Histochemistry 38, 71—83 (1974)

Simplified Control Experiments in the Histochemical Study of Coenzyme-linked Dehydrogenases

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Received November 7, 1973

Summary. Different experiments concerning some of the most critical steps in the histochemical procedure for coenzyme-linked dehydrogenases (fixation procedure, cryoprotection, osmolar protection, substantivity of formazans, "nothing dehydrogenase" reaction, diffusion of enzyme, rediffusion of reduced coenzyme and/or reduced phenazine methosulphate) were carried out in order to improve or introduce simplified control methods by which the in situ localization of enzyme activity can be achieved without the need of expensive equipments.

As a test-object glucose-6-phosphate dehydrogenase was used.

Brief (5 min) prefixation of tissue (liver) at 0–4° C with 1% buffered (pH = 7.2) methanol-free formaldehyde (from paraformaldehyde) gave excellent preservation of morphology during the procedures of freezing, cutting and incubation and caused no inhibition of G6PDH. With the named fixation no improvement was obtained by the simultaneous use of cryoprotection (DMSO) or osmolar protection (sucrose). Finally, the fixation caused an enhancement of Nitro BT penetration into the tissue as well as of formazan substantivity. On the other hand, the brief fixation did not abolish the diffusion of enzyme (proved by different methods) and of reduced coenzyme or reduced phenazine methosulphate.

In a conventional aqueous incubation medium as well as in a gel incubation medium (PVA, grade Bo5/140) the rate of diffusion of reduced coenzyme and/or reduced phenazine methosulphate was investigated by using a special double-section incubation method. The concentrations of Nitro BT, NADP and PMS were balanced against each other and it was concluded that by using a gel medium containing 0.5 mg/ml Nitro BT, 0.1 mg/ml NADP and 0.003 mg/ml PMS, the in situ localization of G6PDH activity could be achieved at the cellular level with an incubation time not exceeding 10 min. With the incubation time mentioned, the "nothing dehydrogenase" reaction was out of the question. The sensibility of the double-section incubation method is discussed and provided that the dehydrogenase in question contains sulphydryl groups in the active enzyme centre, the method seems to exhibit a sufficient high level of sensitivity in the control of the diffusion of the different components operative in the histochemical dehydrogenase procedure.

The recording of the incubation time needed for the appearance of the two formazans (red and blue) is recommended in order to follow the enzymatic reaction rate and the effect of different procedures (fixation, solvents added *etc.*) as well as the rates of "nothing dehydrogenase" reaction, diffusion of reduced coenzyme and/or reduced PMS.

Introduction

In recent years much attention has been paid to the different problems concerned with the histochemical evaluation of coenzyme-linked dehydrogenase activity (Altman, 1972; Pearse, 1972). Although the dehydrogenase histochemistry has reached a high level of sophistication, various problems still exist and have to be settled before the fulfillment of the following two demands has been obtained: 1) the preservation of the quantities of enzymes in a native state inside the tissues and 2) the localization of the enzyme activity maintained in situ.

To meet these requirements one has to accept the necessity of investigating all the different steps in the histochemical procedure not only for each single enzyme but also when using different cells and organs (Andersen and Høyer, 1973).

Since no general method can be ruled out, the aim of the present study has been to reevaluate some of the most critical steps in the histochemical procedure for coenzyme-linked dehydrogenase systems [fixation procedures, cryoprotection, osmolar protection, substantivity of formazans, "nothing dehydrogenase" reaction, diffusion of enzyme, rediffusion of reduced coenzyme, diffusion of reduced phenazine methosulphate (PMS)] and further to improve or to introduce some simplified control experiments, all of which must be regarded as unavoidable prerequisites in the histochemical practice.

As a test object we have used the activity of glucose-6-phosphate dehydrogenase (G6PDH) since this enzyme for long has been proved to be highly diffusible and furthermore is very sensitive to fixation procedures.

Material and Methods

The material comprises 10 (one month old) white female mice (strain CII) and 15 (one month old) white female rats (Wistar strain) kept in standard conditions and avoiding extreme variations in water and salt balance. The animals were sacrified by cervical dislocation or decapitation. The liver was removed quickly and cut into conveniently thin blocks (1–2 mm). The liver specimens were obtained from the lower half of the left lobe.

Initial Cooling and Sectioning. Unfixed as well as fixed blocks were initially cooled in a CO_2-expansion cooler with covering device (Pearse-Slee) and then transferred to the cryostat chamber. In instances, where several blocks had to be used in an experiment, a drop of ice-cold 0.9% NaCl was frozen in the expansion cooler to the block surface to avoid drying during storage.

The specimens were sectioned at −25° C in 4 micron sections in a Pearse-Slee cryostat (type HR). The sections were picked up on slides on which a ring-formed (diameter = 17 mm) groove was engraved by aid of an aqueous HF-solution. In this way the amount of the incubation medium was standardized (corresponds to 5 drops from a Pasteur pipette). To minimize variation in section thickness when different sections had to be compared after incubation two succeeding sections were always placed within the ring.

In order to avoid drying of the sections (possible inactivation of enzyme) incubation was performed instantaneously.

Fixation. The preservation of morphological integrity during initial cooling, sectioning and incubation is an important prerequisite in order to obtain in situ localization of enzyme activities. Accordingly, brief (5 min) fixation of the tissue blocks at 0–4° C was performed. Comparison between sections from unfixed and prefixed tissues were then carried out with reference to morphology and enzyme activities.

Different fixatives were used in accordance with a previous study (Andersen and Høyer, 1973), with variance in concentration and "vehicles" added. Formaldehyde solutions were made from paraformaldehyde as well as from methanol-containing commercial 40% formalin.

After all fixation procedures the specimens were briefly rinsed in cooled 0.9% NaCl.

Cryoprotection. This was carried out in accordance with the previous study (Andersen and Høyer, 1973). The cryoprotective agens dimethyl sulfoxide (DMSO) was partly applied as a constituent of the fixatives, and partly used solitarily in precooling experiments.

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1 List of manufactures: NAD(P) (Sigma); NAD(P)H (Sigma); Nitro BT (Sigma); PMS (Sigma); glucose-6-phosphate (dipotassium salt) (Sigma); DMSO (Schuchardt); PVA (grade Bo5/140) (Wacker).

Osmolar Protection. In an attempt to preserve cellular isoosmolarity (Pease, 1964; Sjøstrand, 1967; Hajós and Kerpel-Fronius, 1970) 0.2 mol sucrose was included in some of the fixatives (Andersen and Høyer, 1973). The introduction of this disaccharide could be a possible source of error (*e.g.* enhancement of "nothing dehydrogenase" reaction, or as showed by Lisý *et al.* (1971) inhibition of some enzyme activity). Therefore the following control experiments were carried out: 1) One section prefixed in a sucrose containing fixative was incubated in a medium containing Nitro BT, NAD/NADP and buffer (all in the same concentration as used in the standard methods for dehydrogenase activity mentioned below), while other sections were incubated in the medium with the coenzyme omitted. 2) A fresh frozen section was incubated in the medium mentioned above and with 0.2 mol sucrose added as a kind of "substrate". Furthermore, a possible inhibitory effect was investigated by comparing the enzyme activity in a) fresh frozen sections, b) sections from tissue prefixed in 1% buffered methanol-free formaldehyde (prepared from paraformaldehyde) and c) sections from tissue prefixed in 1% buffered methanol-free formaldehyde containing 0.2 mol sucrose.

Penetration of Tetrazolium and Substantivity of Formazans. Sections from unfixed and prefixed tissue (1% buffered neutral formaldehyde) as well as from tissue pretreated with 10% DMSO (5 min at 0–4° C) were incubated for 15 min in Nitro BT (0.25 mg/ml) in 0.2 M phosphate buffer (pH = 7.2). Excess of the medium was decanted, and the tetrazolium salt was converted to formazan by the reducing agent sodium dithionite (10%) for 10 min. After a brief rinse in distilled water and embedding in glycerol-gelatine the sections were compared.

After the brief rinse in distilled water another series of sections were placed for 10 min in acetone, concentrated DMSO, 10% DMSO and 1% buffered methanol-free formaldehyde respectively. Following embedding the extend of formazan extraction was noticed in the sections by comparing with sections only rinsed briefly in distilled water.

Incubation and Incubation Media. The incubation was carried out in a thermo-regulated water bath incubator to avoid changes in the incubation medium due to evaporation. Incubation temperature was 37° C. Except for the media containing PMS, the incubation time needed for the initial, visible (as observed in the microscope) activity was recorded for both the red and blue formazans (Andersen *et al.*, 1970; Høyer and Andersen, 1970; Andersen and Høyer, 1973).

When PMS was included, the incubation was performed in red light. The incubation was interrupted after different intervals (*cf.* below).

The pH-values in the incubation media were tested on a pH-meter with a micro electrode unit (radiometer).

A. NAD(P)H-tetrazolium Reductase

1. Incubation Medium. The method of Thomas and Pearse (1961) was used as a standard procedure (but MTT was replaced by Nitro BT and 0.2 M phosphate buffer was used). In addition the concentration of Nitro BT was varied from 0.12 mg/ml to 2.0 mg/ml total incubation medium. Final incubation time: 10 min.

2. Effect of SH-blocking Agent. Some sections were pretreated with N-ethylmaleimide (0.1 M in 0.2 M phosphate buffer at pH = 7.2) for 5 min, while other sections were pretreated for 5 min in the buffer solution only. After incubation in the ordinary incubation medium the sections were compared with non-pretreated sections.

B. Glucose-6-phosphate Dehydrogenase (E.C.1.1.1.49)

1. Incubation Media. As a conventional aqueous medium the method of Thomas and Pearse (1961) was used [but MTT was replaced by Nitro BT and 0.2 M phosphate buffer was used. Further, the concentration of NADP was reduced to 1 mg/ml in order to minimize the "nothing dehydrogenase" reaction (Andersen, 1965)].

In addition, a gel medium containing polyvinyl alcohol (PVA) was used: 1.6 mg/ml (10 mM) glucose-6-phosphate (dipotassium salt) (Sigma), 0.5 ml/ml of 0.05 M phosphate buffer (pH = 7.2) containing 22% w/v PVA (grade B05/140) (Altman, 1971); NADP (1 mg/ml), Nitro BT (to 1 ml total incubation medium was added 0.25 mg Nitro BT dissolved in 0.1 ml 0.2 M phosphate buffer, pH = 7.2); phenazine methosulphate (PMS) (0.003 mg/ml); cyanide (0.66 mg/ml).

Final incubation time: 2, 5, 10, 15, 20, 25 and 30 min.

2. "Nothing Dehydrogenase" Reaction. This was tested by the conventional aqueous incubation medium omitting the substrate and/or coenzyme.

3. Effect of SH-blocking Agent. Sections were incubated in the ordinary medium in which N-ethyl maleimide (0.1 M in 0.2 M phosphate buffer at pH = 7.2) was included. Incubation time: 15 min.

4. Diffusion of Enzyme. Unfixed sections as well as sections from prefixed tissues were preincubated in 0.2 M phosphate buffer (pH = 7.2) for $^1/_2$, 1, 2, 5 and 10 min and then incubated in the conventional aqueous medium.

Furthermore, several sections placed on cover slides were incubated in a Coplin jar with the aqueous medium omitting NADP. After incubation for 10 min the incubation medium was transferred to a N-ethyl maleimide blocked section and NADP (1 mg/ml) was then added.

5. Rediffusion of Reduced Coenzyme (NADPH). In this experiment two sections were placed on the same slide. One of them was cut from tissue prefixed for 5 min in 1% buffered formaldehyde (from paraformaldehyde) (pH = 7.2) and was preliminarily maleimide-blocked in accordance with the method of Høyer and Andersen (1970). The other was cut from corresponding unfixed tissue and then partly superimposed the maleimide-blocked section. Both sections were incubated in the conventional aqueous incubation medium as well as in the standard gel medium mentioned above in order to minimize enzyme diffusion.

Further, the non maleimide-blocked section was cut from tissue prefixed in 1% buffered methanol-free formaldehyde in one series.

In another, the maleimide-blocked control section was cut from fresh frozen tissue.

Incubation omitting cyanide and/or PMS was performed.

The concentration of Nitro BT was varied from 0.12 to 0.5 mg/ml.

Furthermore, the concentration of NADP was varied from 0.05 to 1 mg/ml.

Finally, omitting NADP or using 0.10 mg/ml of NADP the concentration of PMS was varied from 0.003 mg/ml to 0.1 mg/ml.

Postincubation Techniques. Preliminary to mounting in glycerol-gelatine, the sections were postfixed in 1% buffered formaldehyde (from paraformaldehyde) (pH = 7.2) for 15 min (Andersen and Høyer, 1973).

Results

Influence of Type and Concentration of Fixative. In agreement with the recent paper (Andersen and Høyer, 1973) the best preservation of morphological integrity was obtained following the brief prefixation of the tissue blocks with 1% methanol-free buffered (pH = 7.2) formaldehyde (from paraformaldehyde) or 1% buffered neutral formaldehyde (commercial formalin) containing 10% DMSO and 0.2 M sucrose. In this respect, the two fixatives were superior to fresh frozen tissue.

The activities of NAD(P)H-tetrazolium reductases were not affected by the brief prefixation with the named two fixatives.

All the fixation procedures except 1% buffered methanol-free formaldehyde were followed by a decrease in activity of G6PDH. Especially, glutaraldehyde even at low concentrations caused a pronounced decrease in activity.

Finally, the incubation time needed for the initial appearance of formazans was clearly lowered when sections from tissue prefixed in 1% buffered methanol-free formaldehyde were compared with fresh frozen section.

Influence of DMSO. The influence of DMSO on morphological integrity was in accordance with the previous study (Andersen and Høyer, 1973).

In regard to the activity of G6PDH the addition of DMSO to the fixatives presumably had no inhibitory effect.

Influence of Sucrose. From a morphological point of view the addition of 0.2 M sucrose to 1% buffered neutral formaldehyde gave excellent result. On the other hand, no improvement was observed when 0.2 M sucrose was added to 1% buffered methanol-free formaldehyde (from paraformaldehyde) (pH = 7.2).

When sections from tissue prefixed in a sucrose-containing fixative were incubated in a standard medium for NAD(P)-linked dehydrogenases (omitting the substrate proper), a formazan deposition was noticed within 2 min for the red formazan and 5 min for the blue one using NAD, and 4 min and 9 min respectively when using NADP. Incubation in an identical medium, but now further omitting the coenzyme, showed no formazan depositions within 30 min.

When sections from fresh frozen tissues as well as from prefixed tissues were incubated in the standard medium mentioned above, but with 0.2 M sucrose added to the incubation medium, red formazan depositions were noticed within 2 min and blue depositions within 5 min using NAD, respectively 4 and 9 min using NADP.

The incubation time needed for the initial visual formazan productions in the above mentioned test series was identical with the incubation time needed for the visualization of the "nothing dehydrogenase" reaction (cf. below).

No inhibitory effect of sucrose (with the concentration used) on G6PDH could be detected.

Penetration of Tetrazolium and Substantivity of Formazans. The experiments with formazan production initiated by sodium dithionite revealed: In contrast to fresh frozen sections both the sections from prefixed as well as those from DMSO-pretreated tissues showed a stronger formazan deposition. Such depositions were also noticed in the nuclei, although extremely faint compared to the cytoplasmic ones. Post-treatment of the sections with acetone and DMSO (concentrated) caused a nearly equal and pronounced extraction of formazans, while 10% DMSO only caused a slight extraction. In all instances the most pronounced effect was noticed in fresh frozen sections compared with prefixed sections. No extraction at all was noticed using 1% buffered methanol-free formaldehyde (pH = 7.2).

A. NAD(P)H-tetrazolium Reductase. Variation in concentration of Nitro BT (from 0.12 to 2.0 mg/ml) presumably did not influence the reaction rate. However, at concentrations exceeding 1.0 mg/ml formazan production was observed in the incubation medium proper. This applies particularly to the NADPH-containing medium.

The pretreatment of sections in buffered N-ethyl maleimide did not affect the activity of the tetrazolium reductases. Nor was loss of activity observed following preincubation for 5 min in 0.2 M phosphate buffer (pH = 7.2).

B. Glucose-6-phosphate Dehydrogenase. Incubation of sections in the conventional aqueous medium showed an activity pattern in accordance with the distribution of the NADPH-tetrazolium reductase.

"Nothing dehydrogenase" activity was noticed as a red formazan production within 4 min of incubation and as a blue one within 9 min. The "nothing dehydrogenase" activity was abolished by blocking with N-ethylmaleimide.

No activity was seen when omitting the substrate and coenzyme from the incubation medium.

Sensitivity of G6PDH to an SH-blocking agent was demonstrated by a pronounced decrease in activity (no formazan deposition after 30 min) following incubation in standard medium with N-ethyl maleimide included.

Preincubation in 0.2 M phosphate buffer (pH = 7.2) of sections from fresh frozen as well as briefly prefixed tissues showed that within $^1/_2$ min. G6PDH had diffused into the buffer solution to a considerable extent. This was indicated by a pronounced prolongation of the incubation time needed for initial visual formazan production, when the sections subsequently were incubated in the conventional aqueous medium.

The diffusibility of the enzyme was further indicated by diffuse cytoplasmic blue depositions established within 5 min in a maleimide-blocked liver section. This section had been incubated in a medium which: 1) preliminarily had been used for incubation of several liver sections omitting NADP, 2) subsequently was transferred to the maleimide-blocked section and 3) finally received an admixture of NADP.

By using the double section-method and the conventional aqueous incubation medium a diffuse activity pattern was observed in the liver cells of the maleimide-blocked section within 2 min of incubation.

When the concentration of NADP in the standard gel medium was varied, formazan depositions were established in the maleimide-blocked section 1) at 1.0 mg/ml after 2 min of incubation in a distance corresponding to 10 cell diameters from the non maleimide-blocked section, 2) at 0.1 mg/ml after 5 min of incubation in a distance of one cell diameter and 3) at 0.05 mg/ml after 30 min of incubation in a distance of one cell diameter. However, at the same time the formazan production in the non maleimide-blocked section was clearly reduced as compared with the non maleimide-blocked sections using higher concentrations of NADP.

In a corresponding series, omitting PMS, formazan deposition was not seen in the maleimide-blocked section until 30 min of incubation regardless of the NADP concentration. Further, no unspecific formazan depositions (*e.g.* in the cell nuclei) was observed.

Prefixation did not reduce the rediffusion of NADPH from the non maleimide-blocked section. As regards the maleimide-blocked section an increase of formazan precipitation was noticed using a section cut from prefixed tissues as compared with fresh frozen tissues.

An increase in the concentration of Nitro BT to 0.5 mg/ml (using 1.0 mg/ml NADP) was followed by an enhancement of formazan deposition within 2 min in the non maleimide-blocked section. On the other hand, the formazan depositions in the maleimide-blocked section were lowered, indicated by a reduction in the distance to two cell diameters from the non maleimide-blocked section.

The addition of cyanide caused an increase of formazan precipitation in both the sections.

Variation in concentration of PMS revealed: 1) Without NADP in the medium and using 0.003 mg/ml PMS no formazan production could be detected in the maleimide-blocked section within 10 min of incubation. However, using 0.1 mg/ml PMS a very faint red formazan production could be detected in the maleimide-blocked section in a distance corresponding to 2–3 cell diameters from the non

maleimide-blocked section. The non maleimide-blocked sections showed a very faint diffuse red formazan production which increased using the high PMS concentration. 2) With 0.1 mg/ml NADP and using 0.003 mg/ml PMS a very faint red formazan production could be seen in the maleimide-blocked section in a distance corresponding to 2–3 cell diameters from the non maleimide-blocked section within 5 min of incubation. With the high concentration of PMS the distance was 4–5 cell diameters. After 10 min of incubation the distance was 20–30 cell diameters using the high concentration of PMS. In both the sections the red and blue formazan production was extensively enhanced by increasing the PMS concentration and unspecific depositions were seen in the nuclei.

Discussion

In a recent paper (Andersen and Høyer, 1973) the different aspects of fixation in the histochemical dehydrogenase practice have been discussed. It was concluded that the fixation procedure may act as a considerable improvement of the histochemical method provided that the fixation has no influence on the activity of the enzyme system to be studied. From the present study as well as from the previous one it is evident that the brief tissue block fixation in 1% methanol-free formaldehyde (from paraformaldehyde) buffered to pH = 7.2 served well in the maintenance of morphological integrity and further enhanced the penetration of the incubation medium into the sections and finally enhanced the formazan substantivity. The named fixation obviously did not influence the activity of succinate dehydrogenase activity (Andersen and Høyer, 1973), and in the present study no inhibition of G6PDH activity as well as of NAD(P)H-tetrazolium reductase activity was noticed.

On the other hand, the brief fixation does not seem to prevent diffusion of a "soluble" enzyme as G6PDH or diffusion of reduced intermediates (coenzymes).

The problems concerned with the use of DMSO in the histochemical practice have been discussed elsewhere (Andersen and Høyer, 1973). Only a single comment has to be added: In the present study no inhibitory effect on the G6PDH activity was noticed.

Although sucrose has been widely used in osmolar protection of cells or isolated cell particulates in the field of histochemistry, biochemical analysis of enzyme activities as well as in the field of electron microscopy, little is known about the effect sucrose might have on enzyme activities. Lisý *et al.* (1971) observed that succinate dehydrogenase and lactate dehydrogenase seriously could be inhibited if the sucrose was not washed out of the tissue before investigating the enzyme activity. In the present study, the applied concentration of sucrose in the fixative obviously does not seem to influence the activity of G6PDH. Nor was any enhancement of "nothing dehydrogenase" activity noticed. On the other hand, sucrose caused no improvement in preservation of morphology when used in combination with 1% methanol-free formaldehyde buffered to pH = 7.2.

As strongly stated in two recent papers (Høyer and Andersen, 1970; Andersen and Høyer, 1973) it must be recommended to record the localization of both the red and blue formazan, which occur in the reduction of the ditetrazolium

salt Nitro BT. The two formazans have been proven to represent reaction products in the enzymatic reduction of Nitro BT (Eadie *et al.*, 1970; Gabler *et al.*, 1970) and accordingly will reflect sites with different enzyme concentration. Furthermore, recording of the incubation time needed for the appearance of both the red and blue formazan allows the reaction rate to be followed during the incubation which, as showed in the present study as well as in the previous ones (Høyer and Andersen, 1970; Andersen and Høyer, 1973), is an important prerequisite in the investigation of the effect of fixation, diffusion of enzyme or reduced intermediates (coenzyme, PMS), "nothing dehydrogenase" activity as well as influence of components added (*e.g.* coenzyme Q, solvents like DMSO, dimethyl formamide etc.). These effects are not always reflected by the use of a standard incubation time.

Although the conception "nothing dehydrogenase" reaction has been accepted as a serious source of error in the histochemical practice (Zimmerman and Pearse, 1959; Hashimoto and Ogawa, 1961; Schreiber and Simon, 1963; Hashimoto *et al.*, 1964; Andersen, 1965; Shaw and Koen, 1965; Stiller and Hempel, 1970; Wenk, 1970; Pearse, 1972), many authors still make no reference to it. The mechanism underlying the reaction needs further studies, but so far it has been ascribed to sulphydryl groups, glutathion-cytochrome-c-reductase and diffusible alcohol dehydrogenase. Its dependance on pH as well as on the concentration of NAD(P) has been well proven. Furthermore, the reaction is abolished by N-ethyl-maleimide, which unfortunately is also the case for several dehydrogenases. According to Stiller and Hempel (1970) incubation in gel-media has no influence on the reaction, while Wenk (1970) in some tissues noticed a decrease. As showed in the present study the reaction was noticed as a red formazan production after 4–5 min of incubation and as a blue one after 9–10 min using 1 mg/ml NADP.

From the aforementioned it must be concluded that the "nothing dehydrogenase" reaction always has to be taken into consideration, since it may give rise to a considerable part of the formazan production noticed. Further, it has to be investigated for each type of tissue since a general conclusion cannot be drawn. By lowering the NAD(P) concentration and by keeping the pH-value as close to the alkaline side of 7.0 as possible, and by lowering the incubation time the reaction can be avoided or held to a minimum—even in the liver.

An outstanding problem in dehydrogenase histochemistry is diffusion. This may be related to diffusion of enzyme, diffusion of reduced coenzyme and diffusion of reduced PMS. Meanwhile, nearly all attention has been paid to the problem of enzyme diffusion when it was realized that during incubation of fresh frozen sections in aqueous media, parts of the sections may go into solution and give rise to loss of nitrogenous material (Altman and Chayen, 1965; Butcher, 1971; Altman, 1972). The major approach to solve the problem has been the incorporation of inert colloid stabilising polymers into the incubation medium—especially by using polyvinyl alcohol (PVA) as a 20% solution (Altman and Chayen, 1965). Afterwards, the PVA technique has been widely used (Kunze, 1967; Jacobsen, 1969; Dahl and Mellgren, 1970; Wenk, 1970; Wenk *et al.*, 1970a,b; Ritter *et al.*, 1970; Hecker, 1972; Altman, 1972; Chayen *et al.*, 1973). Several drawbacks have been noticed (*e.g.* artifacts resulting from poor mixing of the components of the medium; reduced reaction velocity; reducing capacity of PVA).

Some of these drawbacks have been overcomed by the introduction of a new grade of PVA (Bo5/140) (Altman, 1971) as a 22% w/v solution. Incontestably, the use of PVA means a considerable improvement of the dehydrogenase technique and must be recommended when dealing with a "soluble" enzyme, which can be lost from the section within minutes, when the section is incubated in an ordinary aqueous medium (Kalina and Gahan, 1965; Altman, 1971). This is in agreement with the present study in which the G6PDH had diffused extensively from the sections within 30 sec rendering the in situ localization out of the question due to the formation of reduced coenzyme in the incubation medium.

Contrary, the diffusion of reduced coenzyme and/or reduced PMS has attracted minor attention. In a recent paper (Wenk *et al.*, 1970) it was stated that diffusion of reduced coenzyme and possibly reduced PMS was not avoided by the use of PVA gel-media, but could be reduced to very low values if the coenzyme and PMS concentration were kept to the minimum required for "optimal" activity. Similar problems were observed by McMillan (1966) in a study using a semipermeable membrane to minimize enzyme diffusion. In this paper it was stated that the diffusion of reduced NAD and PMS was minimized in two ways: a) the concentration of Nitro BT was made as high as practical (3.3×10^{-3} M) and b) the rate of the reaction was controlled by choosing a substrate (lactate) concentration (2.0×10^{-2} M) that would consistently give a sharp staining pattern.

The high concentrations of coenzymes (varying from 0.5 to 3 mg/ml incubation medium) used by the majority of authors add considerably to the diffusion problem. Although there are many reasons why high coenzyme levels should be maintained in histochemical media (for discussion see Pearse, 1972), the study made by Wenk *et al.* (1970), as well as the present one show that such a statement has to be reevaluated. In the present study it was evident that high coenzyme level (1 mg/ml) in the presence of PMS (0.003 mg/ml) gave rise to diffusion artifact indicated by distinct formazan deposition in the maleimide-blocked section within 2 min of incubation using the double-section incubation method. Furthermore, the high coenzyme level favours the "nothing dehydrogenase" reaction as mentioned above. Finally, it was noticed that high levels of reduced coenzyme—especially NADPH—caused an unspecific formazan production in the incubation medium if the concentration of Nitro BT exceeded 1.0 mg/ml incubation medium. The last observation is in agreement with the studies made by Sims *et al.* (1971), and Altman (1972) which showed that Nitro BT could be reduced non-enzymatically by NADH and NADPH. These observations mean that in the detection of the NAD(P)H-tetrazolium reductase activity not all of the formazan produced necessarily is formed via the mediation of the tetrazolium reductase (diaphorase) system in the tissue.

Although the present study revealed that in gel-media (omitting PMS) no formazan depositions due to diffusion of reduced NADP were noticed in the maleimide-blocked section (double section incubation technique) even within 30 min and at high (1 mg/ml) level of NADP, this does not mean that such a level can be used without caution. In the maleimide-blocked section not only the G6PDH activity will be abolished, but so will the "nothing dehydrogenase" activity which within 10 min of incubation may give rise to formazan depositions in the non-maleimide-blocked section. On the other hand, the results indicated that the level of reduced NADP which may have diffused from the non-maleimide-

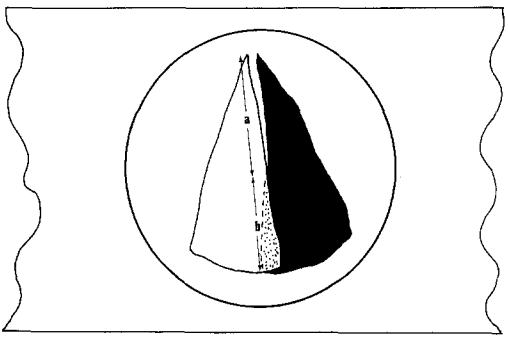

Fig. 1 Diagrammatic representation of the double-section incubation method. The white section represents the "active" section, the black one represents the N-ethylmaleimide blocked section. (a) Zone of separation for the detection of diffusion processes: section → gel-medium → section. (b) Superimposed zone for detection of diffusion: section → gel-medium → section as well as of section → section

blocked section, is of an order too small to act as a substrate for the NADPH-tetrazolium reductase (diaphorase) in the maleimide-blocked section.

In order to by-pass the endogenous NAD(P)H-tetrazolium reductase (diaphorase), phenazine methosulphate (PMS) has been widely used in dehydrogenase histochemistry as an electron transfer intermediate. Meanwhile, its use is encumbered with several drawbacks (for discussion: see Altman, 1972; Pearse, 1972; Wenk *et al.*, 1970). Furthermore, PMS is highly sensitive to light, which was clearly demonstrated in a recent spectrophotometrical study (Marzotko *et al.*, 1973). Within 2 min of exposure its instability in sunlight and ordinary artificial light was noticed, whereas no changes were seen after exposure to darkness or red light. Accordingly, its application in dehydrogenase histochemistry has to be restricted to darkness or red light.

Finally, the application of PMS adds further to the problems of diffusion as mentioned above. With the double-section gel incubation method used in the present study it was evident that the simultaneous application of a low NADP concentration (0.1 mg/ml) and a high level of PMS (0.1 mg/ml) caused a pronounced formazan deposition in the maleimide-blocked section within 10 min of incubation. It is most likely that the deposition was due to diffusion of reduced PMS since the diffusion distance was greatly reduced by an increase in concentration of Nitro BT (from 0.25 mg/ml to 0.5 mg/ml). A similar effect was noticed using a low level of PMS (0.003 mg/ml).

From the aforementioned it seems safely to conclude that by the use of all the named control experiments and especially by using the double-section gel incubation technique, in which the concentrations of coenzyme, PMS and Nitro BT are balanced against each other, a high level of accuracy in the in situ localization of enzyme activity can be achieved at least at the cellular level. For the G6PDH activity this was obtained by using 0.1 mg/ml NADP, 0.003 mg/ml PMS and 0.5 mg/ml Nitro BT and by using an incubation time not exceeding 10 min. Presumably, the double-section incubation method exhibits a sufficient high level of sensitivity to detect the diffusion of the different components operative in the histochemical dehydrogenase procedure (Figs. 1 and 2) provided that the dehydrogenase in question contains sulphydryl groups in the active enzyme centre.

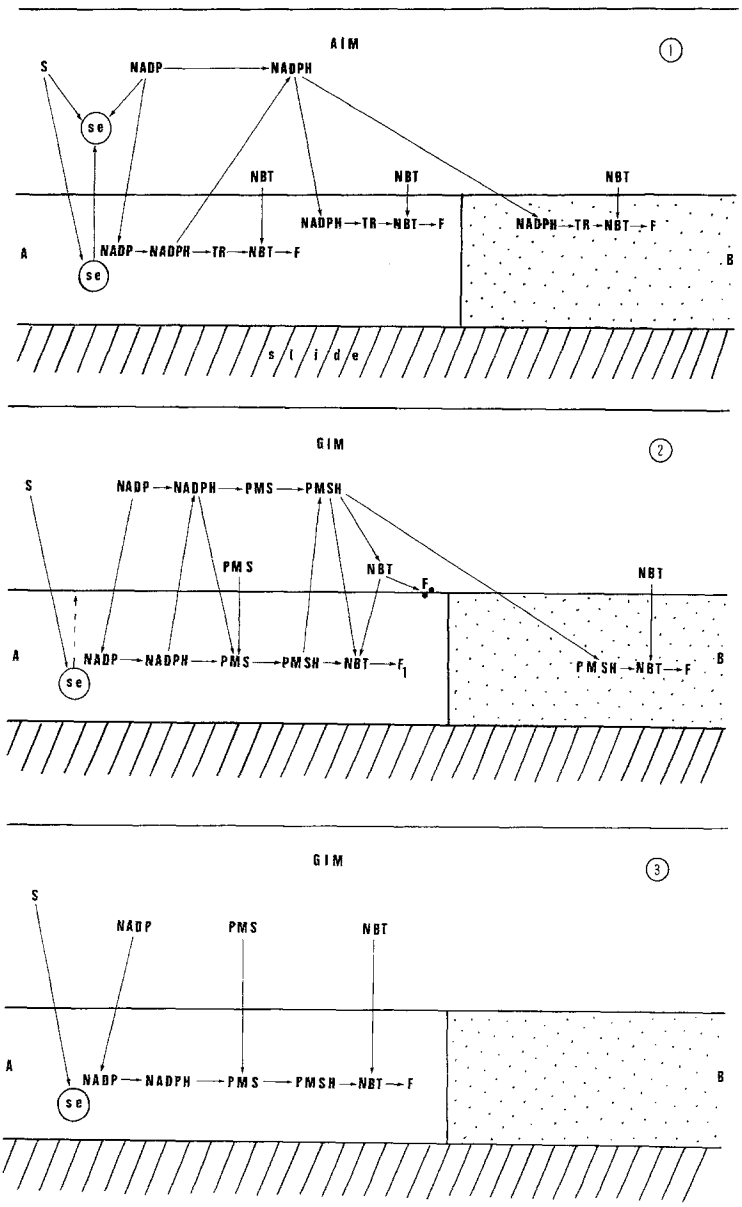

Fig. 2 Diagrams representing diffusion of the different components operative during the histochemical procedure. Arrows represent partly the diffusion and partly the "hydrogen" (hydride ion) transport. Diffusion of components from section to section not included the diagrams. (A) "Active" section, (B) N-ethylmaleimide blocked section. *Diagram 1* illustrates the incubation in an aqueous incubation medium (AIM). *Diagram 2* illustrates the incubation in a gel-incubation medium (GIM) and represents the conditions with high and low concentrations of coenzyme and phenazine methosulphate respectively. *Diagram 3* represents incubation in a gel-medium in which the concentration of Nitro BT, coenzyme as well as of phenazine methosulphate is balanced against each other in order to obtain the in situ localization of dehydrogenase activity. *Abbreviations:* F formazan; F_1 formazan representing the in situ dehydrogenase activity as well as formazan production due to diffusion of reduced coenzyme or reduced phenazine methosulphate; *NADP* nicotinamide adenine dinucleotide phosphate (oxidised form); *NADPH* same in reduced form; *NBT* Nitro BT; *PMS* phenazine methosulphate; *PMSH* same in reduced form; *S* substrate; *se* "soluble" enzyme; *TR* tetrazolium reductase

High levels of Nitro BT should be used with caution, since a rise in concentration from 1 to 2 mg/ml only gave a minimal increase of the reaction rate of NAD(P)H-tetrazolium reductase indicated by a negligible reduction of the incubation time needed for the appearance of the two formazans. This may be due to the toxic effect of the tetrazolium salt.

For the liver in which both the fatty acid biosynthesis as well as hydroxylations (cytochrome P_{450} dependent reactions) are operative, it is important to control all diffusion problems if one wants to follow the rate of NADPH entering the "pathway I" or "pathway II" as described by Altman (1972).

Finally, it must be recommended to carry out all the control experiments not only for each coenzyme-linked dehydrogenase, but also for each type of cell or tissue. The control experiments used in this paper are all easy to perform and no expensive equipments are needed.

References

Altman, F. P.: The use of a new grade of polyvinyl alcohol for stabilising tissue sections during histochemical incubations. Histochemie **28**, 236–242 (1971)

Altman, F. P.: Quantitative dehydrogenase histochemistry with special reference to the pentose shunt dehydrogenases. Progr. Histochem. Cytochem. **4**, 225–273 (1972)

Altman, F. P., Chayen, I.: The retention of nitrogenous material in unfixed sections during incubation for histochemical demonstration of enzymes. Nature (Lond.) **207**, 1205–1206 (1965)

Andersen, H.: Sulphydryl "nothing dehydrogenase" activity and lactic dehydrogenase-$NADH_2$ cytochrome c reductase reactions in tissues of the human foetus. Acta histochem. (Jena) **21**, 120–134 (1965)

Andersen, H., Bülow, F. A. von, Møllgård, K.: The histochemical and ultrastructural basis of the cellular function of the human foetal adenohypophysis. Progr. Histochem. Cytochem. **1**, 153–184 (1970)

Andersen, H., Høyer, P. E.: Studies in succinate dehydrogenase histochemistry. Histochemie **35**, 173–188 (1973)

Butcher, R. G.: Tissue stabilisation during histochemical reactions: The use of collagen polypeptides. Histochemie **28**, 231–235 (1971)

Chayen, I., Loveridge, N., Ubhi, G. S.: The use of menadione as an intermediate hydrogen-carrier for measuring cytoplasmic dehydrogenating enzyme activities. Histochemie **35**, 75–80 (1973)

Dahl, H. A., Mellgren, S. I.: The effect of polyvinyl alcohol and polyvinyl pyrrolidone on diffusion artifacts in lactate dehydrogenase histochemistry. Histochemie **24**, 354–370 (1970)

Eadie, M. J., Tyrer, J. H., Kukums, J. R., Hooper, W. D.: Aspects of tetrazolium salt reduction relevant to quantitative histochemistry. Histochemie **21**, 170–180 (1970)

Gabler, W. von, Wendler, D., Schmidt, W.: Chromatographische und spektrophotometrische Untersuchungen an Nitro-BT und seinen Formazanen. Acta histochem. (Jena) **36**, 295–302 (1970)

Hajós, F., Kerpel-Fronius, S.: The incubation of unfixed tissues for electron microscopic histochemistry. Histochemie **23**, 120–126 (1970)

Hashimoto, T., Kaluza, I. S., Burstone, M. S.: The effect of menadione and phenazine methosulfate on the tetrazolium reduction system under histochemical conditions. J. Histochem. Cytochem. **12**, 797–804 (1964).

Hashimoto, T., Ogawa, K.: Non-enzymatic reduction of tetrazolium salts by sulphydryl groups. Arch. histol. jap. **21**, 239–249 (1961)

Hecker, D.: Über den Einfluß der PVA-Gelinkubation auf den topochemischen Nachweis der löslichen NAD-abhängigen Lactatdehydrogenase. Acta histochem. (Jena) **42**, 324–339 (1972)

Høyer, P. E., Andersen, H.: Specificity in steroid histochemistry, with special reference to the use of steroid solvents. Distribution of 11 β-hydroxysteroiddehydrogenase in kidney and thymus from the mouse. Histochemie **24**, 292–306 (1970)

Jacobsen, N. O.: The histochemical localization of lactic dehydrogenase isoenzymes in the rat nephron by means of an improved polyvinyl alcohol method. Histochemie **20**, 250–265 (1969)

Kalina, M., Gahan, P. B.: A quantitative study of the validity of the histochemical demonstration for pyridine nucleotidelinked dehydrogenases. Histochemie **5**, 430–436 (1965)

Kunze, K. D.: Beitrag zur Histochemie der Laktatdehydrogenase. Histochemie **11**, 350–359 (1967)

Lisý, V., Kováru, H., Lodin, Z.: *In vitro* effects of polyvinylpyrrolidone and sucrose on the acetylcholinesterase, succinate dehydrogenase and lactate dehydrogenase activities in the brain. Histochemie **26**, 205–211 (1971)

Marzotko, D., Warchel, J. B., Wachowiak, R.: Spektrophotometrische Untersuchungen über die Lichtempfindlichkeit von PMS in wäßriger Lösung. Acta histochem. (Jena) **46**, 53–59 (1973)

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

Pearse, A. G. E.: Histochemistry, theoretical and applied, 3rd ed., vol. 2. London: Churchill, Livingstone 1972

Pease, D. C.: Histological techniques for electron microscopy, 2nd ed. New York: Academic Press 1964

Ritter, J., Wenk, H., Gerth, Ch.: Quantitative Untersuchungen zum Diffusionsproblem enzymatisch reduzierter Pyridin-nucleotide; ein Beitrag zum histochemischen Nachweis pyridin-nucleotid-abhängiger Dehydrogenasen. Acta histochem. (Jena) **40**, 64–72 (1971)

Schreiber, G., Simon, H.: Beitrag zur Technik des histochemischen Dehydrogenasen-Nachweises. Acta histochem. (Jena) **15**, 192–193 (1963)

Shaw, C. R., Koen, A. L.: On the identity of "nothing dehydrogenase". J. Histochem. Cytochem. **13**, 431–433 (1965)

Sims, K. L., Weitsen, H. A., Bloom, F. F.: Histochemical localization of brain succinic semialdehyde dehydrogenase-a γ-aminobutyric acid degradative enzyme. J. Histochem. Cytochem. **19**, 405–415 (1971)

Sjøstrand, F. S.: Electron microscopy of cells and tissues, vol. 1. New York: Academic Press 1967

Stiller, D., Hempel, E.: Zum Problem des „nothing-dehydrogenase"-Effektes beim topochemischen Nachweis dehydrierender Enzymsysteme. Acta histochem. (Jena) **35**, 239–252 (1970)

Thomas, E., Pearse, A. G. E.: The fine localization of dehydrogenases in the nervous system. Histochemie **2**, 266–282 (1961)

Wenk, H.: Beitrag zum histochemischen Nachweis löslicher NAD-abhängiger Dehydrogenasen mittels PVA-Gelinkubation. Acta histochem. (Jena) **35**, 380–391 (1970)

Wenk, H., Meyer, U., Ritter, J.: Beitrag zum histochemischen Nachweis pyridin-nucleotid-abhängiger Dehydrogenasen; eine halbquantitative Untersuchung. Acta histochem. (Jena) **38**, 278–292 (1970a)

Wenk, H., Ritter, J., Meyer, U.: Beitrag zum histochemischen Nachweis pyridin-nucleotid-abhängiger Dehydrogenasen; der Einfluß von Coenzym und Phenazinmethosulfat auf die histotopochemische Lokalisation. Acta histochem. (Jena) **37**, 379–396 (1970b)

Zimmermann, H., Pearse, A. G. E.: Limitations in the histochemical demonstration of pyridine nucleotide-linked dehydrogenases ("nothing-dehydrogenase"). J. Histochem. Cytochem. **7**, 271–275 (1959)

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