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CORNELIS J. F. VAN NOORDEN

Histochemistry and Cytochemistry of Glucose-6-Phosphate Dehydrogenase

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Cornelis J. F. Van Noorden

With 40 Figures and 17 Tables

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Contents

1 Introduction

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase; G6PDH; E.C. 1.1.1.49) is an ubiquitous enzyme distributed throughout the animal and plant kingdom, including bacteria, fungi and yeast (NEGI and STEPHENS 1977). The enzyme catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconо-δ-lactone, the first step of the phosphogluconate pathway (pentose phosphate cycle) with a concomitant production of NADPH (LEHNINGER 1975). G6PDH is the rate-limiting, one-way enzyme of pentose phosphate biosynthesis (WEBER 1977 a, b). It was discovered by WARBURG and CHRISTIAN in 1931 in erythrocytes and has been called ‹Zwischenferment› at first. Studies on its working mechanism lead to the identification of the coenzyme $NADP^+$ (LEHNINGER 1975; DIXON and WEBB 1979). The major function of the pentose phosphate cycle is the generation of NADPH as extramitochondrial reducing power necessary for many biosynthetic processes such as the synthesis of fatty acids and steroids (NEWSHOLME and START 1977), the reduction of glutathione (BEUTLER 1977, 1978) and hydroxylating reactions (ALTMAN 1972). The pentose sugars are used for nucleic acid and nucleotide synthesis (LEHNINGER 1975).

Since G6PDH is located in the soluble fraction of the cytoplasm, it is not surprising that up to some years ago relatively little work has been performed on the histochemical and cytochemical localization of this physiological important and widely distributed enzyme (NEGI and STEPHENS 1977; RIEDER et al. 1978). Special care has to be taken in order to prevent loss of nitrogenous material from unfixed tissue sections or cells during incubation for the histochemical demonstration of enzymes (ALTMAN and CHAYEN 1965). The loss of enzymes from the soluble fraction of cells can be considerable within minutes (KALINA and GAHAN 1965; ALTMAN and CHAYEN 1966; MEIJER and DE VRIES 1974; MEIJER 1978). For enzyme histochemical purposes, cryostat sections or cells cannot be fixed very well because destruction of enzyme activity occurs almost immediately (MEIJER and DE VRIES 1974; VAN NOORDEN and TAS 1981; VAN NOORDEN et al. 1982 b; RAAP et al. 1983). Once methods had been developed to prevent loss of enzyme activity from tissue sections and cells, histochemistry and cytochemistry of G6PDH activity made progress and so, in the past few years information about its topochemical localization became available.

Retention of enzyme activity in tissue sections during incubation can be obtained by the addition of inert macromolecules to the medium, like polyvinyl alcohol (ALTMAN and CHAYEN 1965; JACOBSEN 1969; HENDERSON et al. 1978; RIEDER et al. 1978; VAN NOORDEN et al. 1982 b), or collagen polypeptides (BUTCHER 1971 a). A semipermeable membrane interposed between the aqueous or gelled incubation medium and tissue section can be used as well (MCMILLAN 1967; MEIJER and DE VRIES 1974; WEISS et al. 1979), whereas gelled incubation mixtures without interposed membrane have been introduced by FAHIMI and AMARASINGHAM (1964), PETTE and BRANDAU (1966) and RUITENBEEK and SCHOLTE (1976) for the histochemical demonstration of enzyme ac-

tivities in the soluble fraction of cells. VAN NOORDEN et al. (1982 a) have demonstrated G6PDH activity in different kinds of individual cells preventing loss of enzyme molecules by incorporating the cells in the matrix of a thin film of transparent polyacrylamide prior to incubation.

Ultramicrochemical techniques of assay can be employed for a combined approach of localization and quantification of enzyme activities (LOWRY and PASSONNEAU 1972; GALJAARD et al. 1974; JONGKIND et al. 1974; KATZ et al. 1977; TEUTSCH and RIEDER 1979; LOWRY et al. 1980; TEUTSCH 1981). With this methodology, homogenates of very small amounts of tissue (often only a few cells dissected from tissue) are analyzed with scaled-down biochemical methods.

Another combination of localization and quantification of G6PDH activity in tissues and cells, besides ultramicrochemistry, is cytophotometric analysis of tissue sections or individual cells after staining for G6PDH activity (ALTMAN 1969, 1972; BUTCHER 1972; BUTCHER and ALTMAN 1973; VAN NOORDEN et al. 1982 b, 1983 b). Both for a proper localization and quantification of enzyme activity in tissue sections or cell specimen, preparation of the biological material and the histochemical staining reaction should be standardized and validated properly. Preferably correlations should be made between histochemical or cytochemical and biochemical assays (VAN DER PLOEG 1975).

In the present text, methods for the histochemical and cytochemical localization of G6PDH activity and their validity will be discussed. Immunocytochemical and ultramicrochemical techniques for the demonstration of G6PDH will be reviewed as well. Both qualitative and quantitative enzyme histochemical and cytochemical methods will be evaluated using STOWARD's criteria for establishing precision, reproducibility, validity and specificity of the reaction (STOWARD 1980; STOWARD and PLOEM 1982). Finally, applications of histochemistry and cytochemistry of G6PDH activity in cell biology and physiology in general and in the disciplines of genetic diseases, carcinogenesis, reproduction and toxicology in particular will be reviewed. It will be shown that the histochemistry and cytochemistry of this enzyme is a highly important tool in cell science and in the study of diseased tissue.

2 Histochemical methods

Roughly, two different methods are available for the demonstration of G6PDH activity in tissue sections. The one most commonly used is based on the reduction of a tetrazolium salt as final electron acceptor. These colourless or weakly coloured water-soluble compounds can be converted into intensely coloured water-insoluble formazans by reduction ALTMAN 1976 a; WOHLRAB et al. 1979). Ideally, NADPH produced by G6PDH activity is oxidized by a tetrazolium salt and its formazan is precipitated at the site of the enzyme (Fig. 1 A). Potassium ferricyanide is the final electron acceptor in the other method (BERCHTOLD 1979). The end product, copper ferrocyanide, is also water-insoluble and can be observed under the electron microscope.

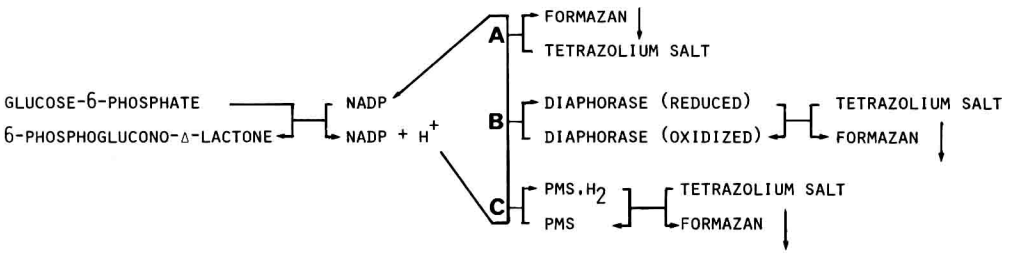


Fig. 1. Schematic representation of tetrazolium salt reduction by glucose-6-phosphate dehydrogenase activity. A, reduction without intervention of an electron transport system; B, reduction via an endogenous electron transport system (diaphorase); C, reduction via an exogenous intermediate electron carrier (PMS).

2.1 Tetrazolium salt methods

2.1.1 Exogenous electron carriers

The reduction of a tetrazolium salt by NADPH does not take place directly at all or occurs only very slowly (ALTMAN 1976 a; BUTCHER 1982 a; VAN NOORDEN and TAS 1982 a, b; BUTCHER and VAN NOORDEN 1984). The redox potentials of tetrazoles are usually too high for the salts to become directly reduced by NADPH (ALTMAN 1976 a) and therefore, either endogenous electron transport systems (Fig. 1 B) or exogenous electron carriers are required for the demonstration of NADPH production by G6PDH activity (Fig. 1 C). Theoretically, BPST is the only tetrazolium salt with a redox potential low enough to be reduced by NADPH (Fig. 1 A; ALTMAN 1976 a, b; CLARK 1980). Endogenous electron transport systems involved are most likely NADPH cytochrome c (P450) reductase (VAN NOORDEN and BUTCHER 1984) and DT-diaphorase (RAAP and VAN DUIJN 1983). Sometimes it is assumed that the mitochondrial respiration chain is involved (RIEDER et al. 1978; MEIJER 1980) but this is highly unlikely, considering that NADPH cannot permeate the membrane of these organelles (BUTCHER and CHAYEN 1968; MAHLER and CORDES 1971). For a proper localization of G6PDH activity one cannot rely on the endogenous electron transport systems because these can well be the rate-limiting factor in the reduction of the tetrazolium salt and therefore, will be demonstrated instead of the primary dehydrogenase activity (GARBARSCH et al. 1978; HEYWOOD and BLACKSHAW 1978; MEIJER 1978; BERCHTOLD 1979; VAN NOORDEN and TAS 1982 a, b).

Several exogenous electron carriers have been introduced in dehydrogenase histochemistry, like phenazine methosulphate (PMS; FARBER and BUEDING 1956; WALKER and SELIGMAN 1961), methylene blue (SINGER and KEARNEY 1954; FABER and BUEDING 1956; BUTCHER and EVANS 1984), menadione (WATTENBERG and LEONG 1960; HASHIMOTO et al. 1964; CHAYEN et al. 1973 c), meldola blue (BURD and USATEGUI-GOMEZ 1973; MÖLLERING et al. 1974; KUGLER and WROBEL 1978) and l-methoxy-

phenazine methosulphate (l-methoxyPMS; Hisada and Yagi 1977; Nakemura et al. 1980; Seidler 1980; van Noorden and Tas 1982 a, b). Menadione and methylene blue should not be used in G6PDH histochemistry because these intermediates cannot be reduced directly by NADPH (van Noorden and Tas 1982 a, b; Raap and van Duijn 1983; Butcher and Evans 1984). It seems likely that the activity demonstrated in the presence of these intermediate acceptors is that of the flavoprotein reductase rather than the dehydrogenase. Meldola blue is not very useful either, although its electron transferring capacity between NADPH and a tetrazolium salt may be comparable with PMS or l-methoxyPMS, when it is used in a not too high concentration (van Noorden and Tas 1982 a; Kugler 1982), but it stains cellular components like DNA and lipids blue. This interferes with the colour of most formazans (Henderson and Loveridge 1981; van Noorden and Tas 1982 a, b; Kugler 1982). Meldola blue also appeared to have little effect in incubation media containing polyvinyl alcohol (Henderson and Loveridge 1981; Butcher and Evans 1984). This may be due to an almost complete binding of this particular dye to the colloid stabilizer (Kugler 1982). Therefore, at the moment only PMS and l-methoxyPMS are really candidates for the role as exogenous electron carrier in G6PDH histochemistry. *In vitro* experiments showed that l-methoxyPMS is a faster carrier than PMS (Hisada and Yagi 1977; Seidler 1980; Hisada et al. 1981; Kugler 1982) and it is also much less light sensitive than PMS.

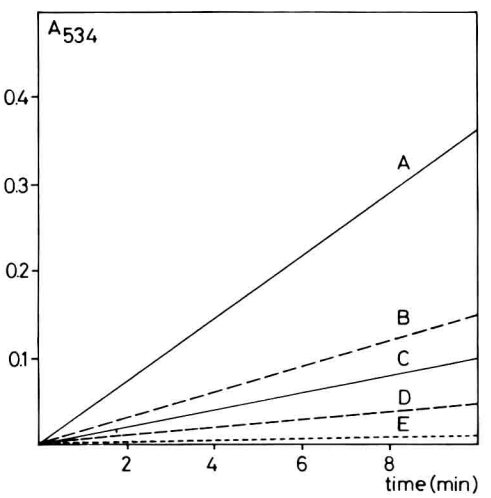


Fig. 2. *In vitro* reduction of tetranitro BT in the presence of either l-methoxyPMS or PMS demonstrated by the increase of absorbance at 534 nm (A_{534}). A, reduction in a solution of 0.5 % (w/v) bovine serum albumin (BSA) and 0.32 mM PMS; B, same as A but with l-methoxyPMS instead of PMS; C, reduction in a solution of 0.32 mM PMS; D, same as C but with l-methoxyPMS instead of PMS; E, reduction in a solution of 0.5 % BSA. Temperature 37 °C, pH 7.45.

Incubations in the presence of l-methoxyPMS give less photochemical reduction of easily reducible tetrazolium salts (Fig. 2; HISADA and YAGI 1977; HISADA et al. 1981; VAN NOORDEN and TAS 1982a; VAN NOORDEN 1983). Moreover, Fig. 2 shows as well that the so-called ‹nothing dehydrogenase› activity due to SH-groups in proteins with the example of bovine serum albumin (cf. SRI VENUGOPAL and ADIGA 1980) does occur when using either l-methoxyPMS or PMS. This kind of ‹nothing dehydrogenase› activity can be inhibited by addition of a SH-group inhibitor to the incubation medium, like N-ethylmaleimide. No SH-group-dependent formazan production can be observed in the absence of electron carriers. This formazan production is higher in the case of PMS than of l-methoxyPMS (Fig. 2). It might well be a reason for a higher formazan production in the absence of substrates and/or coenzymes when PMS is used instead of l-methoxyPMS (NAKAMURA et al. 1980; VAN NOORDEN and TAS 1982a, b). On the other hand, substitution of PMS for l-methoxyPMS in a study of VAN NOORDEN and BUTCHER (1984) did not result in any substantial difference. Nevertheless, it is recommended to use l-methoxyPMS for G6PDH histochemistry because of its (photo)chemical stability and its fast electron transfer.

2.1.2 Tetrazolium salts

The tetrazolium salts which have been used in the past for the demonstration of G6PDH activity often show different kinds of staining patterns and intensities. Recently, the four most widely used tetrazolium salts, i. e. neotetrazolium chloride (NT), nitro blue tetrazolium chloride (nitro BT), tetranitro blue tetrazolium chloride (tetranitro BT) and 2-(2-benzothiazolyl)-3-(4-phthalhydrazidyl)-5-styryl-tetrazolium chloride (BPST) have been compared for their effects on the localization of G6PDH activity under optimal incubation conditions (SINOWATZ et al. 1983; VAN NOORDEN and BUTCHER 1984). Fig. 3 shows that only the use of nitro BT and tetranitro BT resulted in localization patterns in agreement with the sites of G6PDH activity as determined by microbiochemical techniques (TEUTSCH and RIEDER 1979; TEUTSCH 1981) and with cytochemistry of isolated cells (KNOOK et al. 1980; VAN NOORDEN et al. 1984c). From these studies it has been concluded that Kupffer cells, endothelial cells and epithelial cells of bile caniculi show a high G6PDH activity. The staining patterns obtained with tetranitro BT and nitro BT in the presence of l-methoxyPMS or PMS are in agreement with these findings (Fig. 3). BPST-formazan seems to migrate from its site of production due to a lack of firm substantivity, which results in a rather diffuse staining pattern. Similar findings are reported in the literature (GAHAN and DAWSON 1981; VAN NOORDEN et al. 1982b; SINOWATZ et al. 1983).

It has been previously mentioned that, on theoretical grounds, it should be possible that NADPH reduces BPST directly due to the low redox potential of BPST (ALTMAN 1976a, b; CLARK 1980), but Fig. 4 shows this is not the case. In the absence of l-methoxyPMS, reduction of BPST occurs considerably slower and the staining pattern

resembles that of tetranitro BT and nitro BT in the absence of an exogenous electron carrier (VAN NOORDEN and BUTCHER 1984).

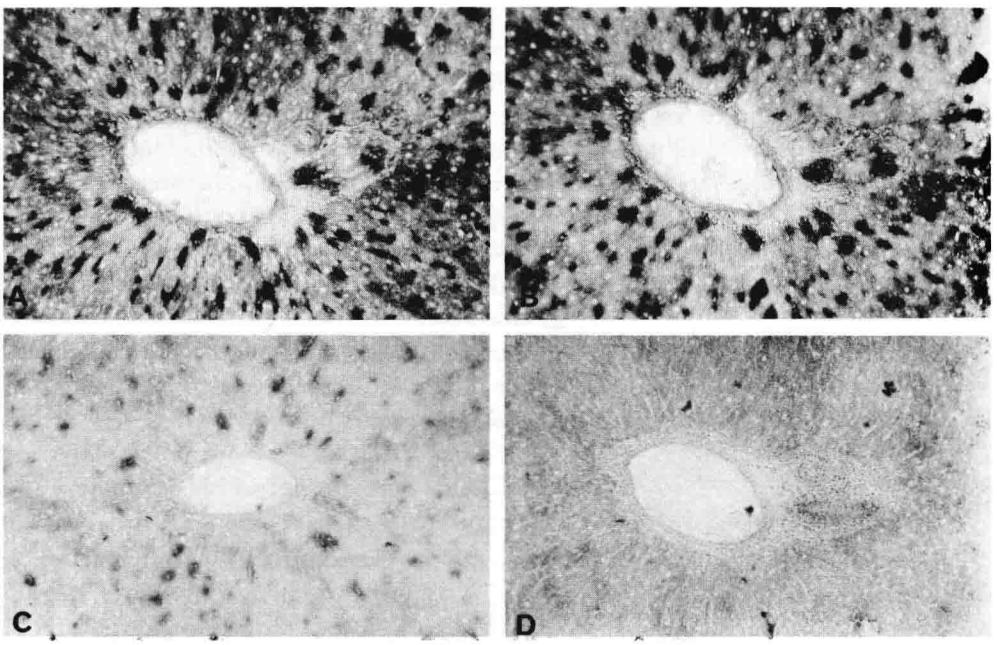


Fig. 3. Adjacent cryostat sections of female rat liver stained for G6PDH activity with four different tetrazolium salts. A, tetranitro BT; B, nitro BT; C, BPST; D, neotetrazolium in the presence of nitrogen. The incubations have been performed in the presence of l-methoxyPMS as electron carrier. x 130.

Staining with NT in the presence of l-methoxyPMS does not result in intercellular differences in staining intensities between e. g. hepatocytes and Kupffer cells (Fig. 3). The pattern is in fact very similar to that obtained in the presence of tetranitro BT in the absence of an electron carrier. This may be due to the large size of NT-formazan granules (ALTMAN 1976 a; SINOWATZ et al. 1983), but it might also be a result of the unavoidable interference of DT-diaphorase or NADPH cytochrome c (P450) reductase as endogenous electron transport systems. Nevertheless, when measuring formazan production in a small area of serial sections of rat liver incubated in the presence of each one of the tetrazolium salts mentioned above, similar reaction rates are obtained with all four salts, when the molar extinction coefficients of the respective formazans are taken into account (Table 1; BUTCHER and VAN NOORDEN 1984). This leads to the conclusion that all four tetrazolium salts can give similar quantitative information of G6PDH activity under optimal incubation conditions, but that the use of nitro BT or tetranitro BT results in the best localization of G6PDH activity. The very fine forma-

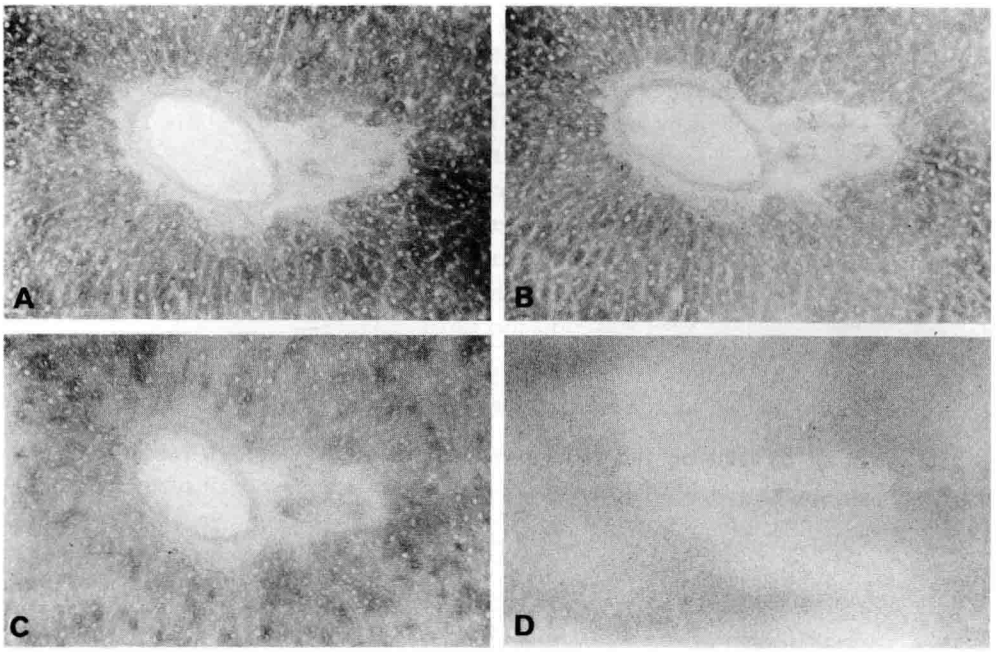


Fig. 4. Serial cryostat sections of female rat liver stained for G6PDH activity with four different tetrazolium salts. A, tetranitro BT; B, nitro BT; C, BPST; D, neotetrazolium in the presence of nitrogen. The incubations have been performed in the absence of an exogenous electron carrier. x 130.

zan granules (ALTMAN 1976 a; VAN NOORDEN et al. 1982 b), the ease of reduction (ALTMAN 1976 a; VAN NOORDEN et al. 1982 b) and the lack of lipid solubility of its formazan (HOROBIN 1982) make tetranitro BT the tetrazolium salt of choice for G6PDH histochemistry under normal conditions.

Table 1. End-point measurements of G6PDH activity in serial sections of female rat liver stained with four different tetrazolium salts. The readings of integrated absorbance are taken from the same areas in the sections at the wavelengths given in Table 9. Reactions were performed for 5 min at 37 °C.

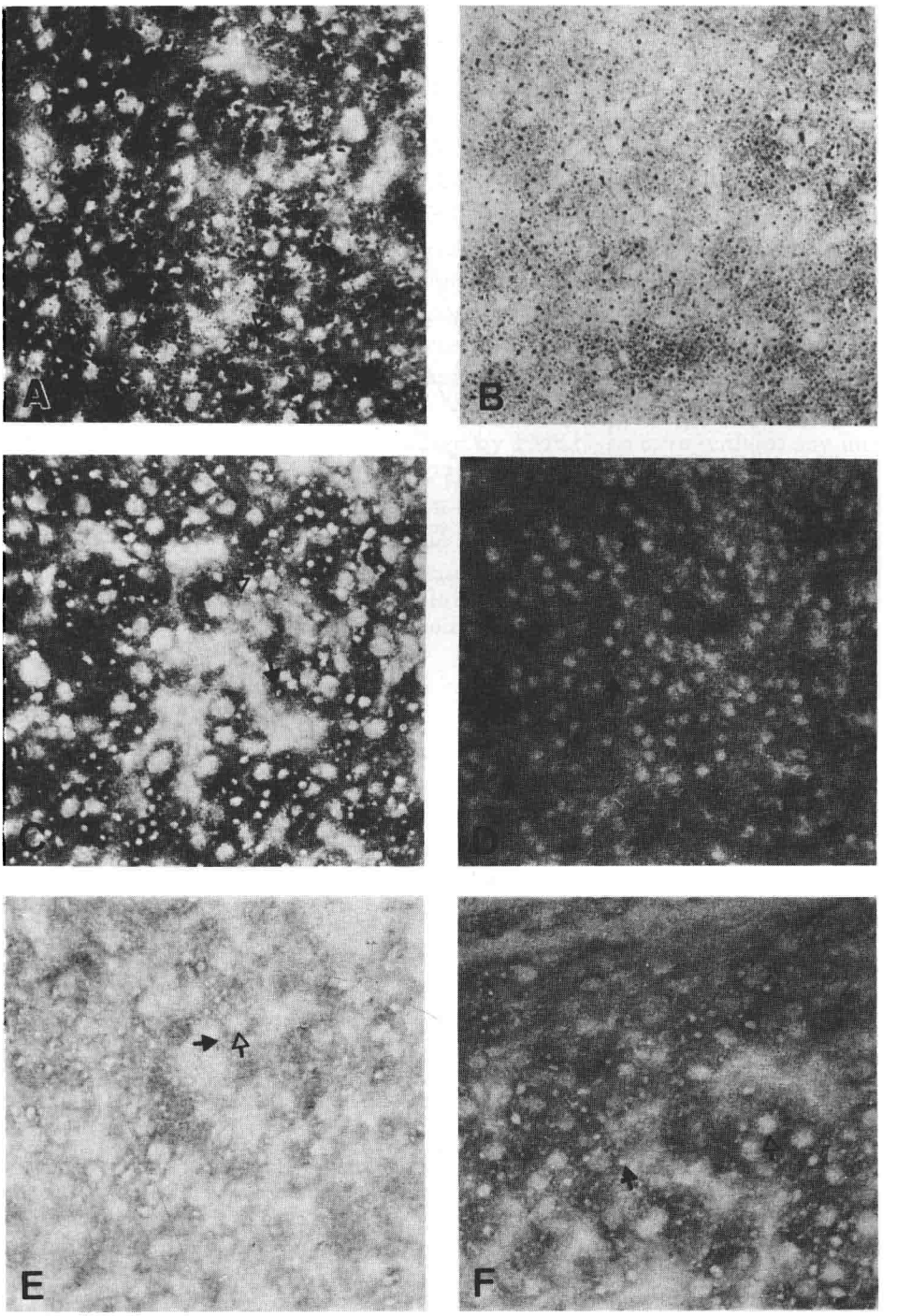
Tetrazolium salt	Increase in integrated absorbance	G6PDH activity (nmol H_2/mm^3)
Tetranitro BT	1280	80
Nitro BT	1190	93
BPST	480	60
Neotetrazolium	530	90

When G6PDH activity is demonstrated in lipid-rich tissues, there might be even some tetranitro BT-formazan diffusion into lipid-water interfaces (HØYER and ANDERSEN 1977; MØLLER and HØYER 1979). ALTMAN et al. (1979) have found a solution for this diffusion problem by using BPST in the presence of nickel chloride. Nickel-chelated BPST-formazan appeared to be extremely lipophobic (Fig. 5). Tetranitro BT- and nitro BT-formazans did not show any chelation with metal ions under histochemical conditions. On the other hand, lipid can be removed before staining by acetone pretreatment of the sections at 0 °C to avoid formazan drift (JACOBSEN 1969; MELLGREN 1971). However, our experience with acetone pretreatment is not very good: it destroys the morphology of the section too much.

2.1.3 The effect of oxygen on the tetrazolium salt reduction

The effect of oxygen on the reduction of tetrazolium salt is a rather confusing matter. The reduction of NT is strongly decreased in the presence of oxygen (ALTMAN 1976 a, c; BUTCHER 1978 a) and this is independent of the presence of PMS or l-methoxyPMS or azide (inhibitor of cytochromes) (VAN NOORDEN and BUTCHER 1984). It is shown by BUTCHER (1978 a) that the *rate* of *in vitro* reduction of NT in an aqueous medium is decreased by the oxygen tension (Fig. 6). On the other hand, it appeared from the same study, that the reduction rate of NT is not decreased substantially in a viscous medium (containing polyvinyl alcohol (PVA)) by the oxygen tension, but a lag phase is increased with time, when more oxygen is present in the atmosphere (Fig. 7). An explanation for the differences between the effect of oxygen in a PVA-containing medium and its effect in a medium without PVA might well be a diminished diffusion of oxygen by PVA. During the first period of incubation of a section, electrons are transferred preferentially to any oxygen present; when the oxygen supply is exhausted at the surface of the section, the electrons are then available to reduce NT and formazan production begins. Apparently, oxygen cannot diffuse quickly enough into the section. The interference of oxygen in the reduction of NT is not limited by diffusion to any significant extent in media without a stabilizer like PVA.

Fig. 5. G6PDH activity demonstrated in lipid-rich tissue of rat ovarian stroma with different tetrazolium salts. A, nitro BT: poor localization; large accumulations of formazan granules are distributed throughout the section, particularly over lipid droplets (solid arrow) and around nuclear envelopes (open arrow). B, MTT + cobalt ions: very poor localization, with extensive accumulations of formazan granules throughout the section. C, tetranitro BT: improved localization, but granular formazan accumulations are still apparent around lipid droplets (solid arrow) and nuclear envelopes (open arrow). D, BPST: better localization as in A–C, but formazan is still accumulating around lipid droplets (solid arrow) and nuclear envelopes (open arrow). E, BPST + cobalt ions and F, BPST + nickel ions: localization greatly improved. Formazan now appears agranular, and there is no indication of accumulation around lipid droplets (solid arrows) or nuclear envelopes (open arrows). x 400. – Reprinted with permission from ALTMAN et al. (1978).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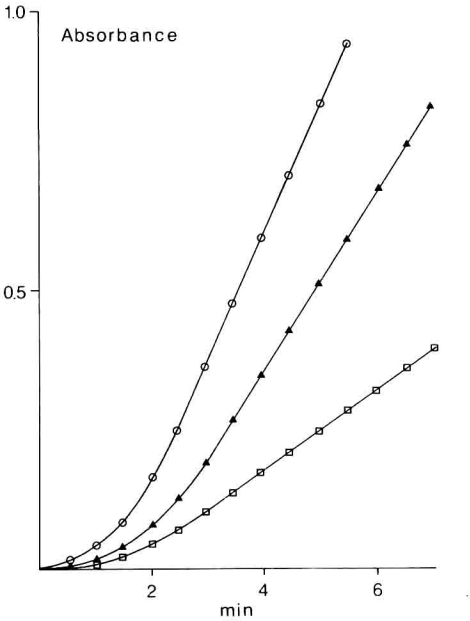

Fig. 6. The effect of oxygen on the rate of formazan production from neotetrazolium by G6PDH activity in an aqueous solution in the presence of PMS. ○—○ 0 % oxygen, ▲—▲ 10 % oxygen, □—□ 20 % oxygen. – Reprinted with permission from BUTCHER (1978).

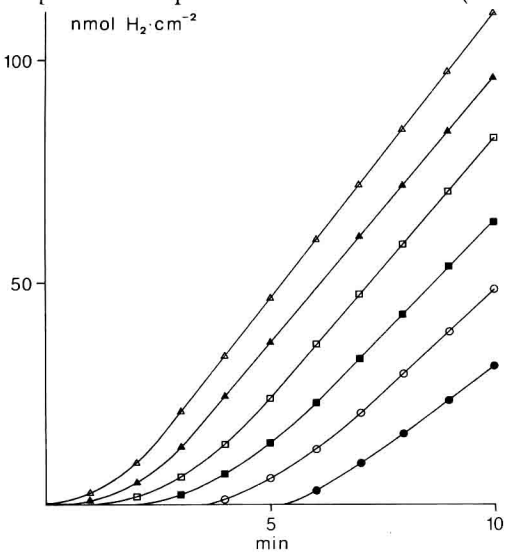

Fig. 7. G6PDH activity in the same pericentral region of serial sections of rat liver incubated on the stage of a cytophotometer at room temperature under different oxygen tensions. The incubations were performed in aqueous media containing polyvinyl alcohol. The final electron acceptor was neotetrazolium and PMS was used as intermediate electron acceptor. △—△ 0 % oxygen, ▲—▲ 10 % oxygen, □—□ 20 % oxygen ■—■ 30 % oxygen, ○—○ 40 % oxygen, ●—● 50 % oxygen. – Reprinted with permission from BUTCHER (1978).