Enzyme cytochemical substrate solutions

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There exist a great number of substrates for enzymes as markers in histology which give final colors in *blue*, *red*, *black*, *grey-black*, *brown* etc.; some of the reactions are electron dense and suitablefor ultrastructural studies (PEARSE AGE, 1980). The stainings have been originally developed for enzyme cytochemistry, but with the introduction of immune reactions in histology many of them became adapted to immuno-enzyme stainings (GRAHAM RC and KARNOVSKY MJ, 1966; HUGON J and BORGERS M, 1966; NAKANE P and PIERCE GB, 1966; NAKANE PK, 1968; KUHLMANN WD, 1970; STRAUS W, 1972; HANKER JS et al., 1977; ITOH K et al., 1979; PEARSE AGE, 1980; BASKIN DG et al., 1982; BONDI A et al., 1982; KUHLMANN WD and PESCHKE P, 1986; SHU SY et al., 1988).

As with histochemical methods in general, attention must be directed toward the correct histological localization of the employed enzyme substrates. One must be aware that manipulation of cells on the one hand and the use of reactive substrates on the other hand is prone to false positive and false negative reactions. Hence, all staining procedures must be rigorously controlled for reliable localization. In the following, some of the widely used cytochemical reactions for immuno-enzyme purposes are described.*

Enzyme substrate for peroxidase (HRP) reaction

Peroxidase stains date back to the investigations of R FISCHEL (1910) on blood cells who used benzidine (diamino-biphenyl) for the demonstration of peroxidase granules in cytological blood cell preparations. Two types of substrates are involved in the peroxidase reaction which are called hydrogen donor and hydrogen acceptor, respectively. Hydrogen donors which will be oxidized by the peroxidase-hydrogen peroxide system are for example mono- and diphenols. Benzidine, o-dianisidine, phenylenediamine or pyrocatechol are common in peroxidase cytochemistry (LOELE W, 1912; GRAHAM GS, 1918; GOODPASTURE EW, 1919; MCJUNKIN FA, 1922; WACHSTEIN M et al., 1959; STRAUS W, 1959; MITSUI T, 1960; STRAUS W, 1964; GRAHAM RC et al., 1965; STRAUS W, 1967; MESULAM MM, 1978; MORRELL JI et al., 1981; LAKOS S and BASBAUM AI, 1986).

Important improvements derived from the introduction of 3,3'-diaminobenzidine (DAB) as hydrogen donor by RC GRAHAM and MJ KARNOVSKY (1966). Oxidation of DAB results in a highly osmiophilic polymer giving high contrast in the elctron microscope. DAB has become one of the most employed chromogens in electron and light microscopy due to its high staining reliablilty and the clear reaction product (FAHIMI HD, 1968; NOVIKOFF AB and GOLDFISCHER S, 1969; NOVIKOFF AB et al., 1972; FAHIMI HD, 1979; PELLINIEMI LJ et al., 1980). The cytochemical product is stable for years, insoluble in organic solvents and sections can be mounted in a standard way. Yet, one has to keep in mind that diffusion artefacts cannot

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* Chromogens and other cytochemical reagents can be toxic and carcinogenic. They must be handled with great care

be always excluded even with a good enzyme substrate (NOVIKOFF AB et al., 1972; SELIGMAN AM et al., 1973).

DAB is useful for the localization of different enzyme activities (SELIGMAN AM et al., 1968) including the indirect detection of hydrogen peroxide-producing oxidases (KUHLMANN WD, 1970; KUHLMANN WD and AVRAMEAS S, 1971) by means of exogenous peroxidase and by means of endogenous catalase (reviewed by HAND AR, 1979; LITWIN JA, 1979). Finally, DAB cytochemistry is very popular in immunohistology for the localization of antigens.

In attempts to optimize peroxidase detection systems, several enhancing procedures have been proposed such as lowering the buffer pH to approach the pH at which HRP is maximally active and the use of imidazole to induce formation of a third electron transfer site in peroxidases, thereby increasing their activity (TU AT et al. 1968; SIMIONESCU N et al. 1975; TIGGEMANN R et al. 1981). Furthermore, color modification of DAB may be applied. It was found that the brown oxidized DAB can be modified by metal compounds. Thus, new colors, enhanced staining intensity and enhanced electron density of the DAB reaction product are achieved by modification of the original DAB procedure (ADAMS JC, 1977; HARDY H and HEIMER L, 1977; ADAMS JC 1981; HSU SM and SOBAN E 1982; NEWMAN GR et al. 1983).

There exist many variations of the original cytochemical procedures which will not be listed. So far, the following descriptions will give an overview of the most applied cytochemical methods.

<table>
<tr><th>Enzyme substrate (HRP)</th><th>Cytochemical feature</th></tr>
<tr><td>0.05% Diaminobenzidine* (DAB) and 0.01% H_2O_2
(substrate of GRAHAM RC and KARNOVSKY MJ 1966)
• DAB stock solution:
50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years)
• H_2O_2 stock solution:
100 µL 30% H_2O_2 dissolved in 3.0 mL distilled water
• Working solution:
500 µL DAB stock dissolved in
4.5 mL Tris-HCl buffer (0.2 M pH 7.2-7.4)** plus
50.0 µL H_2O_2 stock
* 3,3’-Diaminobenzidine tetrahydrochloride
** PBS can be also used</td><td>Brown color
Due to the very insoluble reaction product in both aqueous solution and organic solvents this peroxidase substrate is the most used chromogen for light and electron microscopic studies
Electron dense product (electron microscope)
Enzyme reaction product is further intensified by postfixation in OsO4 (osmium black)</td></tr>
<tr><td colspan="2">Osmium tetroxide and copper sulfate are frequently used for post-chromogenic intensification of DAB reacted slides
• 0.1% Osmium tetroxide solution:
0.5 mL OsO_4 solution (aqueous) plus
9.5 mL distilled water or PBS
- Solution is freshly prepared for immediate use and then discarded
- DAB stained slides are rinsed in distilled water (2 x 2 min), then covered with few drops of OsO_4 solution and incubated for 1-2 min at room temperature
- Slides are rinsed (2 x 2 min) in distilled water, dehydrated and mounted under coverglass as usual</td></tr>
</table>

- 0.5% Copper sulfate solution:
  1.0 g copper sulfate pentahydrate dissolved in
  200 mL PBS or 0.15 M NaCl
  - Solution can be prepared in advance, stored at room temperature and repeatedly reused
  - Slides are placed in a staining jar (COPLIN, HELLENDAHL or SCHIFFERDECKER), rinsed in distilled water (2 x 2 min) and incubated for 1-5 min at 25ºC in copper sulfate solution
  - Slides are rinsed (2 x 2 min) in distilled water, dehydrated and mounter under coverglass as usual

Enzyme substrate (HRP)	**Cytochemical feature**
0.05% Diaminobenzidine (DAB), 0.01% H_2O_2 and 0.05% nickel chloride • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • H_2O_2 stock solution: 100 µL 30% H_2O_2 dissolved in 3.0 mL distilled water • Nickel chloride stock solution: 0.1 g $NiCl_2$ are dissolved in 10 mL distilled water • Working solution: 500 µL DAB stock dissolved in 4.20 mL PBS (0.01 M pH 7.2-7.4) *plus* 250 µL nickel stock *plus* 50.0 µL H_2O_2 stock	**Gray-black color** Enzyme substrate is useful in combination with other substrates for double stainings

Enzyme substrate (HRP)	**Cytochemical feature**
0.05% Diaminobenzidine (DAB), 0.01% H_2O_2 and 0.05% nickel ammonium sulfate • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • H_2O_2 stock solution: 100 µL 30% H_2O_2 dissolved in 3.0 mL distilled water • Nickel ammonium sulfate stock solution: 0.1 g $Ni(NH_4)_2$ $(SO_4)_2$ are dissolved in 10 mL distilled water • Working solution: 500 µL DAB stock dissolved in 4.20 mL PBS (0.01 M pH 7.2-7.4) *plus* 250 µL nickel ammonium sulfate stock *plus* 50.0 µL H_2O_2 stock	**Black color** Enzyme substrate is used for enhancement of stainings in immunohistology

Enzyme substrate (HRP)	**Cytochemical feature**

0.05% Diaminobenzidine (DAB), 0.01% H_2O_2 and 0.05% cobalt chloride, 0.05% nickel ammonium sulfate • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • H_2O_2 stock solution: 100 µL 30% H_2O_2 dissolved in 3.0 mL distilled water • Cobalt chloride stock solution: 0.1% $CoCl_2$ are dissolved in 10 mL distilled water • Nickel ammonium sulfate stock solution: 0.1 g $Ni(NH_4)_2$ $(SO_4)_2$ are dissolved in 10 mL distilled water • Working solution: 500 µL DAB stock dissolved in 3.95 mL PBS (0.01 M pH 7.2-7.4) *plus* 250 µL cobalt chloride stock *plus* 250 µL nickel ammonium sulfate stock *plus* 50.0 µL H_2O_2 stock	**Blue color** Enzyme substrate is used for enhancement of stainings in immunohistology. The substrate is also useful in combination with other substrates for double stainings

Enzyme substrate (HRP)	**Cytochemical feature**
0.03% Chloro-naphthol (CN)* and 0.01% H_2O_2 • CN stock solution: 0.3 g CN are dissolved in 10 mL absolute ethanol aliquoted and stored at -20ºC (stable for months) • H_2O_2 stock solution: 100 µL 30% H_2O_2 dissolved in 3.0 mL distilled water • Working solution: 100 µL CN stock dissolved in 9.8 mL Tris-HCl buffer (0.05 M pH 7.4-7.6) *plus* 100 µL H_2O_2 stock Substrate solution is stable for about 60 min and must be filtered prior to use * 4-Chloro-1-naphthol	**Blue color** The substrate is also useful in combination with other substrates for double stainings

Enzyme substrate (HRP)	**Cytochemical feature**
0.05% Aminoethylcarbazole* (AEC) and 0.01% H_2O_2 • AEC stock solution: 0.1 g AEC are dissolved in 10 mL DMF** aliquoted and stored at 4ºC (stable for years) • H_2O_2 stock solution: 100 µL 30% H_2O_2 dissolved in 3.0 mL distilled water • Working solution: 250 µL AEC stock dissolved in 4.7 mL Acetate buffer (0.05 M pH 5.5) *plus*	**Red color** The substrate is also useful in combination with other substrates for double stainings

50.0 µL H_2O_2 stock * 3-Amino-9-ethylcarbazole ** N,N-Dimethylformamide	

Enzyme substrate (HRP)	**Cytochemical feature**
0.01% BDHC* and 0.05% H_2O_2 • BDHC stock solution: 0.02 g BDHC are dissolved in 10 mL distilled water aliquoted and stored at 4ºC • H_2O_2 stock solution: 100 µL 30% H_2O_2 dissolved in 10.0 mL distilled water • Working solution: 250 µL BDHC stock dissolved in 5.0 mL phosphate buffer (0.01 M pH 6.5) containing 0.03% sodium nitroprusside *plus* 75.0 µL H_2O_2 stock * Benzidine dihydrochloride	**Blue-green color** The substrate is also useful in combination with other substrates for double stainings

Enzyme substrate for peroxidase (HRP) reaction by use of the HRP-GOD coupled method

In neuroanatomical tracing technique, the transport of horseradish (HRP) has been widely used for light and electron microscopic studies. Modifications of the original HRP-DAB method appeared necessary to intensify DAB reactions. One proposed way is the intensification of DAB reactions by metals such as obtained by cobalt or nickel salts. Then, another possibility is the use of glucose oxidase (GOD) and glucose to achieve a continuous release of hydrogen peroxide into the incubation medium instead of direct addition of high concentration of hydrogen peroxide (ITOH K et al., 1979; OLDFIELD BJ et. al., 1983; PARENT JM et al., 1997; SAKANAKA M et al., 1987; SHU SY et al, 1988).

The reaction of the enzyme GOD upon D-glucose results in a continuous release of hydrogen peroxide. It was supposed that this continuous release favors the deposition of DAB reaction products around HRP molecules. This coupled enzyme method together with metal intensification proved to be very successful in bringing out histochemical and immunoreactive structures.

In a similar way, HRP is used as secondary system amplifier and coimmobilized in close proximity of the marker enzyme (GOD) onto the same cellular sites for immunohistological purpose (KUHLMANN WD and PESCHKE P, 1986); see chapter “Enzyme substrate solution for glucose oxidase (GOD) as marker in the coimmobolized GOD-HRP method”.

Enzyme substrate (HRP) **(HRP-GOD coupled method)**	**Cytochemical feature**
0.05% Diaminobenzidine (DAB), 0,2% β -D-glucose 0.04% ammonium chloride containing 0.3-0.5 mg glucose oxidase (GOD) per 100 mL solution • *0.5% Cobalt acetate for pretreatment:* *0.5 g cobalt acetate dissolved in 100 mL Tris buffer pH 7.6* • Ammonium chloride stock solution: 0.4 g NH_4Cl dissolved in 100 mL distilled water • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • β-D-Glucose stock solution: 200 mg D-glucose dissolved in 10 mL distilled water, aliquoted and stored at 4°C • Glucose oxidase (GOD, e.g. 250 U/mg) solution: 3 mg GOD dissolved in 10 mL 0.1 M phosphate buffer pH 7.3 • Working solution: 1 mL DAB stock *plus* 5 mL mL phosphate buffer pH 7.3 *plus* 1 mL glucose stock *plus* 1 mL ammonium chloride stock *plus* 100 µL GOD solution *plus* 1.9 mL 0.1 M phosphate buffer pH 7.3	**Dark blue/black color** Method to increase the sensitivity of the HRP method, useful in combination with other substrates
DAB intensification by metal salts such as cobalt acetate or nickel chloride is frequently in use. In this case, the incubation sequence is as follows: • Incubation in primary antibody, PAP or ABC method as usual and followed by appropriate washings • Sections are rinsed twice in PBS and once in 0.05 M Tris-HCl buffer pH 7.6 (15 min in each) • Place in 0.5% cobalt acetate solution for 10 min • Wash three times in 0.1 M Tris-HCl buffer pH 7.6 and twice in 0.1 M phosphate buffer pH 7.3 (10 min in each) • Incubate in the enzyme substrate solution of the HRP-GOD coupled method (described above) for 1-2 hours at room temperature or for several hours at 4°C (optimal incubation time and optimal temperature will depend on experimental conditions) • Sections are washed three times in 0.1 M phosphate buffer pH 7.3 (10 min in each), rinsed in distilled water (2 x 2 min), dehydrated and mounted as usual	

Enzyme substrate for alkaline phosphatase (AP) reaction

The choice of alkaline phosphatase (AP) as marker can be of advantage in certain conditions when for example the use of peroxidase labeling is not favorable due to difficulties in the inhibition of endogenous peroxidase activities.

Alkaline phosphatase is a generic name for phosphomonoesterases that hydrolyze orthophosphate at an alkaline pH. From the known and purified phospatases, calf intestinal alkaline phosphatase is the label of choice in immunohistology because endogenous activity of non-intestinal AP can be easily inhibited by levamisole. A variety of simultaneous coupling azo dye methods exist. In principle, the phosphate-substrate is hydrolysed and then coupled with a diazonium salt such as Fast Blue BB. Several chromogenic substrates are commercially available, and one can choose between different colors for histochemical AP staining (HUGON J and BORGERS M, 1966; PEARSE AGE, 1968; KUHLMANN WD and MILLER HR, 1971; CORDELL JL et al., 1984; DE JONG AS et al., 1985).

Enzyme substrate (AP)	**Cytochemical feature**
0.01% New Fuchsin and 0.05% Naphthol AS-BI Phosphate • New Fuchsin stock solution: 20 mg New Fuchsin are dissolved in 10 mL 2M HCl and stored at 4ºC • Naphthol AS-BI Phosphate stock solution: 100 mg Naphthol AS-BI Phosphate are dissolved in 10 mL DMF* and stored at 4ºC • Sodium Nitrite and levamisole stock solution: sodium nitrite (final 0.4%) and levamisole (final 20 mM) are dissolved in distilled water and stored at 4ºC • Working solution: 250 µL New Fuchsin stock dissolved in 4.25 mL Tris buffer (0.05-0.01 M pH 8.7) *plus* 250 µL sodium nitrite/20 mM levamisole stock *plus* 250 µL Naphthol AS-BI Phosphate stock solution * N,N'-Dimethylformamide	**Red color**

Enzyme substrate (AP)	**Cytochemical feature**
0.02% BCIP* and 0.03% NBT** • BCIP stock solution: 100 mg BCIP are dissolved in 10 mL DMF**** (light sensitive, store in a brown bottle) • NBT stock solution: 150 mg NBT are dissolved in 10 mL 70% DMF (light sensitive, store in a brown bottle) • Levamisole stock solution: 50 mM levamisole in distilled water • Working solution: 100 µL BCIP stock dissolved in 4.7 mL Tris buffer (0.1 M pH 9.5, 0.005 M $MgCl_2$) *plus* 100 µL NBT stock *plus*	**Blue-violet color**

100 µL levamisole stock * 5-Bromo-4-chloro-3-indolyl phosphate ** Nitro blue tetrazolium *** N,N-Dimethylformamide	

Enzyme substrate (AP)	**Cytochemical feature**
0.25% β-Glycerophosphate and 0.13% lead nitrate • Tris-maleate (0.2 M pH 9.0) stock solution • Sodium β-glycerophosphate stock solution: 125 mg sodium β-glycerophosphate are dissolved in 10 mL distilled water • Lead nitrate stock solution: 100 mg lead nitrate are dissolved in 10 mL distilled water • Working solution: 2.0 mL Tris-maleate stock *plus* 2.0 mL sodium β-glycerophosphate stock *plus* 4.7 mL distilled water *plus* 1.3 mL lead nitrate stock Substrate solution is freshly prepared; lead nitrate should be added very slowly under continuous stirring. The solution is filtered and used immediately	**Black color (light microscope)** Enzyme reaction is visualized as PbS by rinsing the section in diluted ammonium sulfide for 2 min **Electron dense product (electron microscope)** Tissue is postfixed in cacodylate buffered 2% OsO_4 solution

Enzyme substrate for glucose oxidase (GOD) reaction

The fungal enzyme glucose oxidase (GOD) can be employed as a marker enzyme in immunohistology. In mammalian tissue, no endogenous GOD activity is found, thus, making this enzyme an excellent marker in immunohistology with unambiguous staining pattern.

Several cytochemical reactions exist. The activity of GOD is usually visualized with tetrazolium salts and intermediate electron carriers (NACHLAS MM et al., 1957; REISS J, 1966; ALTMAN FP, 1976; SUFFIN SC et al., 1979; CLARK CA et al., 1982). The chromogens Nitro Blue Tetrazolium, Iodophenyl-Nitrophenyl Tetrazolium and Tetra Blue Tetrazolium result in differently colored end products. The different colors make GOD staining useful for double or multi labelling experiments or for combination with HRP as marker.

In order to overcome problems of grained cytochemical staining and background by formazan deposits in the tetrazolium method, staining of GOD by a coupled peroxidase procedure and the use of 3,3'-diaminobenzidine as chromogen has been proposed (KUHLMANN WD, 1970; KUHLMANN WD and AVRAMEAS S, 1971). In this technique, the secondary system enzyme, i.e. HRP, is added to the reaction mixture quite analogous to assays in clinical chemistry (KESTON AS, 1956). The enzyme reaction chain involves hydrogen peroxide which is released during the reaction of GOD with D-glucose and which serves as substrate for HRP in the oxidation of 3,3'-diaminobenzidine yielding a colored product (DAB_{oxid}). Some important aspects in the kinetics of the coupled oxidation reaction of the GOD-HRP type are given by I LUNDQUIST and JO JOSEFSSON (1971).

Enzyme substrate (GOD)	Cytochemical feature
0.05% MTT*, 0.75% D-glucose and 0.01% PMS* • Working solution: 150 mg β-D-glucose dissolved in 20 mL phosphate buffer (0.1 M pH 6.8) *plus* 10 mg MTT add 2 mg PMS just prior to use, mix and filter rapidly [in order to avoid crystallisation of MTT on the slide, add 2 mg cobalt chloride to the substrate], staining is performed in the dark MTT can be replaced by NBT or INT tetrazolium salts * Methyl thiazolyl tetrazolium ** Phenazine methosulfate	**Blue color (MTT)** Other chromogenic substrates: (a) Nitroblue tetrazolium (NBT) to give purple-blue color (b) Tetranitroblue tetrazolium (TNBT) to give black color (c) Iodophenyl-nitrophenyl-phenyl tetrazolium (INT) to give violet color Enzyme substrates are useful in combination with other substrates for double stainings

Enzyme substrate (GOD)	Cytochemical feature
0.05% Diaminobenzidine (DAB), 1% D-glucose containing 0.1-1 mg peroxidase (HRP) per mL solution • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • β-D-Glucose stock solution: 1000 mg D-glucose dissolved in 10 mL distilled water (for a final 1% glucose concentration), aliquoted and stored at 4°C • Peroxidase (HRP RZ 3) stock solution: 10 mg HRP dissolved in 10 mL 0.1 M phosphate buffer (for 0.1 mg peroxidase per mL working solution) • Working solution: 1 mL DAB stock *plus* 3 mL 0.1 M phosphate buffer pH 6.8 *plus* 1 mL D-glucose stock solution *plus* 1 mL peroxidase stock solution *plus* 4 mL 0.1 M phosphate buffer pH 6.8	**Brown color** Enzyme substrate is useful in combination with other substrates for double stainings

Enzyme substrate (GOD)	Cytochemical feature
0.05% Diaminobenzidine (DAB), 1% D-glucose, 0.025% nickel ammonium sulfate containing 0.1-1 mg peroxidase (HRP) per mL solution • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • β-D-Glucose stock solution: 1000 mg D-glucose dissolved in 10 mL distilled water (for a final 1% glucose concentration),	**Black color** Enzyme substrate is used for enhanced GOD stainings and useful in combination with other substrates for double stainings

aliquoted and stored at 4°C • Peroxidase (HRP RZ 3) solution: 10 mg HRP dissolved in 10 mL 0.1 M phosphate buffer pH 7.3 (for 0.1 mg peroxidase per mL working solution) • Nickel ammonium sulfate stock solution: 0.1 g $Ni(NH_4)_2$ $(SO_4)_2$ are dissolved in 10 mL distilled water • Working solution: 1 mL DAB stock *plus* 3 mL 0.1 M phosphate buffer pH 6.8 *plus* 1 mL D-glucose stock solution *plus* 1 mL peroxidase stock solution *plus* 3.75 mL 0.1 M phosphate buffer (0.01 M pH 6.8) *plus* 250 µL nickel ammonium sulfate stock	

Enzyme substrate for glucose oxidase (GOD) in the coimmobilized GOD-HRP method

The cytochemical method involves a two-step enzyme method in which GOD and HRP are coimmobilized onto the same cellular sites by immunological bridging or by the principle of avidin-biotin interaction. This method is by far better than the use of soluble HRP in the substrate mixture (KUHLMANN WD and PESCHKE P, 1986).

In this enzyme-amplification technique, H_2O_2 generated by the reaction of GOD with D-glucose serves as substrate for HRP in the oxidation of chromogens such as 3,3'-diamino-benzidine (DAB). Coimmobilization of both *marker enzyme* (GOD) and *secondary system amplifier* (HRP) in close proximity proved to be of great advantage in immunohistology because the unfavorable properties of soluble HRP in substrate mixtures are avoided. In later studies, the behaviour of kinetic properties of free and immobilized HRP has been studied in histochemical models with DAB as a hydrogen donor substrate showing obvious diffusional constraints in free and immobilized enzyme systems (MALPIECE Y et al., 1980).

Multistep enzyme systems have shown that a tightly coupled enzyme system will work very efficiently inasmuch as a cumulative effect on the efficiency of the overall reaction is achieved (MOSBACH K and MATTIASSON B, 1976). In a similar way, continuous release of hydrogen peroxide in the proximity of HRP might favor the deposition of DAB reaction products around HRP molecules.

The coupled GOD-HRP principle was developed for the use of GOD as marker enzyme in immunohistology because no such endogenous enzyme activity is known in mammalian cells and to profit from the general usefulness of DAB as cytochemical stain. Hence, the coupled GOD-HRP principle can be superior to conventional immunoperoxidase labeling for the localization of biomolecules in tissues which are rich in endogenous peroxidase activitites.

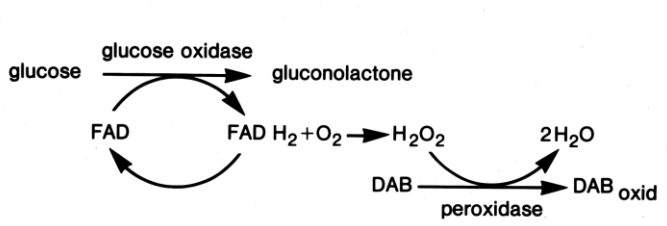

Fig.: Schematics of glucose oxidase (GOD) staining by the two-step enzyme technique using HRP as a secondary system enzyme. Hydrogen peroxide formed in the oxidation of D-glucose is the substrate for HRP and further utilized for the oxidation of DAB to yield a colored product (from Kuhlmann WD, 1970; Kuhlmann WD and Avrameas S, 1971; Kuhlmann WD and Peschke P, 1986)

Enzyme substrate (GOD) **(GOD-HRP coupled method)**	**Cytochemical feature**
0.05% Diaminobenzidine (DAB), 0.2-1% β -D-glucose **0.04% ammonium chloride (optional)** • *0.5% Cobalt acetate for pretreatment is optional: 0.5 g cobalt acetate dissolved in 100 mL Tris buffer pH 7.6* • *Ammonium chloride stock solution is optional: 0.4 g NH_4Cl dissolved in 100 mL distilled water* • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • β-D-Glucose stock solution: 200 mg D-glucose dissolved in 10 mL distilled water (for a final 0.2% glucose concentration), aliquoted and stored at 4°C • Working solution: 1 mL DAB stock *plus* 5 mL mL phosphate buffer pH 7.3 *plus* 1 mL glucose stock *plus* 3 mL 0.1 M phosphate buffer pH 7.3 (*alternatively, 1 mL ammonium chloride stock and 2 ml phosphate buffer are added*)	**Brown color,** **dark blue/black color** GOD-HRP method as alternative to immunoperoxidase staining, useful in combination with other markers and substrates

DAB intensification by metal salts such as cobalt acetate or nickel chloride can be useful. In this case, the incubation sequence is as follows:

- Incubation in primary antibody followed by GOD labeled secondary antibody and PAP complexes/HRP labeled third antibody. In the avidin-biotin method, f.e. biotin-antibody and complexes from avidin-HRP and biotin-GOD may be used. Each step followed by appropriate washings
- Sections are rinsed twice in PBS and once in 0.05 M Tris-HCl buffer pH 7.6 (15 min in each)
- Place in 0.5% cobalt acetate solution for 10 min
- Wash three times in 0.1 M Tris-HCl buffer pH 7.6 and twice in 0.1 M phosphate buffer pH 7.3 (10 min in each)

- Incubate in the substrate solution of the HRP-GOD coupled method (described above) for 30 min to several hours at 4-20°C (optimal incubation time and optimal temperature may be varied according to preliminary experiments)
- Sections are washed three times in 0.1 M phosphate buffer pH 7.3 (10 min in each), rinsed in distilled water (2 x 2 min), dehydrated and mounted as usual

Enzyme substrate for galactose oxidase (GOD) reaction

The same staining principles as that described for glucose oxidase can be used for the detection of galactose oxidase as marker enzyme with the exception that β-D-galactose has to be used as substrate instead of β-D-glucose (KUHLMANN WD and AVRAMEAS S, 1971).

Enzyme substrate for β-galactosidase (β-Gal) reaction

Beta-galactosidase (β-Gal) from *Escherichia coli* (CRAVEN GR et al., 1965) can be conjugated by a variety of cross-linkers to antibodies for immunohistological purposes. β-Gal is a sensitive and useful marker in applications where endogenous enzyme activities (e.g. peroxidases, phosphatases) are a serious problem (BONDI A et al., 1982).

The enzyme catalyses the hydrolysis of β-galactosides including lactose. The histochemical staining is based on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as chromogenic substrate (PEARSON B et al., 1963; HORWITZ JP et al., 1964; LOJDA Z, 1970). β-Gal cleaves the glycosidic linkage in X-Gal, thus producing a soluble and colorless indoxyl monomer. Liberated indoxyl moieties form subsequently a dimmer which is nonenzymically oxidized. Dimerization and oxidation reactions require transfer of an electron which is facilitated by ferric and ferrous ions. The resultant halogenated indigo is a stable and insoluble dark blue compound.

Enzyme substrate (β-Gal)	**Cytochemical feature**
0.05% X-Gal*, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide and 1 mM $MgCl_2$ • X-Gal stock solution: 50 mg X-Gal are dissolved in 1 mL DMF** and stored at -20ºC (stable for at least 1 year) • Substrate buffer stock solution: 0.1 M phosphate buffer containing 6 mM potassium ferricyanide, 6 mM potassium ferrocyanide and 2 mM $MgCl_2$ (store refrigerated, stable for at least 1 year) • Working solution: 100 µL X-Gal stock dissolved in 5.0 mL substrate buffer stock solution *plus* 4.9 mL distilled water * 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside ** N,N-Dimethylformamide	**Blue color** Enzyme substrate is useful in combination with other substrates for double stainings Other chromogenic substrates: (a) 5-Bromo-3-indolyl- β-D-galactopyranoside (b) 6-Chloro-3-indolyl- β-D-galactopyranoside (c) 5-Iodo-3-indolyl- β-D-galactopyranoside (d) 5-Bromo-6-chloro-3-indolyl-β-D-galactopyranoside (e) N-Methylindolyl- β-D-galactopyranoside

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Full citation of publications is given in chapter *References*
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