell or tissue can be reliably distinguished from artifactual changes originating from technical variables.

Many papers presented at the symposium emphasized the great advances in data acquisition systems and in data processing that have occurred recently. It seems clear that quantitative histochemistry and cytochemistry are undergoing a revolutionary development and expansion through these technological innovations. What is needed, in addition, is some similar expansion in the variety of enzymes and other cell components that can be accurately measured by staining procedures. Such developments will clearly serve to strengthen the contributions that quantitative histochemistry and cytochemistry will make to our overall appreciation of cellular and tissue biology; they will also increase the use of the relevant techniques by a much larger group of investigators than at present.

The symposium allowed a group of like-minded persons to get together and discuss the current use and quantitation of histochemical and cytochemical procedures, and to critically examine present standards in the areas of study. No unequivocally clear conclusions were reached that would allow firm recommendations to be made concerning standards to be adopted in future studies. On the whole, however, the symposium gave timely opportunity for valuable interactions to occur between workers with different approaches to quantitative histochemistry and cytochemistry.

In my view, the use of quantitative histochemical and cytochemical methods in pathology will be one of the key developments during the next 10 years and will encourage important and related developments in the related disciplines of cytology and biochemistry.

The future of quantitative histochemistry and cytochemistry looks bright when viewed against the increasing awareness of the importance of precisely relating activity to structure in heterogeneous tissues and cells, and against the rapid advances in microcircuit technology that are making cheaper and more sophisticated instrumentation available.

In writing this preface to the symposium I have been much helped by notes provided by Professor van Duijn, Dr Stoward and Dr Altman. In addition, a post-symposium meeting at Brunel University between these colleagues and Professor Holt was of great assistance to me; I am most grateful to them for their advice and help.

# Introduction

T.F. SLATER

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This symposium was designed to examine in depth the major concepts, principles and practices of quantitative histochemistry. During the presentations or the discussions some of you are sure to say, no doubt with quiet but deep conviction, that you don't think much of the procedures, practices or techniques of Dr X or Professor Y. In such cases, I shall interrupt to ask you to justify your criticism. It is important, in my view, that we do this in order to understand the basis for criticism so that we can then decide whether it is well founded in fact or is based on hearsay or even on private prejudice.

Another aspect of my chairmanship, and one for which I offer no excuse, is that I am largely ignorant of the finer details of the experimental procedures of quantitative histochemistry. My own areas of interest are free radical biochemistry and cancer, so I hope to be educated here in a vigorously developing and important area of scientific enquiry. For a specialized meeting to have such an uninformed chairman has some clear advantages and some disadvantages: the advantages are that I have no histochemical axe—or knife—to grind and I shall be looking at the problems that are raised from a different direction, scientifically, to most of you. The disadvantage is that I will probably be asking very naive questions—but this may be a blessing in disguise if it helps the non-specialist to understand the main points at issue. On this particular point may I ask you to avoid the use of jargon or technical phrases peculiar to you or your group: even if you don't confuse our foreign visitors you will certainly confuse the chairman.

Having stressed that my own scientific expertise is not in the particular area of this symposium, let me hasten to add that I have long recognized the


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tremendous contribution that histochemistry and cytochemistry can make to biochemistry in general and to my own area of study—tissue injury—in particular. The application of histochemical techniques directs much-needed biochemical attention to the variability of cellular activities in organized tissues, and to the dangers inherent in working with homogenized suspensions obtained from complex cellular varieties. Even nowadays, in my opinion, some biochemists are not sufficiently aware of the complex cellular structure of tissues such as lung or liver, or of the spectrum of biological activities of which cells of a similar type are capable, depending on their precise tissue location. Three examples based on my own experience will indicate my interest in this area.

When the amino acid analogue ethionine is given to a rat in an acute dose it produces a fatty liver; chronic dosing produces profound biochemical changes in the liver tissues of the treated animals. Ethionine is also a rather weak carcinogen and I was interested (Slater & Sawyer 1966, Slater & Delaney 1971) in studying the profound changes that occur in liver ATP, liver protein synthesis, liver nucleotide levels, bile flow and so on. However, how can such changes that occur after chronic dosing be interpreted? By that time, the whole architecture and cellular make-up of the liver has changed. Ethionine produces hyperplasia of the bile duct and the proportions of the cells that make up the complex liver tissue change markedly (Rubin *et al.* 1961) so that one ends up looking at a tissue with a completely different cellular structure. This is an example of biochemical changes being associated with a change in tissue organization, and in such cases analysis of homogenates or tissue fractions is difficult if not impossible to interpret.

A similar example, which I discussed here at the symposium on lysosomes in 1963, is the increase in the activity of lysosomal enzymes in the rat mammary gland during early mammary involution. Before 1960 or so, about 16 enzymes had been studied in the various stages of the lactation cycle in the rat. Most of them reached a peak during lactation that coincided with the peak milk production and then fell rapidly during mammary involution. In about 1960 to 1962 a few components—lysosomal enzymes—were found that actually increased in activity in involution, as discussed at the 1963 symposium (Slater *et al.* 1963). It turned out in retrospect that this increase is due to a migration into the mammary gland of a completely different cell type so that in early mammary involution we were analysing a different set of cells to those we were analysing in late lactation. This is an example of biochemical changes being associated with changes in cell type, and these data depended greatly on the application of histochemical procedures for their elucidation.

As a final example of this point, I can mention some recent studies that we have done with the hepatotoxic agent carbon tetrachloride, which requires

metabolism in the liver by cytochrome *P*-450 to a free radical product that is chemically very active. This free radical product (probably the trichloromethyl radical: Slater 1979) destroys some of the parenchymal cell cytochrome *P*-450. However, this destruction is not uniformly spread throughout the lobule but is concentrated very much in the cells of the centrilobular areas. The *P*-450 in these particular groups of cells is almost completely destroyed whereas the *P*-450 in the periportal region is untouched (J. Chayen, J. Johnston, K. Cheeseman and T.F. Slater, unpublished). In this particular case, which was studied using microdensitometry, we found that a biochemical change, which had been studied earlier by gross analysis of microsomal fractions, was actually located within a particular group of cells of the same type in the same organ.

I have described these three examples to explain why, although I am not a specialist in the area of histochemistry and cytochemistry, I have long been sympathetic to it and have valued very much its contributions to biochemistry in general.

The critical use of histochemical and cytochemical procedures raises many questions relating to precision, reproducibility, specificity and validity. Quantitative histochemistry is an area of some considerable controversy, and this symposium is an important opportunity to raise, discuss and evaluate many issues of current interest.

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# Criteria for the validation of quantitative histochemical enzyme techniques

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*Abstract* Some practical criteria are suggested for establishing the precision, reproducibility, validity and specificity of quantitative histochemical techniques used for assaying the activities of enzymes in single cells and tissue sections. To be valid, a technique should ideally pass 12 tests. Principally these involve proving that the mean absorbance or fluorescence of the specific final reaction product (FRP) is related to section thickness, incubation time, substrate concentration and the concentration of enzyme *in situ*. However, the formation of appreciable amounts of non-specific FRP may interfere in the determination of the true enzyme activity. This and other difficulties are illustrated with data obtained from an investigation of Meijer's semipermeable membrane technique for assaying acid phosphatase in unfixed sections of muscle.

It is becoming generally agreed that objective quantitative cytochemical measurements are preferable to subjective qualitative observations for comparing the activities of enzymes *in situ* in different biological systems, particularly in diseased and perturbed tissues. However, there is a growing realization that many of the methods used to obtain quantitative measurements in recent years have not been properly validated, and consequently the data and conclusions arising from their employment in biological investigations may be of little value. One cannot always be sure whether differences in such measurements can be attributed to true changes in enzyme activity or are caused by technical imperfections in the methods adopted. The need has thus arisen for a set of agreed criteria against which applied investigators can judge the precision, reproducibility, validity and specificity of the techniques they wish to use.

Some criteria are suggested in this paper. They are illustrated by reference to one technique, the semipermeable membrane technique, in order to indicate


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some of the difficulties and limitations that may be encountered in their application.

## GENERAL CRITERIA AND DEFINITIONS

Ideally all cytochemical techniques for localizing and assaying enzymes in either sections of tissue or preparations of single cells should be *precise, reproducible, specific* and *valid.*

A *precise* technique may be defined as one in which the specific final reaction product (FRP) arising from its use is deposited only in or on the true *in vivo* subcellular site of the enzyme, and nowhere else.

A technique may be considered *reproducible* if it gives the same results whoever performs it and irrespective of how many times it is carried out, provided that exactly the same procedure is followed each time and that the biological samples are equivalent.

The term *specific* describes a technique which either reveals only the enzyme in question or, if several FRPs are formed, gives rise to a component of the FRP that can be clearly identified and attributed to the activity of the enzyme.

A *valid* technique can be roughly defined as one in which the amount of FRP deposited per unit volume (or other reference parameter) in a particular region of a cell (i.e. the intensity of staining) is related to either the concentration or the specific activity of the enzyme in that region.

Other definitions are possible for each of these terms, but in practice they are limited and rarely absolute.

A technique can be assumed to be precise, reproducible, specific and valid if it meets the following practical criteria.

### *Precision*

(i) Sections retain their morphology and look 'clean'.

(ii) Specific FRP is confined to certain subcellular sites, particularly those predicted from 'biochemical' experiments to contain the enzyme.

(iii) There is positive proof that specific FRP does not diffuse and bind to other subcellular sites.

Fahimi elaborates on these criteria in the next paper. They will not be discussed further here.

### *Reproducibility*

(i) The mean values of measurable parameters (e.g. the mean absorbance or fluorescence emission of FRP) do not vary significantly in repeated experiments.

(ii) The individual measurements of these parameters within a preparation (e.g. the absorbances of the specific FRP at its spectral absorption maximum) statistically form a unimodal population.

One way of testing these two criteria is as follows. Suppose, for a given set of incubation conditions, that the specific FRP formed in $n$ different cells of the same histological type is found to have the following absorbances ($A$) at its spectral absorption maximum ($\lambda_{max}$):

$$A_1, A_2, A_3 \ldots A_n$$

Then the technique is reproducible if it passes the following tests.

(a) The cumulative mean absorbance ($\overline{A}$) and the cumulative coefficient of variation ($\overline{CV}$) of absorbance become constant (to, say, within $\pm 3\%$) when $n$ is above a certain minimum value ($n_{min}$). Although $\overline{A}$ and $\overline{CV}$ may differ in different histological cell types and when experiments are repeated, the value of $n_{min}$ required to give these constant parameters should remain the same.

(b) Statistical tests confirm that $A_1, A_2 \ldots A_n$ do not depart significantly from a normal single distribution.

(c) The same mean absorbances, reaction rates, etc., are obtained, within statistically acceptable limits, for repeated experiments (usually four or five) with the same set of incubation conditions:

$\alpha$) on the same sample,

$\beta$) on other samples (of equivalent physiological status) by the same investigator, and

$\gamma$) in different laboratories.

*Validity*

The following criteria have been suggested or employed at one time or another for establishing the validity of histochemical techniques.

(i) No enzyme is lost from its subcellular site during the procedures required for its visualization, or, if some enzyme is inevitably lost, the loss is small (say $<30\%$), constant and known.

(ii) The specific FRP arising from an enzymic reaction:

(a) has its expected chemical composition, and

(b) can be identified with reasonable certainty in cells and tissues (by, for example, having a characteristic $\lambda_{max}$ similar to that of pure specific FRP in solution).

(iii) There is a stoichiometric relationship between the amount of specific FRP deposited in a cell or section and the amount of primary reaction product (PRP) formed by the enzyme.

$$[\text{Specific FRP}] \propto [\text{PRP}]$$

(iv) The mean absorbance ($\overline{A}$) or fluorescence emission of the specific FRP is proportional to its mean concentration in the cell or section.

$$\overline{A} \propto [\text{specific FRP}]$$

(v) For sections incubated for a constant time ($t$) in media containing an excess of substrate, S, the mean absorbance ($\overline{A}$) of the specific FRP in cells of the same histological type is proportional to the thickness ($l$) of the section up to a certain 'critical' level.

$$\overline{A} \propto l \quad (t, [\text{S}] \text{ constant})$$

It is probably unrealistic to expect a similar proportionality to hold for fluorescence reactions because, among other factors, thicker preparations will reabsorb some fluorescence.

(vi) The rate of increase of mean absorbance or fluorescence emission per unit incubation time ($\overline{A}/t$), i.e. reaction rate, in a particular site is directly proportional to the specific activity or concentration of the enzyme (E) in that site.

$$(\overline{A}/t) \propto [\text{E}] \quad (l, [\text{S}] \text{ constant})$$

(vii) Once specific FRP has begun to form in a cell, its mean absorbance or fluorescence emission increases uniformly with incubation time ($t$), preferably linearly.

$$\overline{A} \propto t \quad (l, [\text{S}] \text{ constant})$$

(viii) On extrapolated absorbance– or fluorescence–incubation time plots, the mean absorbance or fluorescence emission of the specific FRP corresponding to zero incubation time should ideally be zero, or at worst, small but constant for a particular set of reagents and reaction conditions, i.e.

$$\text{When } t = 0, \ \overline{A} = 0 \text{ or constant } (\overline{A}_0)$$

In addition, certain enzymes, particularly those thought to be contained within membrane-bound organelles, may show a lag before FRP begins to be formed.

(ix) The rate of formation of specific FRP ($\overline{A}/t$) in whole cells or sections of

constant thickness is a function of the concentration of substrate, f(S), in the incubation medium.

$$(\overline{A}/t) = \mathrm{f(S)}$$

This means that at low substrate concentrations the amount of FRP formed per unit incubation time increases as the concentration increases, but when the substrate concentration is above a certain level, the reaction rate reaches a constant maximum.

(x) Reciprocal or other suitable plots relating substrate concentrations and the observed reaction rates of an enzyme *in situ* should yield Michaelis constants ($K_m$) comparable to, or possibly higher than, the constants obtained for the same enzyme and substrates in solution.

(xi) The changes in the rates at which specific FRP are formed *in situ* in the presence of low concentrations of enzyme modifiers (especially inhibitors) should be of an order comparable to those exhibited by the enzyme *in vitro*.

(xii) In control sections and preparations of either fresh samples incubated with media lacking an essential ingredient (such as substrate) or boiled samples incubated with complete media, the mean absorbance ($\overline{A}$) or fluorescence of any non-specific FRP is small and, preferably, constant, say not more than about 5–10% of the mean absorbance or fluorescence of the specific FRP.

*Specificity*

Traditionally a technique is judged to be specific if it satisfies the following tests.

(i) No specific FRP is formed in control sections or preparations.

(ii) The reaction conditions (pH, $Po_2$, etc.) that give rise to the maximum rate of formation of specific FRP *in situ* are the same as, or very similar to, those favouring the optimum formation of reaction product in *in vitro* systems.

(iii) Inhibitors and other enzyme modifiers exert their expected effects on the formation of specific FRP in ways suggested by 'biochemical' precedents.

(iv) Potentially interfering enzyme systems have either been suppressed or shown to be absent, or can be distinguished from the enzyme under study.

## APPLICATION OF THE CRITERIA

Inevitably difficulties arise when the criteria enumerated so far are applied to a particular technique. Some criteria are also not quite as sound as they look. I shall illustrate these difficulties with some unpublished data recently obtained

by my colleague, Mrs Basima Ibrahim, while she was studying the validity of Meijer's (1972) semipermeable membrane technique for assaying the activity of acid phosphatase (EC 3.1.3.2) in sections of skeletal muscle (Ibrahim 1978).

This technique reveals three different histological sites of acid phosphatase activity in cryostat unfixed sections of normal skeletal muscle when naphthol AS-BI phosphate is used as the substrate and hexazotized pararosaniline (HPRA) as the simultaneous coupling reagent. The intensely coloured 'granules' scattered inside the muscle fibres constitute the first site (Fig. 1). The diffusely and

FIG. 1. Skeletal muscle (hamster dystrophic gastrocnemius) incubated for acid phosphatase using Meijer's semipermeable membrane technique. ×400. Arrows: intensely stained 'granule' areas.

uniformly stained sarcoplasmic or 'intrafibre' areas form the second site, and certain connective tissue elements between the fibres, which we prefer to call 'interfibre' areas, form the third.

I shall skip the problem of proving at the light microscope level, as distinct from the ultrastructural level, whether the technique is precise in the sense defined earlier, except to comment that the FRP formed within the fibres seems to reflect enzyme localized almost exclusively in the sarcoplasmic reticulum, rather than, as in most other tissues, in lysosomes (Christie & Stoward 1977, Trout *et al.* 1979). My discussion will be confined to considering whether the technique meets the criteria suggested for reproducibility, validity and specificity.

*Reproducibility*

The technique seems highly reproducible since it satisfies the first and third tests set out earlier, but it is inconclusive for the second. For example, 12 measurements of absorbance are sufficient to yield cumulative mean absorbances and coefficients of variation that never vary by more than $\pm 2\%$ in any of the three principal reactive sites, irrespective of how many further measurements are carried out.

In addition, an analysis of variance has revealed that the mean absorbances of successive serial sections incubated for the same time do not differ significantly, and the rates at which specific FRP is deposited in sections in livers from hamsters ranging in age from 2 to 8 months have a coefficient of variation of less than 8%. Thus, the technique passes tests (a) and (c), except that it is not known yet whether it is reproducible in different laboratories.

However, our data for the second test (b) are less satisfactory. We obtained about 40 separate absorbance measurements in each of three reactive sites in a section of normal muscle incubated for an arbitrary time, and examined them in two ways—arranging them in the form of frequency histograms, and analysing their distribution statistically for normality. The histograms (Fig. 2) suggest that the absorbances of the diffuse FRP present in the intrafibre areas appear statistically to be distributed normally, whereas in the more strongly stained 'granule' areas within the muscle fibres, and to a lesser extent in the interfibre connective tissue cells, there seem to be two populations of absorbances, and hence specific FRP. In contrast, an analysis of the absorbance data for skewness using Geary's test shows that these conclusions are probably wrong. For example, the test suggests that the absorbances of FRP in the connective tissue cells do not depart significantly from a single normal distribution.

The very least these analyses and data show is the two mistakes many investigators have made of assuming, first, that three to ten measurements of absorbance are necessarily sufficient for calculating a constant mean absorbance and, second, that the normal distribution of the absorbance measurements can be inferred with absolute confidence by inspection of arbitrarily constructed histograms.

*Validity*

No data are available to indicate whether the membrane technique meets criteria (ii*a*), (iii), (iv) and (xi). On the other hand, unlike most conventional techniques, the membrane technique by its very nature fully satisfies the first criterion (Meijer 1972). It also seems to live up to the remaining criteria, until the supporting data are looked at more closely.

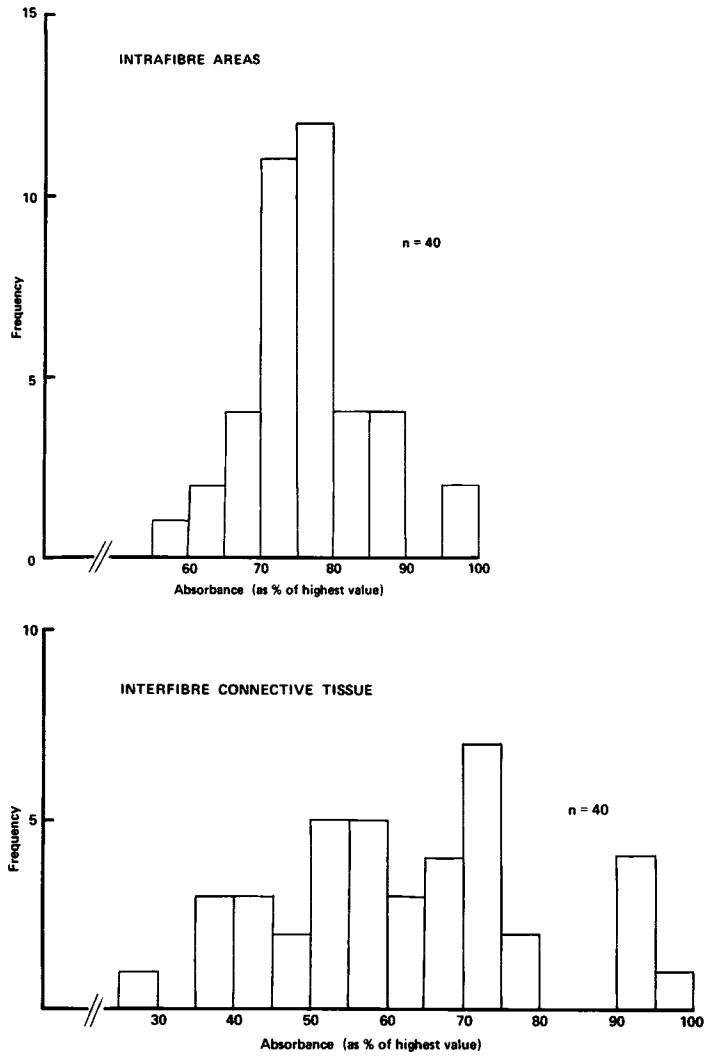


FIG. 2. Distribution of absorbance (at $\lambda_{max}$) of acid phosphatase-specific FRP in 10 $\mu$m section of normal hamster gastrocnemius incubated for 3.5 h. *Above:* intrafibre areas. *Below:* interfibre connective tissue.

Let us consider the fifth criterion, for example. The mean absorbances of the specific FRP in sections of liver certainly increase linearly with section thickness when incubated for a constant time, but sections of skeletal muscle, on the other hand, suddenly cease to produce any more FRP when their thickness is greater than about 16 $\mu$m (Fig. 3). In conventional techniques the linear relationship

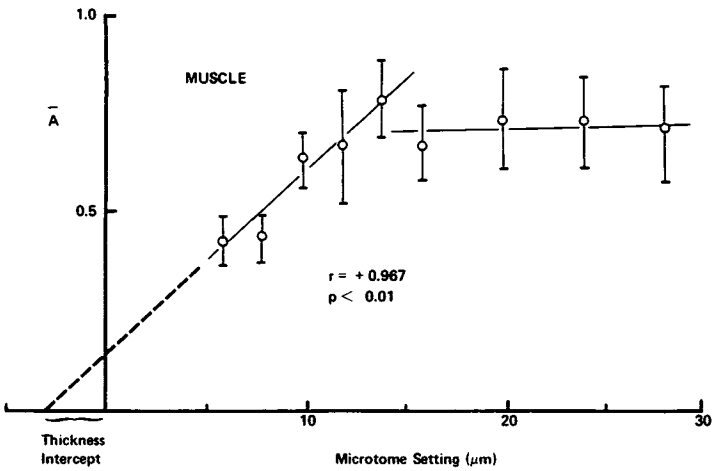


FIG. 3. Variation with cryostat microtome setting (presumed section thickness) of mean absorbance ($\bar{A}$) at $\lambda_{max}$ of acid phosphatase-specific FRP in intrafibre areas of serial sections incubated for 3 h. Linear regression lines drawn.

between absorbance and section thickness sometimes begins to fall off above a certain point which Warburg (1923) called 'Die Grenzschnittdecke': the 'critical section thickness'. For aminopeptidase in kidney, for example, the critical thickness is also about 16 μm (Felgenhauer & Glenner 1966). Consequently, in order to maintain the validity of any technique it is safer to work with sections less than about 12–14 μm thick.

In the standard membrane technique the linear regression lines correlating mean absorbance and section thickness do not pass through the origin (see e.g. Fig. 3). We eventually discovered that this is because the measured absorbance includes a contribution from non-specific FRP.

It is impossible to prove criterion (vi) directly on tissue sections, namely that the rate at which specific FRP is formed is related to the concentration of enzyme within a particular cell. However, we have found that this relationship can be confirmed indirectly if we use model sections prepared from low gelling temperature (LGT) agarose gels containing known amounts of pure enzyme. The advantage of LGT agarose here, compared to the agars and polyacrylamide mixtures which have been used by others, is that it avoids denaturation of the enzyme during the preparation of the gel or artificial tissue mixture.

Fig. 4 illustrates that the membrane technique appears to comply with the seventh criterion satisfactorily and, with the exception of one histological site (the granule areas), with the eighth as well. However, the linear relationship between mean absorbance and incubation time shown in Fig. 4, as has often

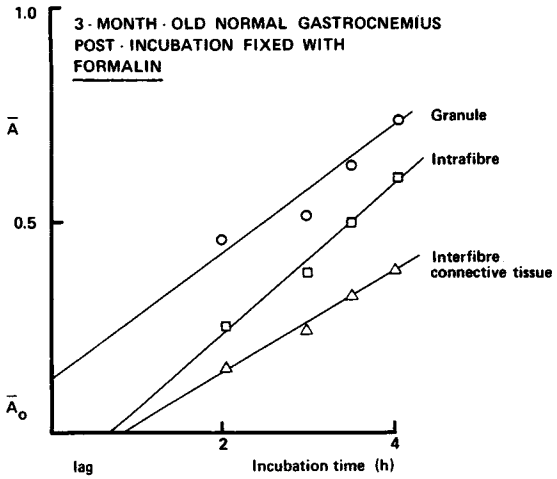


FIG. 4. Linear regression plots of mean absorbance ($\overline{A}$) at $\lambda_{max}$ against incubation time of acid phosphatase FRP in 10 $\mu$m serial sections of normal skeletal muscle incubated with the standard membrane technique. Note the different slopes for the three reactive areas, and the high $\overline{A}_0$ (0.13) for the granule areas.

been found for many other histochemical reactions, may be more fortuitous than real. Indeed there is no absolute necessity for a valid technique to display such a relationship. It is simply more convenient for practical purposes if it does. The following three examples illustrate the danger of accepting linearity uncritically as proof of validity.

(1) Prenna *et al.* (1977) found that although the fluorescence emission of the acid phosphatase PRP (naphthol AS-BI) formed in neutrophils increased remarkably linearly for incubation times up to about 10 min (when incubated with a conventional medium), the fluorescence of the surrounding medium increased exponentially upwards during this period. The moot question, still unresolved, is whether the full amount of specific reaction product formed as a result of the action of the enzyme within the cells is the sum of the reaction products in the cells and the surrounding medium. If it is, the true reaction rate is non-linear.

(2) A reaction rate *in situ* may appear to be linear, but this may be due to the inefficient conversion of the PRP to FRP. If the efficiency is improved, the linearity may be lost. According to Barter *et al.* (1956), for example, under conditions in which only 30% of the inorganic phosphate released by alkaline phosphatase in intestinal brush borders is captured or converted to FRP (calcium phosphate) in the Gomori technique, the dry weight of the deposited FRP increases in proportion to the incubation time. However, when the