**THE EFFECTIVENESS OF OUR COLLOIDAL IRON METHOD
IN THE ELECTRON-HISTOCHEMICAL DEMONSTRATION
OF SULFHYDRYL AND DISULPHIDE WITH PARTICULAR
REFERENCE TO PANCREATIC ZYMOGEN GRANULES**

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The colloidal iron method here introduced is an improved form of the Chèvremont-Frédéric method for the SH+SS groups.

The principal point of improvement is the use of Müller's colloidal iron hydroxide instead of ferric sulphate as the ferric iron reacting with the ferrocyanide ion.

This modification significantly reduced staining contamination, significantly increasing staining contrast; therefore, it made to be possible the electron microscopical demonstration of the protein bound SH+SS groups.

However, strict care must be taken against non-specific reduction caused by the tissue components and their adsorption reactivity.

In the present experiment, this method was applied to the electron microscopic demonstration of the SH+SS groups in rat pancreatic acini, in which the internal portion and the limiting membrane of the secretory granules yielded clearly positive results.

Protein-bound sulfhydryl (SH) and disulphide (SS) groups are essentially found in protein-bound cysteine (-SH), or cystine (-SS).

SH+SS groups are known to have metabolic functions, especially the reversible SH and SS transformations which are important biological mechanisms. Therefore, an ultrastructural demonstration method of reasonable specificity, sensitivity and easy applicability for SH+SS groups is thought to be greatly available because of their vast biological significance.

However, but for a few preliminary reports (1, 2, 3, 4, 5, 6), no histochemical method generally applicable to the electron-microscopical demonstration of SH+SS groups has yet been offered. Therefore, the present authors modified the ferric ferrocyanide method of Chèvremont and Frédéric (7) by applying Müller's colloidal iron hydroxide as the ferric iron reacting with ferrocyanide (8, 9, 10). By virtue of availability of iron's high electron density and of obtaining a better staining contrast under the colloidal condition, the road to the electron-microscopical demonstration of SH+SS groups was opened up.

By applying this modified method to rat pancreatic acini in the present study, we could demonstrate the ultrastructural reactivity patterns of SH+SS groups in

Dedicated to Professor Hideo Takamatsu in memory of his retirement.

the exocrine cellular components, especially in the secretory granules and could confirm their biological significance.

MATERIALS AND METHODS

Experimental materials

Mature Wistar rats of both sexes, reared on a normal laboratory diet ("Oriental" solid feed) and water *ad lib.*, and weighing between 200 gr. and 400 gr., were used. The pancreases were removed from the animals, sacrificed under ether anesthesia; and tissue blocks, cut at about 2 mm × 2 mm × 3 mm, were immediately immersed in a cold 6 per cent formalin solution buffered at pH 7.4 with 0.1 M phosphate. After fixation for 6 to 12 hours, the tissues were rinsed thoroughly in frequent changes of water, and freeze-sectioned at a 50 μ thickness by using the electric freeze for microtome.

Experimental methods

The following staining directions followed:

1. Treat the sections with thioglycollic acid* if necessary to reduce the SS group.
 2. Treat them with N-ethyl-maleimide**, if necessary to block the SH group.
 3. Immerse them in the reagent medium*** for exactly 5 minutes, rinse them in 0.1 per cent acetic acid, and then wash them in several changes of changes of distilled water.
 4. Post-fix them in 1 per cent osmic acid buffered in a 0.1 M phosphate at pH 7.4, and wash them in several changes of distilled water.
 5. Dehydrate them in graded alcohols, and routinely embed them in Epon.
- Some sections were picked up before post-fixation and mounted in glycerin jelly for a light-microscopic check.

Ultrathin sections were double-stained with uranyl acetate and lead.

For comparison, light-microscopic observation was also performed on frozen sections of 10 μ in thickness, stained in the same manner and mounted in balsam.

RESULTS

Light microscopy

SH+SS groups: A clear staining reaction, giving a deep blue colour, was

* Thioglycollate reduction (11): Incubate the section for 4 hours at 37°C in freshly made 0.5 M thioglycollic acid titrated to pH 8.0 with 0.1 N NaOH. Then, wash them in several changes of distilled water, rinse them briefly in 1 per cent acetic acid, and wash them again in several changes of distilled water.

** Maleimide block (11): Treat the sections for 4 hours at 37°C with 0.1 M N-ethyl maleimide in a 0.1 M phosphate buffer at pH 7.4, and follow this by washing them in 1 per cent acetic acid and then in several changes of distilled water.

*** Reagent (9, 10): Add 5 ml. of freshly prepared 1 per cent potassium ferricyanide and 15 ml. of acetic acid to the Müller's colloidal ferric hydroxide solution, which is prepared by adding 36 ml. of a 35 per cent solution of FeCl₃ to 750 ml. of boiling distilled water, which must be immediately cooled through pouring water.

FIG. 1. Electron microscopical demonstration of SH+SS groups. Clear stainability is seen in the internal portion and limiting membrane of the secretion granules, and on the membrane system of the mitochondria and ergastoplasm in the vicinity of the granules. $\times 10,000$.

FIG. 2. Electron microscopical demonstration of SH+SS groups. A finely granular staining pattern is observable in the internal portion of the secretory granules and their limiting membrane. $\times 35,000$.

Fig. 3. Electron microscopical demonstration of SH group alone. A staining pattern similar to that seen in Fig. 1 is shown, but its reactivity is very weak, especially in the internal portion of the granules and in their limiting membrane, compared with that of SH+SS groups. $\times 10,000$.

observed in the whole exocrine portions. The islets also showed a positive but comparatively weak reactivity. As to the acinar cells, the apical portion of the cytoplasm showed a comparatively intense reaction, proportionate to the massive presence of secretory granules, but a strong basilar concentration of stainability occurred frequently. The reactivity of nuclei was nearly absent.

SH group: The histochemical reaction pattern was similar to that of SH+SS groups, but comparatively weak.

Blocking test: The reactivity did not become perfectly negative, but the staining pattern was similar to that of SH+SS groups, although it was very weak.

Electron microscopy

SS+SH groups: The most specific and strong reactions were observed in the secretory granules of the acinar cells (Figs. 1 and 2). Very finely granular reaction products were found very densely deposited in the limiting membrane of the secretory granules and they were also cloudily or dispersedly deposited in their contents. The limiting membrane of the well stained granules appeared as if reaction products were deposited linearly on its surface. The membrane systems of the rough ER and the mitochondria (cristae and limiting membrane) showed a mildly or fairly positive reactivity.

SH group: The secretory granules and the membrane system of the rough ER and the mitochondria showed a similar pattern of reactivity, although it was significantly weak, compared with those of SS+SH groups (Fig. 3).

Blocking test: The stainability was significantly depressed by this test, while

FIG. 4. Paraffin section stained for SH+SS groups. A strong staining reaction is observed in a comparatively apical portion of the acini in coincidence with the presence of secretory granules. $\times 200$.

FIG. 5. Transverse cutting of an epon embedded section stained for SH+SS groups. In practice, the permeability of the reagents is limited to within two or three cell layers from section surface so that the staining reactivity can be suitably positive within those layers.

a very weak reactivity remained, which was considered to be unspecific. The staining pattern was found to be similar to that of the SH+SS groups, although the internal reactivity of the secretory granules was most significantly depressed.

DISCUSSION

The colloidal iron method used in this experiment is an improvement upon the ferric ferricyanide method introduced by Chèvremont and Frédéric (7, 8). The operating principle is the reduction of a fresh solution of ferricyanide in a highly acidic pH (pH 2.4 in the Chèvremont-Frédéric method and pH 1.8 in the present method) by the sulfhydryl group in the tissue. The resulting ferrocyanide combines with ferric iron provided as ferric sulphate in the Chèvremont-Frédéric method, to give a precipitate of insoluble Prussian blue.

Our main modification is the utilization of colloidal ferric hydroxide used by Müller (9, 10) for the demonstration of acid mucopolysaccharides, instead of ferric sulphate or chloride as the ferric iron reagent.

The use of colloidal iron significantly reduced staining contamination, significantly increasing staining contrast (12, 13). Its points of advantage could already be seen on the light microscopic level (Fig. 4.), and our method made possible the demonstration of the protein bound SH+SS groups on the electron microscopic level. The physico-chemical property of ferric iron in a colloidal con-

FIGS. 6-a, b, and c. Frozen sections of $50\ \mu$ thickness taken before postfixation and mounted in glycerin jelly for light-microscopical and initial observation.

In 6-a, a strong staining reaction of SH+SS groups is seen in the whole acinar portion excluding the nuclei and connective tissue. In 6-b, the staining pattern of SH group is similar to that seen in 6-a, but it is far more weak; Fig. 6-c shows that the blocking test is generally effective, but the positive reaction remains faintly, perhaps because of a remaining unspecific reactivity.

dition in the medium may be the main factor yielding the improved results.

However, in utilizing this technique and interpreting the results, the following points must be carefully noted:

- (1) The reagents in the medium are not permeable through more than two to three cell layers from surface of the frozen section. (Fig. 5). Therefore, the reactivity of the SH group must be discriminated within those layers.

- (2) Excluding melanin, lipofuscin, and argentaffin granules which can be

easily discriminated, the specificity of the methods depends on (a) proof of the absence of non-protein reducing groups (14) such as lipids or vitamin C and on (b) the properties of ferricyanide reduction, as far as protein is concerned (for example, some protein groups other than the SH groups, notably tyrosine and tryptophan, as indicated by Pearse (15), are oxidized by ferricyanide).

To overcome these difficulties, a specific blocking test is essential before this method can properly be used for the demonstration of SH group (Figs. 6a, 6b, and 6c.). In practice, it was found that the results obtained with the Chèvremont-Frédéric method closely resemble those obtained with Bennett's RSR method on the light microscopical level (15, 16, 17).

In the present experiment, N-ethyl maleimide was used as a specific SH-blocking reagent and the blocked staining results were compared with those before blocking test.

As already described, a weakly positive stainability was observed even after the blocking procedure. The mechanisms involved in such a reaction remain to be investigated.

(3) Furthermore, the question arises as to whether the reagents such as the colloidal iron, bind themselves to the cell surface or to other cell components in some unspecific mode, for example, electrostatically (18, 19). For the solution of this problem, the following experiment was performed:

Either the colloidal ferric iron or the potassium ferricyanide was omitted from the reacting medium. In either case an unspecific binding of the reagent with the tissue components or with the section surfaces could not be observed.

Thus, the greater part of the reactivity of the cellular structures, especially the internal portion and the limiting membrane of the secretory granules, could be regarded as being that of the SH+SS groups.

This method, then, is a useful means, not only of demonstrating the ultrastructural mode of the protein bound SH+SS groups in the secretory granules, but also of investigating the dynamic aspects of these groups with respect to their biosynthesis, concentration process, and movement, as well as to the excretion of the secretory granules.

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