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Methods of microphotometric assay of succinate dehydrogenase and cytochrome *c* oxidase activities for use on human skeletal muscle

SALLY L. OLD and MARGARET A. JOHNSON*

Muscular Dystrophy Group Research Laboratories, Regional Neurological Centre, Newcastle General Hospital, Westgate Road, Newcastle-upon-Tyne NE4 6BE, UK

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Summary

Microphotometric assay media for the measurement of succinate dehydrogenase (SDH) and cytochrome oxidase activities in sections of human skeletal muscle have been developed. The optimal constitution of these media was determined experimentally. Factors investigated include the effects of substrate concentration, pH, use of different electron acceptors and electron donors, influence of intermediate electron carriers and tissue-stabilizing agents, effects of inhibitors, the extent of endogenous and non-specific reactions and the linearity of the reactions during the time course of the assays. Optimal assay media (SDH) contained 130 mM succinate, 1.5 mM Nitro Blue tetrazolium, 0.2 mM phenazine methosulphate and 1.0 mM sodium azide in 0.1 M phosphate buffer, pH 7.0. Cytochrome oxidase was optimally assayed in media containing 4 mM diaminobenzidine and 100 μM cytochrome *c*. Reactions in individual muscle fibres were found to be linear for incubation times up to 10 min in SDH assays and for more than 15 min in cytochrome oxidase determinations. Some potential uses of these microphotometric assays in the investigation of human metabolic muscle disorders are discussed.

Introduction

Defects of respiratory chain enzymes in human skeletal muscle comprise an important class of metabolic myopathies. Disorders involving different individual respiratory complexes have been recorded (for review, see Di Mauro *et al.*, 1985). Of these, Complex II (succinate dehydrogenase) and Complex IV (cytochrome *c* oxidase) abnormalities are most readily amenable to histochemical investigation. Numerous methods for the qualitative demonstration of their activities, and several techniques for their quantitative determination, are already in existence. However, many of the microphotometric assay methods published previously for succinate dehydrogenase (Butcher, 1970; Nolte & Pette, 1972; Pette, 1981) and for cytochrome *c* oxidase (Ballantyne & Bright, 1979) have been developed for tissues other than skeletal muscle or in species other than man.

The aim of this study was to devise assay methods specifically for use on human tissue, with particular reference to the investigation of deficiencies of Complex II and Complex IV using sections of skeletal muscle. These methods were designed to be reliable, reproducible and suitable for routine use in a diagnostic laboratory.

Materials and methods

Tissue samples

Human m. quadriceps biopsies were obtained for diagnostic purposes. Biopsies shown to be normal by histological and biochemical criteria were used. Electromyographic and clinical assessment of patients was also taken into consideration in the selection of these normal control samples.

Tissue preparation

Muscle blocks were orientated transversely on filter paper strips and frozen using isopentane cooled to about −150° C in liquid nitrogen. Frozen muscle was stored in heat-sealed polythene packets at −196° C until required. Sections, 8 μm-thick, were cut at −20° C using a Reichert Frigocut 2800N cryostat microtome with a motor-driven cutting mechanism. Sections were picked up on slides such that approximately 0.5 mm clearance between section and coverslip was maintained in succinate dehydrogenase assays; for cytochrome *c* oxidase, the clearance was about

* To whom correspondence should be addressed.

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1 mm to ensure an adequate supply of molecular oxygen (see the Results section). This was achieved by means of strips of electrical tape positioned on either side of the section; coverslips were held in place by double-sided Sellotape strips placed over the electrical tape. Until used, sections were stored at +4° C, thus avoiding additional cycles of freezing and thawing. Sections and incubation media were equilibrated to 25° C before incubation.

Incubating media

The optimal constitution of assay media was determined experimentally for succinate dehydrogenase and cytochrome *c* oxidase. Factors investigated include the effects of substrate concentration, the use of different electron acceptors or electron donors, the influence of pH and reaction time, the use of intermediate electron carriers, the effect of inhibitors and the use of tissue-stabilizing agents. Details of optimal media determined as a result of these experiments are given in the Results section.

Sources of reagents

The following reagents were obtained from Sigma: Nitro Blue tetrazolium (NBT), Tetranitro Blue tetrazolium (TNBT), Thiazolyl Blue tetrazolium bromide (MTT), phenazine methosulphate (PMS), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), 3,3'-diaminobenzidine (DAB), cytochrome *c* (Type III, from horse heart), catalase (from bovine liver), malonic acid (sodium salt), thenoyltrifluoroacetone (TFA). Other chemicals, including sodium succinate, sodium azide and sodium cyanide, were obtained from BDH Chemicals Ltd.

Microphotometry

The absorbances (*A*) of the final reaction product (FRP) were measured using a Zeiss UMSP30 microphotometer equipped with a scanning stage linked to a Hewlett-Packard Series 200 microcomputer. Core measurements were made of a central zone about 25 μm in diameter in each muscle fibre assayed. A substage aperture corresponding to 40 μm at the level of the specimen was used to restrict stray light. Zero absorbance was calibrated using the measurement obtained through slide, section, coverslip and incubation medium immedately after addition of the latter. All assays were made at 25 ± 1° C. Succinate dehydrogenase reaction product was measured at 574 nm and that of cytochrome *c* oxidase at 450 nm.

Assay system

Rates of enzyme activity were determined in samples consisting of 20 individual muscle fibres in any one measuring cycle. This sampling protocol is broadly comparable to that adopted by Reichmann & Pette (1982). A FISCAN computer program allowed cycles of absorbance measurements to be made at intervals varying from 10 s upwards. Usually 10 cycles of meaurements were made in each assay procedure. An MDATA program computed the reaction rate (slope) for each of the 20 muscle fibres. This program also allowed the linearity of the reaction to be determined by regression analysis and by inspection of data from individual muscle fibres plotted graphically.

Reaction rates are expressed as $\Delta A \times s^{-1}$, measured at the appropriate absorption maximum of the FRP. The *mean reaction rate*, calculated from each sample of 20 muscle fibres, was used in the comparisons involved in the experimental determination of optimal incubation conditions. An identical sample of 20 fibres was used throughout any one experiment; the location of the sample area was recorded by means of a Polaroid photomicrograph of the unreacted section taken using differential interference contrast optics.

Results

SUCCINATE DEHYDROGENASE (SDH) ASSAY MEDIA

Choice of tetrazolium salt

Initial experiments involved the selection of final electron acceptor (tetrazolium salt). In early studies of dehydrogenase activity in skeletal muscle, Nitro Blue tetrazolium (NBT) has been shown to bind selectively not only to mitochondria but also to sarcoplasmic reticulum where non-specific reduction may occur (Brooke & Engel, 1966). MTT (thiazolyl blue tetrazolium bromide) does not suffer from this disadvantage (Pearse, 1972) and has therefore been used extensively in qualitative work on dehydrogenases in skeletal muscle. However, the formazan produced from MTT requires chelation by cobalt ions as otherwise it will not form an amorphous reaction product. The possibility that cobalt was inhibitory to SDH activity was tested using concentrations of this cation ranging from 0 to 500 mM, in a medium consisting of 1.5 M MTT, 130 mM sodium succinate in 0.1 M phosphate, pH 7.0. The addition of 5 mM cobalt chloride resulted in an approximately 50% decrease in SDH activity; this was decreased to about 30% normal by 50 mM cobalt and to about 5% by 500 mM cobalt. Since at least 5 mM cobalt is required in MTT-containing media to prevent the formation of large crystalline arrays of formazan reaction product, it is concluded that MTT is unsuitable for quantitative assays of SDH activity.

The tetrazolium salts NBT and TNBT (Tetranitro Blue tetrazolium) were compared for the prevalence of non-specific reactions obtained with each. At a concentration of 1.5 mM of either NBT or TNBT in 0.1 M phosphate buffer, pH 7.0, non-specific reactions (in the absence of substrate) were about 25% with each of these final electron acceptors. Since TNBT provided no significant advantage in this respect, subsequent experiments were conducted using NBT.

Effects of phenazine methosulphate on reaction rate

Phenazine methosulphate (PMS) has been widely used as an intermediate electron carrier in dehydrogenase reactions (Brody & Engel, 1964; McMillan, 1967; Nolte & Pette, 1972; Kugler, 1982; Van Noorden & Tas, 1982). Since it mediates more efficient electron transfer from coenzyme or prosthetic group to tetrazolium salt, its effect is to increase the product of FRP. Concentrations of PMS ranging from 0 to 2.0 mM were

tested in incubation media containing 130 mM succinate, 1.5 mM NBT and 0.1 M phosphate buffer, pH 7.0. It was found that the addition of PMS alone exerted no significant effect on reaction rate (Fig. 1). However, in conjunction with terminal respiratory chain block, achieved using 1 mM cyanide or azide, a large increase in the rate of FRP formation was observed.

Having established that PMS (plus respiratory chain block) increased the total reaction rate, the effect of PMS on non-specific reaction product formation (in the absence of substrate) was also investigated. It was seen that this component of the reaction also increased with increasing PMS concentration (Fig. 2). The factor that determined the choice of PMS concentration (0.2 mM) was that at this concentration the relative proportion of non-specific reaction was at its minimum level.

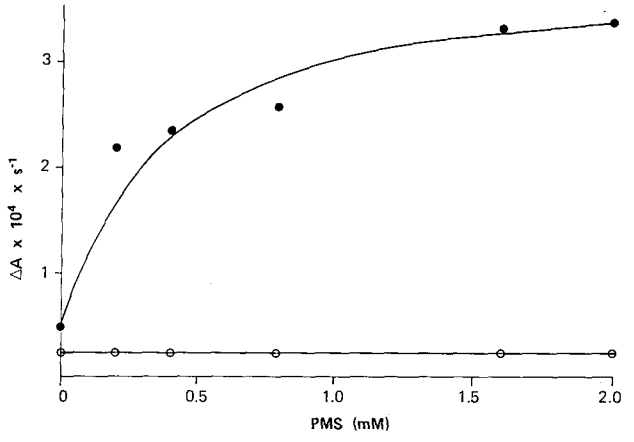

Fig. 1. The relationship between mean reaction rate ($\Delta A_{574} \times s^{-1}$) of SDH and concentration of PMS. Open symbols (○) show reaction without terminal respiratory chain block (1 mM cyanide). Closed symbols (●) show reaction in the presence of 1 mM cyanide. Each symbol in this and succeeding graphs represents mean reaction rate in identical samples of 20 muscle fibres.

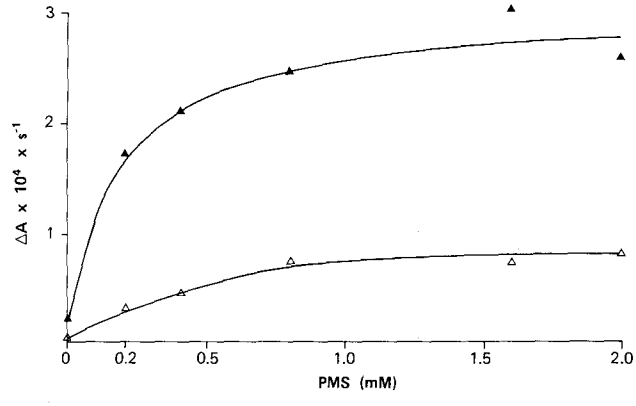

Fig. 2. Comparison of reaction in the presence of 130 mM succinate (▲) and in the absence of succinate (△). Both reactions increase with increasing concentration of PMS (+1 mM cyanide) but at 0.2 mM PMS the proportion of non-specific reaction is at its lowest.

Effect of NBT concentration rate

The effect of varying the tetrazolium salt concentration in the incubation media was investigated, both in the absence of PMS and using an optimal concentration of 0.2 mM PMS plus 1 mM cyanide as determined previously. It can be seen from Fig. 3 that, in the absence of PMS, a gradual increase in FRP formation was seen up to a concentration of 15 mM NBT. However, in the presence of PMS, an optimal reaction rate was achieved at a concentration of approximately 1.5 mM NBT.

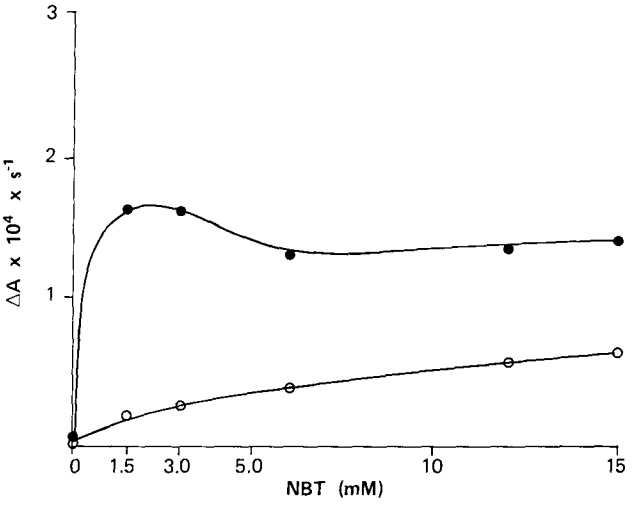

Fig. 3. Effect of NBT concentration on mean reaction rate. In the absence of 0.2 mM PMS (+1 mM cyanide) (○), the reaction increases gradually to a concentration of 15 mM NBT. In the presence of PMS and cyanide (●), a peak is seen at 1.5 mM NBT and a slight decline thereafter.

Effect of magnesium ions on reaction rate

Magnesium is a cofactor of SDH and is required for full catalytic activity. Mg^{2+} ions are often provided exogenously in incubation media, although it is possible that enough endogenous Mg^{2+} is present in tissue sections to promote optimal activity. Concentrations of $MgCl_2$ varying from 0 to 10 mM were added to assay media but resulted in no significant increase at any of the concentrations tested. It was concluded that the exogenous provision of Mg^{2+} was unnecessary in this assay system.

Effect of EDTA

The chelating agent EDTA has been claimed to aid the penetration of either substrate or electron acceptors, or both, into the mitrochondrial matrix. Concentrations of EDTA varying from 0 to 10 mM were investigated but no effect on reaction rate was seen.

Effects of tissue-stabilizing agent

Polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) have been used in previous microphotometric

assays of dehydrogenases to prevent diffusion from the tissue sections of FRP or of the enzyme itself (Altman, 1980). This is undoubtedly important in assays of soluble dehydrogenases such as lactate dehydrogenase, but probably less so in the case of membrane-bound enzymes such as SDH. In the present study, various concentrations of PVA and PVP (0–12.5%, w/v) were investigated. No significant increase was seen when compared to the reaction in the absence of these substances. In fact a steady decline was seen with both additives. Using 12.5% PVA this decrease was about 75% normal rate; with 12.5% PVP, the rate decreased to approximately 55% normal values.

Effect of substrate concentration on reaction rate

The apparent K_m in this assay system was estimated using a range of substrate concentrations up to 10 mM. Fig. 4 shows a double-reciprocal plot (Lineweaver–Burk) of substrate concentration against reaction velocity, in which K_m for succinate is 1.35 mM. In more recent experiments, a computer program giving a transformation of direct linear plots was used to calculate K_m.

Cytochemical incubation media for SDH traditionally employ high substrate levels (about 100 K_m). These could be decreased by at least 50% without apparent decrease in reaction rate. However, in the majority of experiments in the current study a succinate concentration of 130 mM was used.

Effects of malonate (a competitive inhibitor) and thenoyltrifluoroacetone (a non-competitive inhibitor) on reaction rate

The competitive inhibitor malonate was studied, using concentrations of up to 50 mM in conjunction with succinate concentrations up to 10 mM. The maximal velocity ($V_{max.}$) of the reaction was unchanged in the presence of this inhibitor but the K_m was increased: 3.5 mM in the presence of 5 mM malonate. This is characteristic of the effects of a competitive inhibitor. The non-competitive inhibitor thenoyltrifluoroacetone (TFA) was used in concentrations varying from 0 to 100 μM. In media containing 130 mM succinate, SDH activity was decreased to about 40% by 25 μM TFA with no further decrease when higher concentrations were used. TFA blocks the transfer of electrons from succinate to ubiquinone and subsequent experiments in the presence and absence of PMS were carried out to determine whether the presence of this electron carrier interfered with the action of the inhibitor. However, there was no significant difference in the degree of inhibition obtained plus or minus PMS. The effect of TFA on the reaction in the absence of substrate was also investigated to exclude the possibility that the inhibitor interfered with the reduction of tetrazolium salt. No decrease in the non-specific reaction was seen in the presence of 50 μM TFA and it was concluded that its mode of action in tissue sections, as in isolated mitochondrial preparations, is to inhibit electron transfer from the prosthetic group of SDH to ubiquinone.

Effect of section thickness on the rate of FRP formation

Sections varying in thickness from 4 to 16 μm were cut at −20° C using the motor-driven cutting cycle of the Reichert Frigocut cryostat microtome. Figure 5 shows that the rate of FRP deposition is directly proportional to section thickness over this range. Routinely, sections 8 μm thick were used for SDH assays of human skeletal muscle.

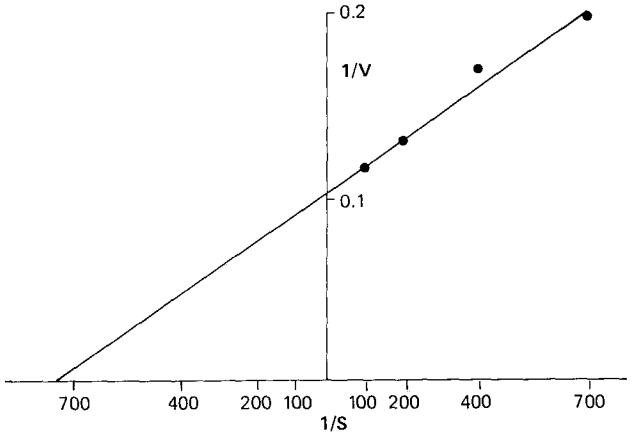

Fig. 4. Lineweaver–Burk (double-reciprocal) plot of reaction velocity vs substrate concentration. Velocity is expressed as $\Delta A \times 10^4 \times s^{-1}$; substrate concentration is expressed in mM. An apparent K_m of approximately 1.35 mM was obtained for succinate using samples consisting of a mixture of metabolic fibre types.

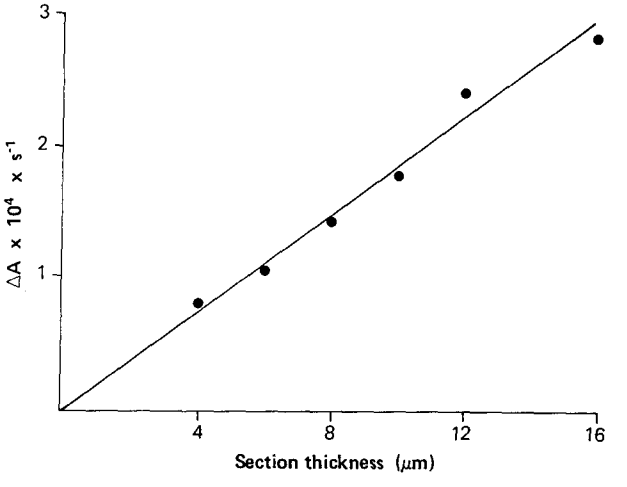

Fig. 5. The effect of section thickness (μm) on the rate of FRP deposition. This relationship is linear over the range tested.

Effect of pH on reaction rate

The pH response of the SDH reaction was investigated over a pH range of 6.0–9.0. A broad peak was obtained at pH 7.0–8.0. In routine use, pH 7.0 was adopted in order to maintain maximal comparability with parallel biochemical SDH determinations using mitochondrial fractions (Sherratt *et al.*, 1988).

Linearity of reaction and incubation time

Computer-generated graphs of absorbance data derived from individual muscle fibres were used to assess the linearity of the reaction. The time taken to complete one cycle of measurements on each of 20 muscle fibres was 6–7 s. The time interval between the start of successive measuring cycles could therefore be as little as 10 s. However, for SDH assays in human muscle a 60 s interval was used routinely, with no departure from linearity over the total time course of the assay which was 540 s (10 measuring cycles, of which the first was at time zero).

A representative graph of absorbance against time is shown in Fig. 6. This is an exact copy of a computer-generated graph of the original absorbance values obtained and is therefore the test reaction before subtraction of control values. For this test reaction, the correlation coefficients (r) were generally ≥ 0.995, often higher in high-activity fibres but sometimes lower in low-activity fibres. Subtraction of control values in individual fibres led to values of r that were as high, if not higher, than those in the raw absorbance data. Instrument error is fairly constant but will be more apparent in measurements of low absorbances in low-activity fibres. Because of this very short measuring cycle times will be more prone to this type of error and offer no obvious advantages. In addition, non-specific reactions in substrate-free controls appear to predominate during the initial phase of incubation (see also Van Noorden *et al.*, 1985). Optimal incubation time must therefore be within the linear phase of the reaction but should not be unduly short.

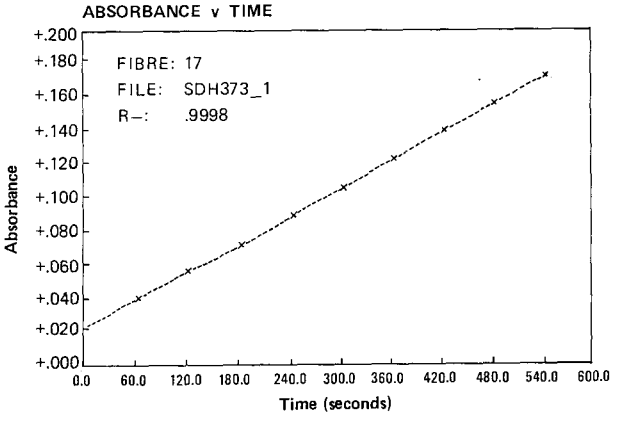

Fig. 6. Copy of an original computer-generated graph of increase in absorbance vs time seen in one individual muscle fibre during the course of an SDH assay. The reaction can be seen to be linear ($r = 0.9998$) over the time course of the assay. The graph shows absorbance values in test reaction before subtraction of control values. The absorbance at time 'zero' may not always be 0 because calibration of zero absorbance (through slide, section and medium) is made in the first fibre of the sample and initial absorbance readings in subsequent fibres will differ slightly from this.

Non-specific reaction product formation

The binding of NBT to sarcoplasmic reticulum and its non-specific reduction in skeletal muscle has already been mentioned. In the absence of PMS, non-specific reduction in the absence of substrate could comprise about 25% total reaction product. However, the inclusion of PMS decreased the *relative* proportion of reaction product attributable to non-specific reduction to approximately 15–17%. The extent of the non-specific reaction did not vary appreciably from fibre to fibre whereas specific SDH activity varied according to fibre type (see below). In SDH assays of human muscle, we therefore calculated the mean non-specific reaction rate, based on a sample of 20 fibres, and subtracted this value from the total reaction rates obtained for each individual muscle fibre. This was less time consuming than providing individual control values for each of the fibres assayed (usually >100 fibres per assay).

SDH activity in individual fibre types in human skeletal muscle

In routine use, SDH assays were carried out using samples of at least 100 muscle fibres (i.e. at least five tissue sections with 20 fibres assayed in each). Serial sections were used to demonstrate the activity of myofibrillar ATPase after preincubation at pH 4.6 (Brooke & Kaiser, 1970). This allowed the classification of individual fibres into metabolic types: Type 1 (slow-twitch, oxidative), Type 2A (fast-twitch, oxidative/glycolytic) and Type 2B (fast-twitch, glycolytic). Although there was considerable overlap in reaction rates between fibre types, as also described by Reichmann & Pette (1982), a clear activity gradient of Type 1 > 2A > 2B was seen in normal human muscle. The amount of variability within individual fibre types is reflected in the relatively large standard deviations in a typical assay (Table 1). However, differences between the fibre types were statistically significant.

CYTOCHROME OXIDASE ASSAY MEDIA

Choice of electron donor

Two major types of incubation medium have been used for the qualitative and quantitative demonstration of cytochrome oxidase activity. The earlier method (Burstone, 1960) employs a combination of aryl amines which react in the presence of cytochrome *c* and cytochrome oxidase (cytochrome aa_3) to produce an azine reaction product. The mechanism of this

Table 1. Microphotometric assay of SDH activities in individual fibre types.

*Fibre type**	*Mean activity* ($\Delta A_{574} \times 10^4 \times s^{-1}$) ± S.D.	
Type 1	2.71 ± 0.36	
Type 2A	1.86 ± 0.38	
Type 2B	1.42 ± 0.34	($n = 100$)

*Fibre type was determined in serial sections in which myofibrillar ATPase was demonstrated (Brooke & Kaiser, 1970).
Student's t test: Type 1 vs 2A, $P < 0.001$; Type 2A vs 2B, $P < 0.001$; Type 1 vs 2B, $P < 0.001$.

reaction is incompletely understood, the reaction product cannot be reliably localized to mitochondria, needs cobalt or other cations such as nickel or cadmium in order to form a stable end product, and has an affinity for tissue lipids.

The introduction of 3,3'-diaminobenzidine (DAB) as an electron donor for the demonstration of cytochrome oxidase (Seligman *et al.*, 1968) resulted in a much more reliable method. DAB acts as an electron donor in the respiratory chain at the level of cytochrome *c*, the reduced cytochrome *c* generated being reoxidized by cytochrome oxidase. Oxidative polymerization and cyclization of DAB gives gives rise to an insoluble indamine polymer which can be shown to be localized in mitochondrial cristae (Seligman *et al.*, 1968). Initial spectrophotometric studies in our laboratory showed an absorption maximum at about 450 nm for the DAB oxidation product.

Effect of electron donor (DAB) concentration

Increasing concentrations of DAB from 1 to 6 mM were incorporated into media containing 80 μM cytochrome *c* in 0.1 M phosphate buffer, pH 7.0. Figure 7 shows the effect of DAB concentration on reaction rate. Although higher rates could be obtained using 5–6 mM DAB, 4 mM was selected for use in routine assays. This was dictated by practical considerations; stock solutions of 10 mM DAB in 0.1 M phosphate buffer, pH 7.0, cannot be stored frozen without DAB precipitating from solution on thawing. Stock solutions of 5 mM did not pose this problem and allowed addition of substrate and other components to give a final concentration of 4 mM. This concentration is considerably higher than that used in the original Seligman medium (about 1.4 mM). It was not possible to define a plateau of activity with increasing concentrations of DAB because at concentrations higher than 10 mM, precipitation during incubation became a problem and interefered with the reaction.

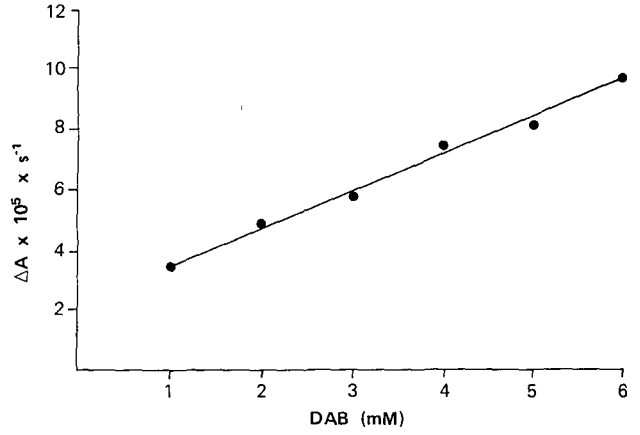

Fig. 7. The relationship of electron donor (DAB) concentration and rate of cytochrome oxidase reaction. This was found to be linear over the range of DAB concentrations tested.

Effect of oxygen concentration

Since the rate of electron transfer is dependent upon an adequate supply of the final electron acceptor, molecular oxygen, care was taken to ensure that aerobic conditions were maintained throughout the time course of the assay. This was achieved by increasing the volume of incubation medium used, and hence the amount of dissolved oxygen available by approximately twofold. The distance between coverslip and section was increased from the usual 0.5 mm to 1.0 mm using additional strips of electrical tape (see the Materials and methods section). Under these conditions, a uniform reaction intensity was seen acrosss the whole tissue section whereas, using a smaller volume of medium, a zone of lower activity was sometimes visible in the centre of the tissue section due to oxygen depletion.

Effect of substrate concentration

Concentrations of cytochrome *c* varying from 0 to 120 μM were investigated in media containing 4 mM DAB in 0.1 M phosphate, pH 7.0. Figure 8 shows the effect of this increasing substrate concentration on reaction rate. It can be seen that maximum activity is not achieved using less than 80 μM cytochrome *c* in this assay system. This concentration is somewhat higher than that (about 50 μM) cited in the original description of the method for qualitative light and electron microscopic demonstration (Seligman *et al.*, 1968). It appears (Fig. 8) that the activity reaches a plateau at 80–120 μM and subsequent studies using substrate concentrations higher than this did not result in further increase in activity.

It is also apparent that in view of the finding that

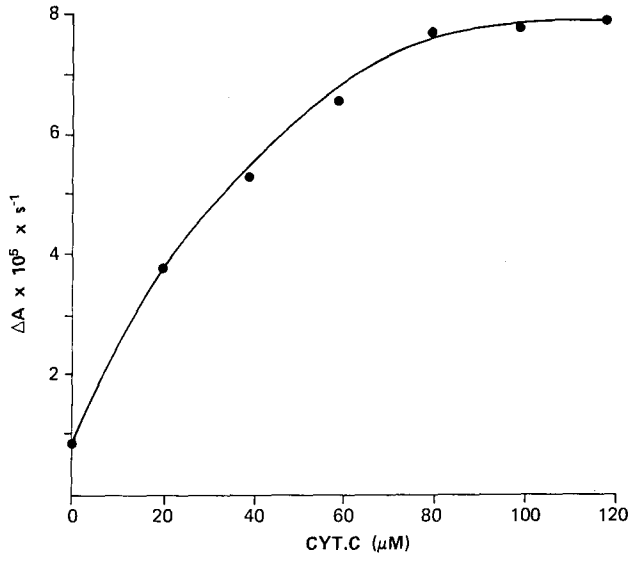

Fig. 8. The effect of cytochrome *c* concentration (μM) on reaction rate. It can be seen that maximal activity is achieved with >80μM cytochrome *c*.

relatively high cytochrome *c* concentrations are necessary for the demonstration of optimal activity, previous suggestions that cytochrome *c* need not be added exogenously are misleading (Nachlas *et al.*, 1958; Ballantyne & Bright, 1979).

Oxidation of endogenous cytochrome c

If no additional cytochrome *c* is present in the incubation medium, oxidation of endogenous cytochrome *c* occurs, giving rise to an appreciable reaction. A comparison of reaction rates obtainable with (a) endogenous cytochrome *c* and (b) 80 μM exogenous cytochrome *c* in the presence of varying concentrations of electron donor (DAB) are shown in Fig. 9. The rate of the reaction in the presence of 80 μM added cytochrome *c* is approximately five times that observed due to endogenous cytochrome *c* oxidation.

In any valid assay method, substrate concentration must be carefully regulated, and it is obvious that this is impossible if endogenous cytochrome *c* alone is used. Even in normal tissues this concentration will be variable and in pathological tissues it may be severely depleted. Unless sufficient exogenous cytochrome *c* is added, the assay will be a measure of cytochrome *c* plus cytochrome aa_3 concentrations rather than a valid assay of cytochrome aa_3 activity.

Effects of specific inhibitors

The extent of the non-specific (non-enzymic) reaction was demonstrated using the terminal respiratory chain inhibitors azide and cyanide. Cyanide (1 mM) and azide (2.5 mM) provided equal inhibition of cytochrome oxidase activity. Compared with the inhibition achieved using 2.5 mM azide, 1 mM azide gave 97% inhibition. No further inhibition was seen on increasing the concentration of azide beyond 2.5 mM.

In Fig. 10, the reaction obtained using endogenous cytochrome *c* plus or minus 5 mM azide gives a comparison of the extent of non-specific 'background' reaction and the reaction due to oxidation of endogenous cytochrome *c*.

Effect of catalase concentration

If hydrogen peroxide is generated in the tissue to be assayed for cytochrome oxidase activity, this will act as a substrate for any peroxidases present. The DAB in the incubation medium will act as an electron donor in

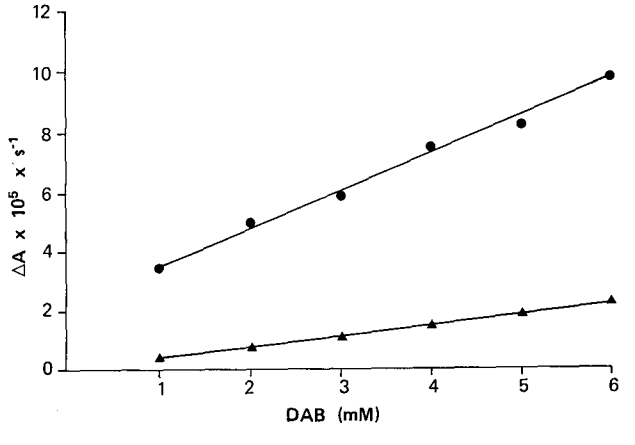

Fig. 9. Comparison of the reaction in the presence of 80 μM cytochrome *c* (●) and in the absence of added substrate (▲) with varying concentrations of electron donor (DAB). The oxidation of endogenous substrate also shows a linear relationship with electron donor concentration.

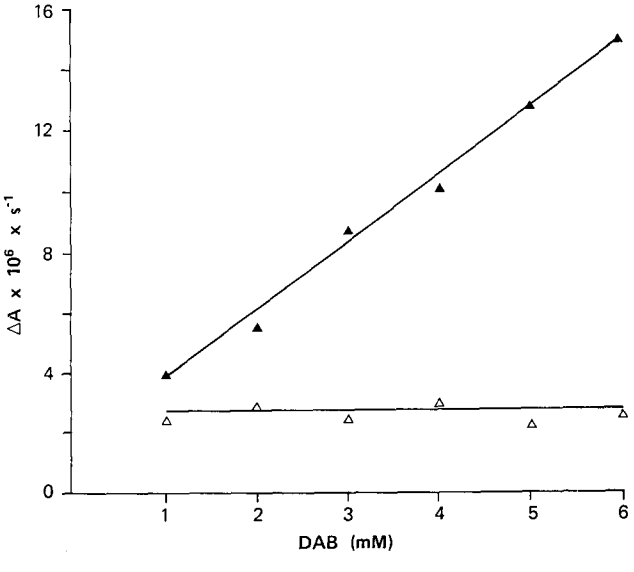

Fig. 10. Comparison of the reaction in the absence of added substrate (endogenous cytochrome *c* oxidation) (▲) and the non-specific reaction obtained in the presence of 5 mM azide (△). Note that these reactions are an order of magnitude lower than those shown in Fig. 9.

such a system, resulting in reaction product due to the presence of peroxidase and not cytochrome oxidase. The original method of Seligman *et al.* (1968) recommended the inclusion of 2 μg ml^{-1} of catalase (about 4 IU ml^{-1}) to avoid possible contaminating reactions from this source. If tissue peroxidases were in fact contributing to the total reaction product, it would be expected that omission of catalase would give rise to an increase in FRP.

This possibility was tested by incorprating concentrations of catalase varying from 0 to 15 μg ml^{-1} in media containing 4 mM DAB, 80 μM cytochrome *c* in 0.1 M phosphate, pH 7.0. No significant difference in reaction rate was seen using media with or without catalase and it was concluded that, in human muscle under these assay conditions, peroxidatic activity does not contribute to total FRP formation.

Effect of pH on reaction rate

Initial experiments were made using 0.1 M phosphate, pH 7.0, as diluent for media components. This pH was chosen becaue it is cited in most currently used biochemical spectrophotometric methods (Cooperstein & Lazarow, 1951; Smith, 1955). It was thought possible, however, that the reaction between electron donor (DAB) and cytochrome *c* might show a different pH optimum from that of the reaction between the oxidase and cytochrome *c*. The response of the DAB–cytochrome aa_3 assay system to pH (range 5.0–8.5) was therefore investigated. The resulting pH–response curve is shown in Fig. 11. A less narrow peak was obtained than that seen in determinations by polarographic or spectrophotometric methods but the pH optimum is strictly comparable.

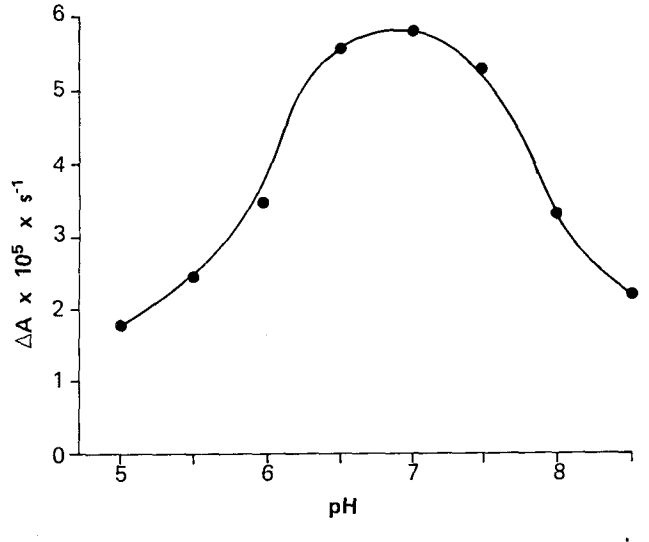

Fig. 11. The pH–response curve for cytochrome oxidase using DAB as electron donor.

Linearity of reaction and incubation time

The reaction was found to be linear for incubation times up to at least 20 min. Routinely, using human muscle sections, the time interval between successive measuring cycles was set at 100 s, which gave a total assay time of 900 s. Figure 12 shows a computer-generated graph of the reaction in an individual muscle fibre; correlation coefficients were generally >0.995. Since the non-specific reaction (in the presence of 5 mM azide) comprised <5% of the total reaction it was ignored in routine assays.

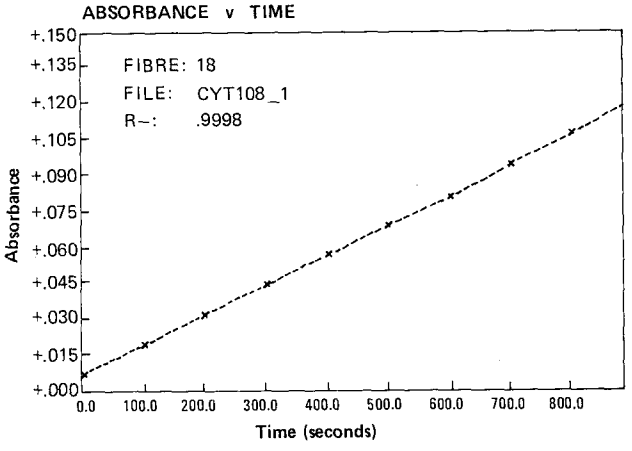

Fig. 12. Copy of an original computer-generated graph of increase in absorbance against time in a single muscle fibre during the course of a cytochrome oxidase assay. The reaction can be seen to be linear ($r = 0.9998$) over the time course of the assay. The control reaction in the presence of 5 mM azide was always very low (<5%) and subtraction of this value did not affect the degree of linearity of the reaction.

Cytochrome oxidase activity in individual fibre types

As with SDH activity, the gradient of cytochrome oxidase activity followed the pattern: Type 1 > 2A > 2B in normal human muscle. The variability shown by individual fibres of a given fibre type was considerable, with overlap of values between fibres of Types 1 and 2A and between Types 2A and 2B (Table 2) but there were statistically significant differences between the fibre subpopulations.

Discussion

The techniques described in this paper largely satisfy the criteria for the validation of quantitative histochemical enzyme methods set out by Stoward (1980). However, it is stressed that these assays have been developed specifically for use on human skeletal muscle and there is no reason to assume their validity in other tissues or in other species. For example, the higher mitochondrial oxidative enzyme activity in the skeletal muscle of laboratory mammals as compared

Table 2. Microphotometric assay of cytochrome oxidase activities in individual fibre types.

*Fibre type**	*Mean activity* ($\Delta A_{450} \times 10^5 \times s^{-1}$) ± S.D.	
Type 1	10.26 ± 1.44	
Type 2A	6.54 ± 1.39	
Type 2B	3.91 ± 1.13	($n = 100$)

*Fibre type was determined as in Table 1.
Student's t test: Type 1 vs 2A, $P < 0.001$; Type 2A vs 2B, $P < 0.001$; Type 1 vs 2B, $P < 0.001$.

with man may mean that linearity of FRP formation will be maintained for shorter periods due to faster accumulation of FRP in amounts that interfere with further enzyme activity. Pette (1981) has suggested that a linear reaction is maintained for only brief periods (about 2 min) in assays of SDH in rat and rabbit muscles. However, this view has been challenged by Blanco *et al.* (1988) who found that linearity was maintained for more than 8 min in their SDH assays of cat diaphragm muscles. They pointed out that the non-specific reaction, in the absence of substrate, is higher during the initial incubation period than in subsequent phases and that unduly short incubation times should be avoided. This reiterates the view expressed by Van Noorden *et al.* (1985). In their muscle fibre assays, Blanco *et al.* 1988 used an image-processing system (Castleman *et al.*, 1984) for measuring increasing absorbance. This development appears to offer an acceptable alternative to microphotometric methods for both end point and kinetic enzyme determinations in tissue sections.

Blanco *et al.* have recommended the use of azide instead of cyanide as respiratory chain inhibitor since the proportion of the reaction due to non-specific reduction of NBT was found to be less. The use of methoxyphenazine methosulphate (mPMS) rather than PMS was also advocated as a further measure to decrease non-specific reactivity. The proportion of non-specific reaction which they encountered using PMS + cyanide, PMS + azide, mPMS + cyanide and mPMS + azide was about 35%, 25%, 25% and 15% respectively. It is apparent from these data that non-specific binding and reduction of NBT constitute more of a problem when using feline muscle than in human muscle. In our assays, non-specific reactions rarely comprised more than 17% total reaction even using PMS and cyanide.

In this study the K_m for succinate with regard to separate fibre types was not especially investigated, though initial results would tend to confirm the findings of Nakae and Shono (1984) that the various fibre types display similar K_m values. The value of 1.35 mM found in the present study is closed to that commonly cited in biochemical sources (e.g. Conn & Stumpf, 1976, for bovine heart) but higher than values obtained cytochemically in rat gastrocnemius (about 0.5 mM) by Nakae and Shono (1984) or in cat diaphragm (approximately 0.3 mM) by Blanco *et al.* (1988).

In assays of cytochrome oxidase using DAB as electron donor, the levels of non-specific reaction were very low (<5%). In order to assess them, the reactions in the presence and absence of the specific inhibitor azide were compared. The cytochrome oxidase reaction in the absence of exogenous substrate is not a measure of non-enzymic FRP production since most of the reaction seen under these circumstances is due to the oxidation of endogenous substrate. The importance of providing an adequate concentration of exogenous cytochrome *c* in assays of cytochrome oxidase is paramount. Under conditions of suboptimal cytochrome *c* concentration the oxidation of endogenous substrate, the concentration of which is variable, will assume increased importance and the assay will cease to give any valid estimation of the activity of the oxidase. The results of previous work using rabbit heart and brain and a mixture of aryl amines an electron donors (Ballantyne & Bright, 1979) are puzzling in this respect. These authors stated that no significant increase in cytochrome oxidase activity was seen when up to 80 μM exogenous cytochrome *c* was added to media, as compared with the reaction obtained through oxidation of endogenous substrate only. One possible explanation of this finding is that *p*-aminodiphenylamine and *p*-amino-*p'*-methoxydiphenylamine, the electron donors used, are inefficient in this function. Under such circumstances, electron transfer from cytochrome *c* to cytochrome aa_3 may proceed at a much faster rate than the transfer of electrons from amine to cytochrome *c*. Using such a system, independence of endogenous cytochrome *c* concentration and demonstration of full catalytic activity of the oxidase are unlikely to be achieved.

The work described in the present paper has concentrated on the development of assay media the optimal constitution of which have been determined experimentally. There has been a tendency in the past for histochemical media to include components because of theoretical rather than practical considerations. In SDH assay media, EDTA, Mg^{2+} ions, PVA and PVP are examples; in cytochrome oxidase media the same can be said of the addition of catalase. The theoretical basis of their inclusion is unexceptionable but in practical terms they do not aid the demonstration of full catalytic activity and may in some cases be inhibitory. It is, however, stressed that the lack of activity-enhancing effect seen in our assay systems

may not necessarily obtain in other contexts, e.g. in other tissues or species. We aimed to develop assay media that would be simple and straightforward to prepare and use in a routine diagnostic laboratory. Because of this, we made considerable use of deep-frozen stock solutions with aliquots of each media component stored in polythene vials until required. This minimized any variability in the constitution of the media and since the number of individual media components was also kept to a minimum this helped to eliminate error due to inaccuracy in pipetting etc.

It was concluded in this study that an appropriate medium for the microphotometric determination of SDH in human skeletal muscle consisted of 130 mM succinate, 1.5 mM NBT, 0.2 mM PMS and 1.0 mM azide in 0.1 M phosphate buffer, pH 7.0. The medium for the assay of cytochrome oxidase activity was also made up in 0.1 M phosphate, pH 7.0, and contained 4 mM DAB and 100 μM cytochrome *c*.

These assay methods have numerous applications in the study of human metabolic myopathies. Many patients who suffer from deficiencies of respiratory chain enzymes, in particular cytochrome oxidase, are infants or small children. In several disorders, multiple biopsies may be necessary in order to monitor the extent of the effect in skeletal muscle (Di Mauro *et al.* 1985) and microphotometric assays are obviously useful in this context since they require so little tissue. SDH is a useful 'reference enzyme' in many of these studies since it is sometimes preferable to express the activity of an affected enzyme relative to that of SDH rather than as an absolute value. In some cytochrome oxidase deficiencies the defect is confined to a subpopulation of muscle fibres (Johnson *et al.*, 1983) and assays made on muscle homogenates may fail to detect any abnormality, especially in the early stages of the disorder (Sherratt *et al.*, 1988). The ability to make enzyme determinations in single muscle fibres is invaluable in this respect (Reichmann, 1988) and results can be correlated with immunocytochemical data on enzyme subunit proteins in the same individual fibres (Johnson *et al.*, 1988). These techniques provide useful ways of investigating respiratory complexes at the cellular level.

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References

ALTMAN, F. P. (1980) Tissue Stabiliser Methods in Histochemistry. In *Trends in Enzyme Histochemistry and Cytochemistry*, pp. 81–101 Amsterdam: Excerpta Medica.

BALLANTYNE, B. & BRIGHT, J. E. (1979) Comparison of kinetic and end-point microdensitometry for the direct quantitative histochemical assessment of cytochrome oxidase activity. *Histochem. J.* **11** , 173–86.

BLANCO, C. E., SIECK, G. C. & EDGERTON, V. R. (1988) Quantitative histochemical determination of succinic dehydrogenase activity in skeletal muscle fibres. *Histochem. J.* **20**, 230–43.

BRODY, I. A. & ENGEL, W. K. (1964) Effects of phenazine methosulfate in histochemistry. *J. Histochem. Cytochem.* **12**, 928.

BROOKE, M. H. & ENGEL, W. K. (1966) Nitro blue tetrazolium: selective binding within striated muscle fibres. *Neurology* **16**, 799–806.

BROOKE, M. H. & KAISER, K. K. (1970) Three 'myosin adenosine triphosphatase' systems. The nature of their pH lability and sulfhydryl dependence. *J. Histochem. Cytochem.* **18**, 670–2.

BURSTONE, M. S. (1960) Histochemical demonstration of cytochrome oxidase with new amine reagents. *J. Histochem. Cytochem.* **8**, 63–70.

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

CASTLEMAN, K. R., CHUI, L. A., MARTIN, T. P. & EDGERTON, V. R. (1984) Quantitative muscle biopsy analysis. In *Computer-Assisted Image Analysis Cytology* (edited by GREENBERG, S. D.) pp. 101–16. Basel: Karger.

CONN, E. E. & STUMPF, P. F. (1976) *Outlines of Biochemistry*. 4th edn. pp. 157–94. New York: John Wiley.

COOPERSTEIN, S. J. & LAZAROW, A. (1951) A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.* **189**, 665–70.

DI MAURO, S., BONILLA, E., ZEVIANI, M., NAKAGAWA, M. & DE VIVO, D. C. (1985) Mitochondrial myopathies. *Ann. Neurol.* **17**, 521–38.

JOHNSON, M. A., KADENBACH, B., DROSTE, M., OLD, S. L. & TURNBULL, D. M. (1988) Immunocytochemical studies of cytochrome oxidase subunits in skeletal muscle of patients with partial cytochrome oxidase deficiencies. *J. Neurol. Sci.* **87**, 75–90.

JOHNSON, M. A., TURNBULL, D. M., DICK, D. J. & SHERRATT, H. S. A. (1983) A partial deficiency of cytochrome *c* oxidase in chronic progressive external ophthalmoplegia. *J. Neurol. Sci.* **60**, 31–53.

KUGLER, P. (1982) Quantitative dehydrogenase histochemistry with exogeneous electron carriers (PMS, MPMS, MB). *Histochemistry* **75**, 99–112.

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

NACHLAS, M. M., CRAWFORD, D. T., GOLDSTEIN, T. P. & SELIGMAN, A. M. (1958) The histochemical demonstration of cytochrome oxidase with a new reagent for the Nadi reaction. *J. Histochem. Cytochem.* **6**, 445–5.

NAKAE, Y. & SHONO, M. (1984) Kinetic behaviour of succinate dehydrogenase of three fibre types in skeletal muscle. I. Effects of temperature and competitive inhibitor. *Histochem. J.* **16**, 1207–18.

NOLTE, J. & PETTE, D. (1972) Microphotometric determinations of enzyme activity in single cells in cryostat sections. I. Applications 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**, 567–76.

PEARSE, A. G. E. (1972) *Histochemistry, Theoretical and Applied.* 3rd edn. pp. 880–920. London: Churchill Livingstone.

PETTE, D. (1981) Microphotometric measurement of initial reaction rates in quantitative enzyme histochemistry *in situ*. *Histochem. J.* **13**, 319–27.

REICHMANN, H. (1988) Enzyme activity measured in single muscle fibers in partial cytochrome *c* oxidase deficiency. *Neurology* **38**, 241–9.

REICHMANN, H. & PETTE, D. (1982) A comparative microphotometric study of succinate dehydrogenase activity levels in Type I, IIA and IIB fibres of mammalian and human muscles. *Histochemistry* **74**, 27–41.

SELIGMAN, A. M., KARNOVSKY, M. J., WASSERKRUG, H. L. & HANKER, J. S. (1968) Non-droplet ultrastructural demonstration of cytochrome oxidase activity with a polymerising osmiophilic reagent, diaminobenzidine (DAB). *J. Cell Biol.* **38**, 1–14.

SHERRATT, H. S. A., WHATMOUGH, N. J., JOHNSON, M. A. & TURNBULL, D. M. (1988) Methods for study of normal and abnormal skeletal muscle mitochondria. *Methods Biochem. Anal.* **33**, 243–336.

SMITH, L. (1955) Spectrophotometric assay of cytochrome *c* oxidase. *Methods Biochem. Anal.* **2**, 427–34.

STOWARD, P. (1980) Criteria for the validation of quantitative histochemical enzyme techniques. In *Trends in Enzyme Histochemistry and Cytochemistry* (edited by EVERED, D. & O'CONNOR, M.) pp. 11–31. Amsterdam: Excerpta Medica.

VAN NOORDEN, C. J. F., KOOIJ, A., VOGELS, I. M. C. & FREDERIKS, W. M. (1985) On the nature of the 'nothing dehydrogenase' reaction. *Histochem. J.* **17**, 1111–18.

VAN NOORDEN, C. J. F. & TAS, J. (1982) The role of exogenous electron carriers in NAD(P)-dependent dehydrogenase cytochemistry studied *in vitro* and with a model system of polyacrylamide films. *J. Histochem. Cytochem.* **30**, 12–20.