A Modified Method of Fine-Granular Cationic Iron Colloid Preparation: Its Use in Light and Electron Microscopic Detection of Anionic Sites in the Rat Kidney Glomerulus and Certain Other Tissues

Takuro Murakami, Takehito Taguchi, Aiji Ohtsuka, Kazuo Sano, Tetsuji Kaneshige, Robert L. Owen and Albert L. Jones

Departments of Anatomy (Prof. T. Murakami) and Surgery (Prof. S. Teramoto), Okayama University Medical School; Okayama Kosei Hospital (Dr. T. Sano) and Okayama Chuo Hospital (Dr. T. Kaneshige), Okayama, Japan; and Cell Biology and Aging Section (Prof. A. L. Jones), Veterans Administration Medical Center, San Francisco, California, U. S. A.

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Summary. Ferric chloride, when boiled with hydrazine hydrate and cacodylic acid, is converted into a fine cationic iron (ferric hydroxide) colloid which consists of 0.5-1.5 nm electron-dense granules, and gives a distinct Prussian blue reaction. This colloid allows light and electron microscopic detection of ionized anionic sites in tissues at a wide pH range of 0.8-7.6. It is smaller in size and more stable, and assures longer and greater staining of tissues, especially at low pH levels, than the iron colloid prepared with sodium or ammonium cacodylate by SENO and his associates (1983, 1984, 1985). Some light and electron micrographs of the rat kidney, spleen and other organs stained with our colloid are presented as examples. These micrographs confirm that the glomerular podocyte end-foot surface facing the Bowman’s capsular space is strongly negative-charged. They also show that almost all lymphoid cells around the arteries in the splenic white pulp and thymic cortex contain strongly negative-charged nuclei and that the distal convoluted and collecting urinary tubules are more negative-charged than the proximal convoluted tubules.

Ferric hydroxide, when dissolved in an acetic acid solution, is converted into a cationic iron colloid useful for light microscopic detection of tissue anionic sites such as acid mucopolysaccharides, though it must be dialyzed before use (Hale, 1946). Such a coarse cationic iron colloid can be prepared by dissolving ferric chloride in a mixture of glycerin and ammonia (Rinehart and Abul-Haj, 1951) or by pouring ferric chloride into boiling distilled water (Müller, 1955; Mowry, 1958, 1963). SENO and his associates improved these methods, and prepared a fine cationic iron colloid widely available for both light and electron microscopic detection of tissue anionic sites, including acid mucopolysaccharides, without dialysis at a pH range of 1.6-7.6, pouring ferric chloride into a boiling sodium cacodylate solution (SENO et al., 1983a). They further prepared a finer cationic iron colloid with better permeation into the tissues or with greater staining intensity of the tissues by boiling a mixture of ammonium cacodylate and ferric chloride (AKITA et al., 1984; SENO et al., 1985). Our recent experiments have
shown that a much finer cationic iron colloid with a more widely useful pH range and also with greater staining intensity of the tissues can be produced by boiling a mixture of hydrazine hydrate, cacodylic acid and ferric chloride (Murakami et al., 1986a, b, c). The details of these experiments are described in this paper.

MATERIALS AND METHODS

Hydrazine hydrate was added to 0.1 M cacodylic acid solution until the pH value reached 7.2–7.4. One volume of 0.1 M ferric chloride solution was added to ten volumes of this hydrazine hydrate-cacodylic acid solution. The hydrazine hydrate-cacodylic acid-ferric chloride mixture thus prepared was boiled for a few minutes (see below), and cooled to room temperature. For staining, the boiled and cooled hydrazine hydrate-cacodylic acid-ferric chloride mixture was diluted with 2 volumes of the hydrazine hydrate-cacodylic acid solution (pH 7.2–7.4) and divided into four parts, the pH values of which were adjusted to 1.0, 1.8, 4.0 and 7.0 with 1–10 N HCl or 0.1 M NaHCO₃ solution (see below).

Small blocks of kidney and spleen were isolated from adult Wistar rats which had been fixed by arterial perfusion with 0.1 M cacodylate-buffered 2–4% glutaraldehyde or 10% formalin solution (pH 7.2–7.4), Bouin’s solution or Pearse’s fixative containing saturated picric acid, 4% glutaraldehyde and 5% acetic acid. The blocks were refixed in these fixatives for about 6 hrs, embedded in paraffin, and cut into sections 10–15 µm in thickness. The sections were immersed in a series of xylol and ethanol, and washed in distilled water.

The sections were then incubated in the boiled and diluted hydrazine hydrate-cacodylic acid-ferric chloride mixture (see above) for 30 min or longer at room temperature. After this staining, the sections were rinsed in distilled water, immersed for 30 min or longer in a mixture of 1% K₄Fe(CN)₆ and 1% HCl for the Prussian blue reaction, rinsed in distilled water, post-stained with 0.1% nuclear fast red in 5% aluminium sulfate, embedded in balsam, and observed with a light microscope.

Some of the glutaraldehyde-fixed kidney blocks were cut into very thin pieces 0.2–0.3 mm in thickness. These pieces were incubated in the boiled and diluted hydrazine hydrate-cacodylic acid-ferric chloride mixture (see above) for 6 hrs at room temperature. They were post-stained with 1% osmic acid, embedded in epoxy resin, cut into ultra-thin sections (silver-gray), and observed, without any additional metal staining, with a transmission electron microscope.

Some physicochemical properties of the boiled and diluted hydrazine hydrate-cacodylic acid-ferric chloride mixture were tested at various pH values. The absorption spectrum was estimated with a spectrophotometer. This estimation was done also before boiling. The electric charges were examined with a paper electrophoretic analyzer. Reactions with anionic and cationic substances were tested by the column chromatographic method of Seno and his associates (Akita et al., 1984; Seno et al., 1985), using ion-exchange resin particles (anionic particles: IR–120 and CG–50; cationic particle: IRA-400) (Röhm and Hass, PA, USA). Furthermore, the granulations were observed with a transmission electron microscope after dispersion on the collodion-coated micromeshes and freeze-drying.
RESULTS

The hydrazine hydrate-cacodylic acid-ferric chloride mixture was yellowish and displayed, in the spectrophotometric analysis, a marked absorption spectrum or band at 470 nm, showing the formation of ferric cacodylate complexes. This mixture, when boiled, turned from its yellowish tint to deep reddish brown and lost its marked absorption band at 470 nm, which indicated the formation of ferric hydroxide. The mixture thus boiled gave a distinct Prussian blue reaction and carried, in the paper electrophoretic analysis, a positive electric charge at a pH range of 0.8-7.6. The column chromatographic affinity test showed that the boiled mixture reacted with the strongly anionic particle IR-120 (IR-SO$_3^-$) at a pH range of 0.8-7.6 and with the carboxyl particle CG-50 (IR-COO$^-$) at a pH range of 4.0-7.6. Neither reaction nor affinity of the boiled mixture with the cationic particle IRA-400 (IR-NH$_3^+$) was observed at the examined pH range of 0.8-7.6. The transmission electron microscopic examination revealed that the boiled mixture consisted of fine electron-dense granules of 0.5-1.5 nm in size (more strictly, 1.0-1.5 nm at pH value 1.0, 0.8-1.2 nm at pH value 4.0, and 0.5-1.0 nm at pH value 7.0). These findings apparently confirm that the boiled mixture is a cationic iron colloid, more strictly, a cationic ferric hydroxide colloid.

Under the light microscope, the sections of the kidney and spleen fixed with glutaraldehyde, formaldehyde, Bouin's fluid and Pearse's fixative showed principally the same reactions to the staining with the boiled hydrazine hydrate-cacodylic acid-ferric chloride mixture (cationic ferric hydroxide colloid, see above). Regardless of the fixatives, the kidney sections stained at pH values 1.0-1.8 showed a distinct Prussian blue reaction in the glomerulus except for its nuclei reddened with nuclear fast red; the proximal convoluted urinary tubules were stained red with nuclear fast red, though their nuclei were stained either red or reddish-blue; the distal convoluted and collecting urinary tubules were stained reddish-blue, though their nuclei were stained distinctly blue (Fig. 1A). The connective tissues were barely stained at pH value 1.0, but stained rather blue at pH value 1.8 (Fig. 1A). At pH value 4.0, almost all elements of the glomerulus, including the nuclei, and the nuclei of the distal convoluted and collecting tubules were stained distinctly blue; the proximal convoluted tubule was stained either red or reddish-blue, though its nuclei were stained either blue or bluish-red (Fig. 1B). The erythrocytes suspended in the blood vessels, including the glomerular capillaries, were only slightly stained blue at pH value 4.0. At pH value 7.0, all urinary tubules and their nuclei were stained blue; overall diffuse blue staining was observed in all segments except the erythrocytes in the blood vessels stained red or reddish-blue (Fig. 1C). In the spleen sections stained at pH values 1.0-1.8, the lymphoid cells—especially their nuclei—around the central artery or its branches (or in the inner zone of the white pulp) showed a marked and selective Prussian blue reaction; almost all lymphoid cells in other areas of the white pulp were stained rather red with nuclear fast red, though the reticular cells were stained slightly blue (Fig. 2A, D). At pH value 4.0, the Prussian blue reaction in the white pulp was enhanced; slight blue staining was noted in many presumable reticular cells and macrophages, in the red pulp (Fig. 2B). At pH value 7.0, diffuse blue staining was observed in all segments, including the red pulp; the nuclei of the cells in the white and red pulps were stained also blue (Fig. 2C).

Transmission electron microscopy of ultra-thin sections allowed detailed analysis of either the reaction sites of the cationic iron colloid or its granules. In the glomerular sections from the kidney pieces stained at pH values 1.0-1.8, a dense distribution of
electron dense fine granules of about 1.0-1.5 nm was noted on the podocyte end-foot surface facing the Bowman's capsular space; few granules were observed in other segments except the outmost area (lamina rara externa) of the basement membrane where some granules were suspended (Fig. 3A). In the specimens stained at pH value 4.0, granules of 0.8-1.2 nm were distributed more densely on the podocyte end-foot surface facing the Bowman's capsular space; reaction of the granules with the lamina externa increased. Some granules were found sporadically in other areas of the basement membrane, on the surface of the capillary endothelium, and within the podocyte end-feet and capillary endothelial cells (Fig. 3B). In the specimens stained at pH value
7.0, the granules showed rather low electron densities, appearing 0.5–1.0 nm in size. They were distributed over the entire surfaces of the podocyte end-feet and capillary endothelium, in all areas of the basement membrane, and also within the podocyte end-feet and capillary endothelial cells (Fig. 3C). However, the staining of the podocyte end-foot surface facing the Bowman’s capsular space was not so intense as those observed at pH levels 1.0–4.0.

DISCUSSION

This paper describes a modification of the method by Seno and his associates for preparation of a fine cationic iron colloid (Akita et al., 1984; Seno et al., 1985). This modified method is characterized by the use of hydrazine hydrate and cacodylic acid: more strictly, hydrazine hydrate (see below). The physicochemical properties, including affinities with the ion-exchange resin particles, of the colloid obtained (see above) are similar to those prepared with ammonium cacodylate by Seno and his associates.
However, the use of the hydrazine hydrate and cacodylic acid has the advantage of producing a more stable colloid which assures longer staining at lower pH levels than the colloid of Seno and his associates (Akita et al., 1984; Seno et al., 1985). Our preliminary experiments in this study have shown that the colloid of Seno and his associates is useful for 6 hr staining at pH value 1.8, but not suitable for staining at pH value 1.0 as it immediately loses its deep reddish brown tint (or effectiveness in tissue staining) at this pH value. In contrast, our colloid prepared with hydrazine hydrate and cacodylic acid can be used for 24 hrs or longer at pH value 1.8, and for 8 hrs or longer at pH value 1.0. Furthermore, it should be noted that our colloid is very fine. Seno and his associates described the granules of their colloid as 1.0 nm in diameter (Akita et al., 1984; Seno et al., 1985). However, our supplementary transmission electron microscopy of rat kidney specimens stained with their colloid have shown that their granules are 1.5-2.5 nm in size at pH values 4.0-7.0 and larger than our granules. Our granules are also finer than those induced by simple boiling (Müller, 1955; Mowry, 1958, 1963) or prepared with sodium cacodylate (Seno et al., 1983a), glycerine and ammonia (Rinehart and Abul-Haj, 1951), or acetic acid (Hale, 1946).
The staining or reacting patterns of our colloid in tissues, when observed under a light microscope, are similar to those of the colloid of Seno and his associates (Akita et al., 1984; Seno et al., 1985). However, the staining intensity of our colloid is greater than that of the colloid of Seno and his associates (Akita et al., 1984; Seno et al., 1985). This may be noted clearly by the fact that our colloid distinctly stains the nuclei of the distal convoluted and collecting urinary tubules blue at pH values of 1.0–1.8. Such a distinct blue reaction for these structures at low pH levels has not been observed in specimens from Seno and his associates (Akita et al., 1984; Seno et al., 1985). A few differences can also be noted in transmission electron microscopy, especially in the specimens stained at pH value 7.0. Firstly, the colloidal granules of Seno and his associates have a distinct electron density with clear delineation at pH value 7.0 (Akita et al., 1984; Seno et al., 1985), though our granules are rather obscurely delineated and also poor in electron density at pH value 7.0. Secondly, many granules are found within the podocyte end-feet and capillary endothelial cells of our specimens stained at pH value 7.0, though such marked intracellular distribution of granules can not be recognized in the specimens prepared by Seno and his associates at pH value 7.0 (Akita et al., 1984; Seno et al., 1985). These differences may be due to the fact that our granules are finer and more permeable than those of Seno and his associates (Akita et al., 1984; Seno et al., 1985), and react more strongly with the intracellular matrices or cell organelles, including the nuclei. The finer granules may give lower electron densities in transmission electron microscopy.

It should be further noted in transmission electron microscopy that our granules are distributed more densely on the podocyte end-foot surface facing the Bowman’s capsular space than those of Seno and his associates. For example, the granules of Seno and his associates were loaded in 3–4 layers on the surface at pH value 4.0 (Seno et al., 1985), whereas our granules comprised 5–6 layers at this pH value (Fig. 3B). This finding, together with that obtained in light microscopy (see above), confirms that our colloid has greater staining intensity than that of Seno and his associates (Akita et al., 1984; Seno et al., 1985). As far as comparison among the specimens stained at pH values 1.8–7.0, suspension of the granules in the glomerular basement membrane is denser in the specimens of Seno and his associates (Akita et al., 1984; Seno et al., 1985) than in ours (Fig. 3). This may be attributed to the fact that our granules, non-specifically suspended in this membrane, are washed out or removed more easily in the successive washings (see above) than those of Seno and his associates (thicker than ours, see above) (Akita et al., 1984; Seno et al., 1985).

The light microscopic appearance of the kidney sections stained with our colloid at pH levels 1.0–1.8 also resembles others stained with alcian blue (Quintarelli et al., 1964a, b) or a dialyzed or coarse iron colloid (Müller, 1955; Mowry, 1958, 1963). However, our colloid as well as that of Seno and his associates (Akita et al., 1984; Seno et al., 1985) stains the specimens much more deeply and clearly than alcian blue or dialyzed iron colloids, including those of Hale (1946) and Rinehart and Abul-Haj (1951). The electron microscopic appearance of the glomerular vascular wall stained with our colloid at low pH levels is also similar to those obtained by perfusion staining with alcian blue (Behnke and Zelander, 1970), ruthenium red or other cationic substances (Kanwar and Farquhar, 1979; Reale et al., 1983). The electron microscopic findings in the glomerular vascular wall obtained by intravenous injection of highly concentrated and pH 7.3–adjusted polycationic polyethyleneimine (Andrews and Bates, 1985) are similar to those obtained by immersion staining of fixed tissues with the pH 7.0–adjusted colloid of Seno and his associates (Akita et al., 1984; Seno et al., 1985), but differ from ours in
that the intracellular matrices in our specimens are strongly stained (see above).

It is noteworthy that the lymphoid cells in the inner zone of the splenic white pulp are stained distinctly blue at pH levels 1.0–1.8 (Fig. 2A, D). As far as we know, no one has reported such a gathering of strongly acid or negative-charged lymphoid cells in the splenic periarterial area. However, an electrophoretic analysis of the suspended cells from the mouse spleen, thymus and lymphnode has shown that the T lymphocytes carry stronger negative charges than do the B lymphocytes (Mehrisi and Zeiller, 1974). This analysis suggests that the strongly negative-charged lymphoid cells in the spleen are T lymphocytes though it remains unknown as to which subpopulations or subsets they belong (Boesen and Hokland, 1982; Matutes and Catovsky, 1982) or whether they are migrating cells or not (Brelinska et al., 1984). It is also noteworthy that the distal convoluted and collecting urinary tubules are more negative-charged (or begin to react at lower pH levels) than the proximal convoluted urinary tubules (Fig. 1A, B). It is well known that urine is acidified in the distal convoluted urinary tubules. The enhanced negative charge of the distal convoluted and lower urinary tubules, including the collecting tubules, may have an advantage of protecting these tubules against such acidified urine.

Our colloid can be used widely for clear detection of anionic sites also in other organs or tissues. For example, such strongly negative charged lymphoid cells as
observed in the spleen were noted also in the thymus, lymphnode and blood, though they were not noted in the bone marrow (Fig. 4). Some secretory cells closely associated with blood capillaries in the pituitary anterior lobe and almost all secretory cells in the pituitary intermediate lobe showed a marked Prussian blue reaction at pH values 1.0-4.0. It was suggested in the liver specimens stained at pH 4.0 that the liver cells are more negative-charged as they are followed more peripherally in the liver lobules.

With the use of our colloid, the staining of tissues occurred usually more intensely at higher pH levels. However, the staining of some tissue elements such as the podocyte end-foot surface facing the Bowman's capsular space was rather weaker at pH value 7.0 than at pH value 1.0, 1.8 or 4.0. This may be caused partially by a local reduction in the number of ionized anionic groups in tissues at pH values 7.0, probably due to their conjugation with cationic groups, including the amino groups (QUINTARELLI et al., 1964a, b; SENO et al., 1985), as well as partially by decreased ionization of our colloidal granules at pH value 7.0. It is well-known that weakly anionic (or acid) groups such as carboxyl groups are hardly ionized at low pH levels. This shows that the nuclei of the distal urinary tubules and the splenic periartrial lymphoid cells, as well as the glomerular podocyte end-foot surface facing the Bowman's capsular space, selectively stained at pH values 1.0-1.8, contain strongly anionic (or acid) groups such as sulfate groups. The affinity test with the ion-exchange resin particles (see above) shows that our colloid reacts with ionized sulfate groups at a pH range of 0.8-7.6, and with ionized carboxyl groups at a pH range of 4.0-7.6. This means that our colloid stains sulfate groups at pH values lower than 4.0, sulfate and carboxyl groups of acid polysaccharides, including mucopolysaccharides, at pH level 4.0, and all the sulfate and carboxyl groups, including weakly acid proteins, at pH level 7.0. Affinities or reactions of our colloid with phosphate, sulfhydryl and other anionic groups have not been tested.

Our colloid is finer and more permeable than that of SENO and his associates (AKITA et al., 1984; SENO et al., 1985) (see above). However, the blocks for transmission electron microscopy should be sliced into very thin pieces for staining even when our colloid is used, since it permeates into the tissues only 0.1-0.2 mm per 3 hrs at room temperature. Immediate or perfusion fixation is recommended as the cells or tissues fixed after their death or degeneration are sometimes stained diffusely blue even at low pH levels. In light microscopy, counter-staining with nuclear fast red or aluminium-nuclear fast red complex (SEN et al., 1983a, 1985; AKITA et al., 1984) is recommendable since it contrasts the Prussian blue reaction (ROMEIS, 1948). The aluminium-nuclear fast red complex is monocationic and stains the tissue anionic sites which are left unstained with our polycationic iron colloid. It should be further mentioned that the pH value of the hydrazine hydrate-cacodylic acid solution (see above) should be adjusted to 7.2-7.4. If adjusted to lower pH levels, larger granules may be obtained. Adjustment of this solution to pH values higher than 8.0 may prepare finer colloidal granules. These, however, carry a negative electric charge (isoelectric point: pH 7.8-7.9), and stain some tissue elements such as the straight portion of the urinary tubule differentially blue.

Our colloid, as that of SENO and his associates (SEN et al., 1983a-c, 1985; TSUJI et al., 1984a, b; AKITA et al., 1984), can be used in perfusion experiments for the detection of anionic sites on living endothelial cell surfaces. Puncture perfusion of the colloid into living tissues may allow the detection of anionic sites of the living tissues, including the connective tissues and their cells. Furthermore, the colloid can be used for the conductive staining of biological specimens in non-coated scanning electron microscopy.
When colloid treatment at pH value 7.0 is done before tannin-osmium impregnation, well highlighted and also well contrasted scanning images are produced, enabling highly magnified observations of tissues (MURAKAMI et al., 1986a). Tissue elements selectively stained with the colloid at low pH levels may produce selective information both in back-scattered scanning electron microscopy and in X-ray microanalysis.

As described above, the present modification is characterized by the use of hydrazine hydrate, which is more strongly basic than the previously used ammonia (RINEHART and ABUL-HAJ, 1951; AKITA et al., 1984; SENO et al., 1985). It is believed that hydrazine hydrate transmutes the ferric cacodylate complexes (see above) into finer and more stable granules than ammonia. In our preliminary experiments, we used other strongly basic agents such as thiocarbohydrazide and p-phenylenediamine as a substitute for hydrazine hydrate. However, these agents reduced the ferric chloride and formed undesired deposits not useful for a colloid. Some amino acids such as glycine were available as buffers or substitutes for cacodylic acid, but produced a cationic iron colloid with a limited use at a pH range of 2.0–4.0. Phosphate buffer was deemed not useful as it reacted with ferric chloride and formed undesired precipitates.

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Prof. Takuro Murakami, M.D.
Department of Anatomy
Okayama University Medical School
2–5–1, Shikata-cho, Okayama
700 Japan