Histochemical Methods for Protein-bound Sulphydryl and Disulphide Groups

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With 2 plates (figs. 1 and 2)

SUMMARY

Histochemical methods for the localization of protein-bound sulphydryl groups were compared in a critical fashion. As far as localization is concerned, several of the methods published were found to be satisfactory. However, from the point of view of approximate visual quantitation, the results are confusing. The two best methods for the histochemical detection of protein-bound sulphydryl groups are the tetrazolium method and Barneett and Seligman’s dihydroxydinaphthyldisulphide technique.

In the course of investigations on the islets of Langerhans by the tetrazolium and the dihydroxydinaphthyldisulphide (DDD) methods it was found that the results obtained by these two techniques were not strictly identical. This discrepancy prompted a comparative study of various methods for the demonstration of protein-bound sulphydryl and disulphide groups.

Both sulphydryl and disulphide groups occur in various proteins. The former can be demonstrated directly by sulphydryl reagents while the latter must be first reduced to sulphydryls. The present paper will deal only with the demonstration of the sum of sulphydryls and disulphides for the following reason.

Sulphydryl compounds are quite sensitive to even mild oxidative agencies such as exposure to air, and are converted to disulphides. The only fixative which is claimed to preserve sulphydryls is acid alcohol (80% alcohol containing 1% trichloro-acetic acid). This mixture is a poor fixative which has a powerful cytolytic effect (e.g. red cells are largely destroyed; myoglobin is leached out of muscle) and a solvent action on many proteins such as insulin. Whatever sulphydryl is preserved by it is probably only a fraction of that originally present. In addition, an unpredictable percentage of the sulphydryl content will be oxidized in the course of cutting, floating, and drying the sections. For this reason, a positive reaction after fixation by acid alcohol is significant, but a negative reaction is meaningless. On the other hand, mercury salts are very powerful precipitants of proteins and also form insoluble mercaptides with sulphydryl compounds. On account of these features, they are considered excellent histological fixatives, especially in combination with formaldehyde. However, Lugol’s solution, used for the removal of mercury precipitates, will oxidize sulphydryls to disulphides. There is every reason to believe that sulphydryl groups produced by reduction in sections of mercury-fixed tissues will closely represent the total of sulphydryls plus disulphides.

originally present in the tissues, provided that reduction is reasonably quantitative, as it appears to be if the right reductant is employed (Mirsky and Anson, 1934–5).

Survey of Histochemical Methods for Sulphydryl Groups

**Fixation.** As mentioned, mercury bichloride formalin mixtures are excellent fixatives for —SH groups. However, theoretically, regular formalin should also be a good fixative since the reaction between cysteine and formaldehyde, yielding thiazolidine carboxylic acid, is readily reversible (Ratner and Clarke, 1937). Barnett and Seligman (1954) actually found that entirely satisfactory results are obtained with plain formalin.

The liberation of sulphydryl groups. The sections, after protection with dilute collodion following the second alcohol, are run down to water and immersed in Gram-Lugol’s solution in order to decompose mercaptides and thiazolidine carboxylic acid, respectively. Excess iodine is removed with a dilute solution of thiosulphate or bisulphite and the slides are washed under the tap.

For the reduction of disulphide groups, thioglycolic acid, alkanized to pH 7 to 8 (Rudall, 1952; Barnett and Seligman, 1954) or pH 11 (Hardy, 1952) is usually recommended. In the present studies it was found that thioglycolic acid can be replaced by thioglycerol (‘thiovanol’, Evans Chemetics, Waterloo, N.Y., U.S.A.), which offers the advantage that it requires no neutralization. Two to 3 ml of thioglycerol is dissolved in about 40 ml of distilled water and 10 ml of a 0.5 borate buffer at pH 8.5 to 9.1 (the latter is essentially a solution of borax) is added. The sections are left in this mixture at room temperature or at 37° for overnight. Subsequently, they are washed in several changes of large amounts of distilled water. Washing in tap water, as mentioned by Barnett and Seligman (1954), is not recommended on account of the danger of partial oxidation, especially in cities with a chlorinated water-supply. The sections are then kept in dilute (about 0.5%) acetic acid until use (within a few hours at most).

Histochemical reactions proper. (a) The nitroprusside reaction, introduced by Buffa (1904), is based on a fleeting purple-red colour given by sulphydryl groups in an alkaline medium containing nitroprusside; its mechanism is poorly understood. Neither the original method nor its modification by Giroud and Bulliard (1932) gave a shade of sufficient intensity in thin paraffin sections.

(b) The ferric ferricyanide test (Chévremont and Fréderic, 1943) is based on the formation of Prussian or Turnbull’s blue or of a mixture of both. The composition of the blue precipitate is not definitely known since sulphydryl groups may reduce either or both components of the mixture (ferric salt and ferricyanide). However, it would appear that the reduction of ferricyanide is more important since a mixture of uranyl nitrate and ferricyanide will give a brown coloration (uranyl ferrocyanide) of identical localization but much less brilliance.
The reagent mixture must be prepared fresh but its composition is not critical. Any combination of about equal parts of 0.2 to 1% ferric ammonium sulphate and potassium ferricyanide solutions will do. Lillie and Burtner (1953) recommend a mixture of 10 ml. of 1% ferricyanide, 75 ml. of 1% ferric chloride, and 15 ml. of water. The addition of a small amount of dilute acetic acid is optional. Ferric ammonium citrate, originally recommended by the writer (1952), should not be used because in the presence of citrate the sensitivity of the method is markedly lowered, and in the case of an excess of citrate no reaction is obtained. Some batches of ferric ammonium citrate contain a large excess of citrate.

This test, as it is well known, is not entirely specific. It will stain some other reducing substances such as melanin (Staemmmler, 1924) and enterochromaffin granules (Laskey and Greco, 1948) promptly and intensely. However, for reason of different localization, these substances are not likely to be confused with sulphhydryl groups. A major disadvantage of the Chêremont–Frédéric method is that it lacks a definite end-point. The longer the tissue is treated with the reagent mixture the more intense and diffuse the reaction will become. By comparison with sections in which the sulphhydryl groups had been blocked it was found that the optimum compromise between intensity and specificity of the reaction is obtained by a treatment of between 3 and 6 minutes at room temperature. The sections are washed in repeated changes of dilute mineral acid, then under the tap, counterstained as desired, and mounted in balsam.

(c) The mercaptide reaction is based on the formation of mercaptides by the treatment of the sections with salts of heavy metals, specifically mercury or cobalt. The mercaptide is either coloured and as such directly visible or converted into black metal sulphide or demonstrated by its high absorption under the electron microscope (Bahr and Moberger, 1954).

The cobalt mercaptide method of Tahmisian and Brues (1950) was found not to yield sufficient contrast for practical purposes. The first staining method involving mercurial mercaptide was developed by Bennett (1948; but unfortunately, Bennett’s reagent is not available on the market; its synthesis is difficult and the yield is very low. The writer has prepared several batches by an identical method and yet the results were not uniform. Some of the batches were excellent; others gave a rather diffuse staining of almost all tissue elements (especially of acidophil structures) without any specificity for sulphhydryl groups, or no staining at all. As a rule, alcoholic solutions of good preparations had a somewhat duller, slightly brownish shade, in contrast to the brilliant orange shade of unsatisfactory batches. The writer has also synthesized a number of organic mercurials with a better shade than that of Bennett’s reagent; histochemically, however, they all lacked specificity. This is very queer since in test-tube experiments they proved to be powerful inhibitors of sulphhydryl enzymes. A new mercaptide method was described by Mauri, Vaccari, and Kaderavek (1954). This utilizes a water-soluble azo dye obtained by the coupling of diazotized p-acetoxy-mercurianiline with
H-acid in an alkaline medium. Results secured with this mercurial will be discussed later.

(d) The reduction of tetrazolium salts by —SH groups was first applied to histochemical use by Barnett and Seligman (1952a) and subsequently used by Pearse (1953), Rogers (1953), Barnett and Seligman (1954), and Formisano and Montagna (1954). In vitro studies of the reaction were published by Roberts (1954) and Findlay (1955).

Not all tetrazolium salts are suitable for histochemical purposes. Pearse noticed that triphenyltetrazolium is not reduced by tissues although it is reduced slowly in the test tube (Findlay). 2,3-diphenyl-5-methyltetrazolium is not reduced by —SH groups even in the test tube (Roberts).

Both Pearse and Barnett and Seligman use relatively high pH values (12.8 and about 11, respectively) and add cyanide to the reagent mixture, in order to reduce —SS— groups. According to Findlay, cyanide also increases the efficiency of reduction by —SH groups. These highly alkaline solutions are somewhat destructive to tissue sections. Fortunately, it was found that neotetrazolium is reduced quite efficiently by —SH groups in the presence of cyanide at pH values as low as 8.5 to 8.8; in fact, even at 8.2 to 8.4, although at a considerably slower rate. Iodophenyl-nitrophentetrazolium (Rogers), which was not tried in the present studies, should be usable even around pH 7.4. However, —SS— groups are not readily reduced by cyanide below pH 11, and for this reason it is necessary to use thioglycolate or thioglycerol in a preliminary step.

By trial and error, the following method was found to yield uniformly satisfactory results:

Incubate sections reduced by thioglycerol for 1 to 2 hours at about 50° C in the following solution: dissolve 1 g of sodium cyanide in 10 to 15 ml of water; add a drop of phenolphthalein solution and enough M (6%) acetic acid to cause almost complete decolorization (about 15 ml will be needed). Add about 20 ml of a 0.5 M borate buffer at pH 8.5 to 8.8. Dissolve about 25 mg of neotetrazolium chloride in 5 ml of alcohol and pour it into the buffered cyanide solution. The mixture can be used many times. It may be kept in the refrigerator for several weeks and should be filtered before every use.

Sites of —SH groups will show up in shades of dull reddish-purple. The sections can be counterstained with haematoxylin and should be mounted in glycerol jelly.

At this point it should be mentioned that tellurous salts are also reduced by —SH compounds, with the formation of a black precipitate of elementary tellurium (Bersin and Logemann, 1933). However, this method did not work in histochemical experiments.

(e) Barnett and Seligman's DDD method (1952 a and b) is based on the attachment of naphtol groups to protein sulphhydrils. The naphtol is then demonstrated by azo-coupling in a second step.

It was found that the alcohol concentration of 30%, as specified by the
Protein-bound Sulphydryl and Disulphide Groups

authors, is important. Significantly higher concentrations of alcohol will result in more or less marked reduction in the intensity of the stain. The reagent mixture should be prepared fresh because stale solutions have a tendency to stain in a darker, redder shade but more diffusely and with less selectivity.

The diazonium salt recommended in the original procedure (diazo blue B, tetrazotized o-dianisidine) gives a variety of shades, ranging from blue to red. At sites of very high —SH concentration (hair cortex) the shade is blue; at other sites, however, it is hard to evaluate the staining in a semi-quantitative way because the equivalence of the shades is not clear. For instance, it is impossible to tell whether a dark red shade or a pale blue one is indicative of a higher —SH concentration. Actually, the variety of shades, while pleasing aesthetically, is confusing rather than informative. For this reason, it would appear that a diazonium salt giving a single shade might be preferable. A number of commercial diazonium salts proved to be usable; diazo red RC (diazotized o-chloro-o-anisidine), which yields a brilliant red hue, was generally utilized in the present work.

(f) Finally, a number of procedures should be mentioned which have not been tested in the present study.

Some of the methods are, no doubt, highly specific, such as the naphthol-maleimide method of Seligman, Tsou, and Barrnett (1954) or the naphthol-iodoacetamide technique of Barrnett, Tsou, and Seligman (1955). Others, like the methods adumbrated by Danielli (1950), appear potentially useful; however, they have not been put in practice so far. Techniques depending on oxidation with peracetic acid (Pearse, 1951; Lillie, Bangle and Fisher, 1953 and 1954; Scott, 1953; Braun-Falco and Rathjens, 1954) are neither sufficiently specific nor sensitive enough for the histochemical detection of —SH groups.

The problem of specificity. It is relatively easy to test histochemical methods for their specificity as —SH reagents since there is a number of good blocking reactions known.

Blockade by mercaptide formation is the least reliable of the tests. HgCl₂, a powerful protein precipitant, will react with —SH as well as with —NH₂ groups (Bjerrum, 1950). For instance, acidophilia which, no doubt, is due to —NH₂ groups, is distinctly reduced by treatment of the tissue with a dilute solution of a mercuric salt. Mercury blockade of both —SH and —NH₂ groups is reversible by —SH substances. In the present studies it was found that acidophilia suppressed by HgCl₂ treatment can be restored in a few minutes by amine buffers (e.g. glycine) around pH 8.5, while the —SH groups remain non-reactive after this treatment.

Conversion of —SH groups into —SS— by mild oxidants such as Lugol’s solution is reasonably effective and selective (Anson, 1940). However, this method can be used only if the reagent proper does not possess a reducing action. By reduction (e.g. alkaline cyanide) the blockade is readily reversed. Iodoacetate (Dickens, 1933; Rapkine, 1933) and ethyl-N-maleimide
Gomori—Histochemical Methods for

(Friedmann, Marrian, and Simon-Reuss, 1949) are efficient and almost completely specific blocking agents for —SH groups, both in the test tube and histochemically. To these, chloropicrin (Ray, Guha, and Das, 1919; Chevremont and Frédéric, 1943) (about 0.5% in 30% alcohol; 3 to 4 hours at room temperature) may be added.

It must be mentioned that alkaline cyanide solution may partially reverse the blockade effected by the last-mentioned substances, especially on greatly prolonged exposure. At 1 hour, blocked sections show no reaction at all with the tetrazolium method; from 90 minutes on there is a slowly increasing coloration of highly reactive structures (especially hair cortex), and this may attain considerable intensity in about 4 hours. However, this is not likely to cause error since a staining time of 60 to 90 minutes need never be exceeded.

Material and Methods

A large number of tissues, mainly of two species, man and the rat, was used. In the beginning, the fixative was a mercury bichloride–formalin mixture (1 part of concentrated formalin, 2 parts of a saturated solution of mercury bichloride, and 3 parts of water); however, practically all of the later material was fixed in plain 1:10 to 1:5 formalin. A number (3–12) of different tissues were processed together and embedded in a single paraffin block so that uniform treatment of all tissues was assured. The sections were handled according to the original specifications of the respective authors except for modifications as described in the preceding passages.

Results (see figs. 1 and 2)

As far as localization proper is concerned, identical pictures were obtained by all the procedures, except the method of Mauri, Vaccari, and Kaderavek, which will be discussed separately. The specificity of all methods, except the last-mentioned one, as judged by completely negative reactions after blockade, was found to be satisfactory. However, a marked difference between relative intensities of staining of various structures is observed with the individual methods. Generally speaking, Bennett’s, Chevremont–Frédéric’s, and the tetrazolium method give a high contrast, some structures being intensely coloured, others much less so or not at all. With the DDD method, staining is more diffuse, and contrasts distinctly less marked. For instance, the intensely reactive beta cells of the islets of Langerhans stand out in high contrast against the unstained alpha cells with the first three methods, while with the

Fig. 1 (plate). Pancreatic islet of guinea-pig (consecutive serial sections; not necessarily corresponding areas). Nuclear staining, wherever present in this fig. and in fig. 2, is due to haematoxylin or nuclear fast red.

A, neotetrazolium method. Beta cells stand out in good contrast. (Green filter.)
B, ferri-ferricyanide method. Good contrast. (Orange filter.)
C, Bennett’s method. Good contrast. (Dark blue-green filter.)
D, DDD method. Low contrast between alpha and beta cells. (Green filter.)
FIG. 1

G. GOMORI
FIG. 2

G. GOMORI
Protein-bound Sulphydryl and Disulphide Groups

DDD method the difference is much less striking, the beta cells being stained with moderate intensity and the alpha cells somewhat more lightly.

In addition to this general difference, other discrepancies of a more specific nature were also seen. Three of them will be described briefly.

1. There is very little staining of stratified squamous epithelium (except the keratin layer itself) by the tetrazolium method; somewhat more by the DDD method, and a fairly intense reaction is obtained with Bennett’s and Chèvremont–Frédéric’s techniques.

2. Certain keratin structures such as the cortex of hair, a dense core in the horny layer of the lingual papillae in the rat, and the deepest layer of keratin in the skin of the human palm and sole, are stained very dark by both the tetrazolium and DDD techniques; these same areas are stained very much less intensely, in fact, often not at all by Bennett’s and the ferric ferricyanide methods. It appears likely that the reagents used in the latter two methods penetrate dense keratin only with difficulty. Another interesting discrepancy in the staining of the keratin layer was reported by Findlay.

3. Red cells are stained in a very pale shade by the tetrazolium and ferric ferricyanide methods, and much more intensely by the DDD method. Staining by the Bennett technique cannot be evaluated because of the intrinsic yellowish cast of red cells.

The Mauri–Vaccari–Kaderavek method gives the most diffuse staining of all. Certain acidophil structures such as red cells, eosinophil granules, muscle, and keratohyalin granules, stand out in an especially intense shade. Cell nuclei are well stained. On the other hand, hair cortex and beta cells are almost undifferentiated. Surface keratin stains unevenly. Staining is prevented by treating the section with a dilute solution of HgCl₂, and this effect is reversed promptly by a dilute solution of cysteine or, somewhat more slowly, by a glycine buffer at pH 8.5 to 9.8. Borate or carbonate buffers at the same pH will not work.

While the staining by the Mauri–Vaccari–Kaderavek reagent cannot be explained on the basis of its being an acid dye, it is not specific for —SH groups either. The reagent is, in all likelihood, a true organic mercurial which happens to combine with the very prevalent and widespread —NH₂ groups as well as with —SH groups.

FIG. 2 (plate). A–D, filiform papillae of the rat’s tongue (consecutive serial sections).
A, neotetrazolium method. Keratinized portions react very intensely; epithelium pale. (Green filter.)
B, DDD method. Pattern essentially similar to that in A, except that the epithelium is darker. (Green filter.)
C, ferri-ferricyanide method. The most intensely reacting area is in the upper part of the prickly layer. The keratinized portions are practically unstained. (Orange filter.)
D, Bennett’s method. The pattern is very similar to that in C. (Dark blue-green filter.)
E–F, skin of human palm.
E, neotetrazolium method. The horny layer is stained moderately intensely, except the superficial and deep layers, which are fairly dark. (Green filter.)
F, ferri-ferricyanide method. The keratin layer is uniformly stained in a fairly deep shade, except a narrow band in the deepest layer, which is unstained. (Orange filter.)
Gomori—Histochemical Methods for

DISCUSSION

According to the data presented, a number of histochemical methods can be used for the localization of protein-bound —SH groups. However, since the relative staining intensities of various structures depend on the method used, semiquantitative visual evaluation of the results is not a reliable procedure. As long as there are no independent criteria available by which it could be decided whether the high-contrast methods or the relatively low-contrast DDD technique reflect a truer picture of the actual quantitative relationships, the choice of the method to be used will be up to the personal preference of the individual worker. From a purely practical point of view, Bennett's method will be, at least for the time being, out of the reach of most laboratories, owing to the unavailability of the reagent. The ferric ferricyanide method has the disadvantage of lacking a dependable end-point. For the reasons mentioned it would appear that at present the best available histochemical methods for protein-bound —SH groups are the tetrazolium and DDD techniques.

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Protein-bound Sulphydryl and Disulphide Groups

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