Fine Anionic Iron Colloid and Its Use in Light and Electron Microscopic Detection of Cationic Sites in the Rat Kidney

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Summary. Ferric chloride, when boiled with ammonium thiocyanate, ammonia and cacodylic acid, is converted into a fine anionic iron colloid which consists of 1–1.5 nm electron dense granules and gives a distinct Prussian blue reaction. This colloid allows light and electron microscopic detection of ionized cationic sites in tissues at a pH range of 4.0–9.0. Light and electron micrographs of the rat kidney stained with this colloid are demonstrated. These micrographs indicate that the foot processes of glomerular podocytes and the brush border of the proximal convoluted tubule contain positively-charged groups in their intracellular matrices, and that the foot process plasma membrane fronting the Bowman's capsular space has positively-charged groups. The latter finding, together with our previous study (MURAKAMI et al., 1986), suggests the co-existence of cationic and anionic sites on this foot process surface.

Cationic iron colloid staining is useful for the detection of acid mucopolysaccharides, sialic acids and other anionic sites in tissues (HALE, 1946). The staining has been improved by many authors (RINEHART and ABUL-HAJ, 1951; MÜLLER, 1955; MOWRY, 1958, 1963; SENO et al., 1983a; AKITA et al., 1984; SENO et al., 1985; MURAKAMI et al., 1986). Among them, SENO and his associates prepared fine cationic iron colloid by boiling a mixture of ammonium cacodylate and ferric chloride (AKITA et al., 1984; SENO et al., 1985). A recent study of ours has shown that a mixture of cacodylic acid, hydrazine hydrate and ferric chloride, when boiled, induces a finer cationic iron colloid (MURAKAMI et al., 1986). These fine cationic iron colloids are available for electron microscopy as well as light microscopy (AKITA et al., 1984; SENO et al., 1985; MURAKAMI et al., 1986).

For the detection of cationic sites in tissues, anionic iron colloid is useful. A mixture of ferric oxide and potassium ferrocyanide (GASIC et al., 1968) or commercially available chondroitin sulfate-iron colloid (Blutar: Dainippon-Seiyaku, Osaka) (SENQ et al., 1983b) has been used for this purpose. However, because of their large granular size (5–20 nm), these colloids can be applied only for cell surface analysis. We have recently prepared a fine anionic iron colloid (1.0–1.5 nm) with a wide range of application for both light and electron microscopic detection of tissue cationic sites, including the intracellular ones. The present paper describes its preparation and use in studying the rat kidney.
MATERIALS AND METHODS

Distilled water containing 28% ammonia was added to 0.1 M cacodylic acid solution until the pH value reached 7.2-7.4. One volume of 1M ammonium thiocyanate solution and one volume of 0.1M ferric chloride solution were added to ten volumes of this ammonia-cacodylic acid solution. The original mixture thus containing ammonium thiocyanate-ferric chloride-ammonia-cacodylic acid was boiled for a few minutes (see below), and cooled to room temperature. This mixture was then diluted with two volumes of the ammonia-cacodylic acid solution (pH 7.2-7.4) and divided into three parts, the pH values of which were adjusted to 4.0, 7.0 or 9.0 with 1-10 N HCl or 28% ammonia water (see below).

Small blocks of the kidney were isolated from adult Wistar rats which had been fixed by arterial perfusion with 0.1M cacodylate-buffered 2-4% glutaraldehyde or 10% formalin solution (pH 7.2-7.4), Bouin’s solution or Pearse’s fixative (pH 1.5-2.0). The Pearse’s fixative contained saturated picric acid, 4% glutaraldehyde and 5% acetic acid. The blocks were refixed in the original fixatives for about 6 hrs, embedded in paraffin, and cut into sections of 5-10 µm in thickness, immersed in a series of xylol and ethanol, and washed in distilled water.

The sections were then incubated in the boiled and diluted ammonium thiocyanate-ferric chloride-ammonia-cacodylic acid 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 eosin, embedded in balsam or Entellan New (E. Merck, Darmstadt, FRG), and observed with a light microscope.

Some of the glutaraldehyde-fixed kidney blocks were cut with razor blades or with a vibratome into thin slices of a 0.2-0.3 mm thickness. These slices were incubated in the boiled and diluted ammonium thiocyanate-ferric chloride-ammonia-cacodylic acid mixture (see above) for 6 hrs at room temperature. They were post-stained with 1% osmic acid, embedded in an epoxy resin, cut into ultra-thin sections (silver-gray), and observed, without any additional metal staining, with a transmission electron microscope. During this process, osmium staining was sometimes omitted.

Some physicochemical properties of the boiled and diluted mixture were tested at various pH values. The electric charges were examined with a U-tube electrophoretic analyzer. The reactions with anionic and cationic substances were tested by the column chromatographic method according to Seno and his associates (Akita et al., 1984; Seno et al., 1985), using ion-exchange resin particles (cationic particles: IR-400 and IR-45; anionic particles: IR-120 and IR-84) (Amberlite Resin: Röhm and Haas, PA, USA). The granulations were further observed with a transmission electron microscope after their dispersion on the collodion-coated micromeshes and freeze-drying.

RESULTS

The ammonium thiocyanate-ferric chloride-ammonia-cacodylic acid mixture, when boiled, turned from its yellowish hue to reddish brown. This boiled mixture gave a distinct Prussian blue reaction with potassium ferrocyanide, and carried, in the electrophoretic analysis, a negative charge at a pH range of 3.5-9.5 (Fig. 1). The column chromatographic affinity test showed that the boiled mixture reacted with strongly
basic or cationic particles (IR-400: functionally acts as ammonium) and weakly basic or cationic particles (IR-45: functionally acts as polystyrene polyamine) at a pH range of 3.5-9.5. Neither reaction nor affinity of the boiled mixture with the acid or anionic particles (IR-120: functionally acts as sulfate; IR-84: functionally acts as carboxylate) was observed at the examined pH range of 3.5-9.5. The transmission electron microscopic examination revealed that the boiled mixture consisted of fine electron-dense granules of 1.0-1.5 nm in size (Fig. 2). These findings apparently confirmed that the boiled mixture was an anionic or negative-charged iron colloid.

Under the light microscope, the sections of the rat kidney fixed with glutaraldehyde, formaldehyde, Bouin's fixative and Pearse's fluid showed principally the same reactions to the staining with the boiled ammonium thiocyanate-ferric chloride-ammonia-cacodylic acid mixture (fine-granular anionic iron colloid). The kidney sections stained at pH value 4.0 showed a distinct Prussian blue reaction in the glomerulus and the distal convolution of the urinary tubules; the brush border in the proximal convolution was stained blue, though the cytoplasm was stained red or bluish-red; all nuclei of the glomeruli and urinary tubules were diffusely stained blue except for the spotty unstained nucleoles (Fig. 3A). At pH value 7.0, the glomerulus, the distal convoluted urinary tubule and the brush border of the proximal convoluted urinary tubule were stained blue; the nuclei, except the nucleoles, were likewise stained blue (Fig. 3B). At pH value 9.0, the nuclei and the macula densa of the distal convoluted urinary tubule were stained distinctly blue; the glomerulus, distal convoluted urinary tubule and
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The collecting tubule were stained reddish-blue; the brush border of the proximal convoluted urinary tubule was stained reddish-blue, while the cytoplasm was stained red (Fig. 3C).

Transmission electron microscopy of ultra-thin sections, especially those from the blocks without osmium counterstaining, allowed the detailed and exact analysis of the reaction sites of the anionic iron colloid or its granules. In the glomerular sections from the rat kidney slices stained at pH value 4.0, electron dense fine granules of anionic iron colloid were diffusely distributed within the podocyte foot processes (Fig. 4A, B). The plasma membrane of the foot processes facing the Bowman’s capsular

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Fig. 3. Light micrographs of the glutaraldehyde-fixed rat kidney sections stained with our boiled and diluted ammonium thiocyanate-ferric chloride-ammonia-cacodylic acid mixture (see text) at pH values 4.0 (A), 7.0 (B) and 9.0 (C). Note that the nuclei and distal convoluted urinary tubules (d) show a distinct Prussian blue reaction at a pH range of 4.0–9.0, and that the glomeruli (g) and the brush border of the proximal convoluted urinary tubules (p) are stained blue at pH values 4.0 and 7.0. A–C: ×360
Fig. 4. Transmission electron micrographs of ultra-thin sections obtained from glutaraldehyde-fixed rat kidney pieces, stained with the boiled and diluted ammonium thiocyanate-ferric chloride-ammonia-cacodylic acid mixture (fine anionic iron colloid) at pH values 4.0 (A, B), 7.0 (C, D) and 9.0 (E, F), and post-stained with osmic acid (B, D, F) or not osmicated (A, C, E) (see text). Note that numerous electron dense fine granules of the anionic iron colloid are distributed in the foot process cytoplasm and its plasma membrane facing Bowman’s capsular space at pH values 4.0 and 7.0. f Podocyte foot process, e endothelial cell. A–F: ×100,000
space was intensely stained with the anionic iron colloid. The basal areas of the slit membrane between the foot processes distinctly reacted with the colloidal granules. Diffuse distribution of the colloidal granules was observed in the lamina rara externa and interna of the basement membrane and in the endothelial cells. In the specimens stained at pH value 7.0, the diffuse distribution of electron dense granules of anionic iron colloid was observed within the podocyte foot processes and their plasma membrane; in the endothelial cells, a slight distribution of the colloid granules was observed; the lamina rara externa, lamina densa and lamina rara interna of the basement membrane were not stained (Fig. 4C, D). In the specimens stained at pH value 9.0, few granules of the colloid were found in any components of the glomerulus (Fig. 4E, F).

DISCUSSION

The present paper describes a new method for the preparation of a fine granular anionic iron colloid. This method is characterized by the use of ammonium thiocyanate, cacodylic acid and ammonia. Ammonium thiocyanate is of special importance in this preparation. A simple mixture of ferric chloride-cacodylic acid-ammonia, without ammonium thiocyanate, is converted into a cationic iron colloid (Akita et al., 1984; Seno et al., 1985).

Ferric oxide-potassium ferrocyanide (Gasie et al., 1968) and chondroitin sulfate-iron (Seno et al., 1983b) colloids have been used for histochemical or cytochemical detection of cationic site in the tissues. However, these anionic iron colloids are so coarse or large in granular size (5-20 nm) that they cannot be used for block staining. In contrast, our anionic iron colloid is fine and allows block staining for electron microscopy.

Our anionic colloid is stable in a pH range of 4.0-9.0. At pH levels less than 3.4, the colloidal granules dissolve, discharge free ferric ions, and finally form a thiocyanato-iron(III) complex not useful as an anionic colloid. The thiocyanato-iron(III) complex formation is easily noted as the colloid is markedly darker red. At pH levels higher than 9.6, the colloid loses its stability and precipitates.

In the kidney specimens stained with the anionic chondroitin sulfate-iron colloid by Seno and his associates, no tissue component gave a positive Prussian blue reaction at pH value 7.0 (Seno et al., 1983b). However, the specimens stained with our anionic iron colloid at pH value 7.0 gave a distinctive Prussian blue reaction in the glomerulus, the distal convolution, the brush border of the proximal convolution and the nucleus. This difference may be due to the granular sizes (Seki, 1961). It is believed that our colloidal granules are sufficiently small (1.0-1.5 nm in size) to easily permeate into the tissues or react with their cationic sites.

At pH value 9.0, the appearance of the kidney sections stained with this anionic iron colloid was similar to that stained with the cationic iron colloid prepared with cacodylic acid and ammonia (Akita et al., 1984; Seno et al., 1985) or cacodylic acid and hydrazine hydrate (Murakami et al., 1986). This similarity may be due to the electric charge conversion of the cationic colloids from positive to negative at pH values higher than 8.0 (Hazel and Ayres, 1931; Murakami et al., 1986).

It should be noted that the glomerulus is distinctly stained blue with our anionic iron colloid at pH values 4.0-7.0, and that at these pH values, the colloidal granules are distributed in the plasma membrane and intracellular matrices of the podocyte foot processes. This shows the existence of basic groups in these structures, which are
positively charged in the pH levels lower than 7.0. It has been shown that the foot process surface facing the Bowman’s capsular space is weakly stained with the cationic iron colloids at pH level 7.0, and strongly at pH level 4.0 (Send et al., 1985; Murakami et al., 1986). The present staining, namely the marked deposition of our anionic granules on the foot process surface, clearly reveals the exact occurrence of cationic groups even on the foot process surface, and gives evidence to support the view that the weak stainability of the foot processes with the cationic colloids at pH level 7.0 is caused by conjugation of the cationic groups with the anionic groups on the foot process surface (Quintarelli et al., 1964a, b; Seno et al., 1985; Murakami et al., 1986).

It should be further noted that the brush border in the proximal convoluted tubule is stained blue with our anionic iron colloid at pH values 4.0 and 7.0, and that at these pH values, the colloidal granules are mainly distributed within the microvilli of the epithelial cell (Fig. 5). This shows the existence of enhanced cationic groups within the microvilli. Previous cationic iron colloid staining has shown that the surface coat of the microvilli of the proximal tubule is anionic or negatively charged (Seno et al., 1983a). The cationic sites demonstrated here in the intracellular matrices in the microvilli, especially those beneath the cell membrane, may act to anchor the anionic surface coat onto the microvillous surface.

It is also noteworthy that the distal convolution of the urinary tubule was distinctly stained blue with our anionic colloid in a pH range of 4.0–9.0, while its proximal convolution shows only slight reaction in a pH range of 4.0–7.0. This suggests that the
distal convolution has stronger, more numerous cationic sites than the proximal one. Our previous cationic iron colloid staining has also shown that the distal convolution is provided with enhanced anionic sites which are ionized at a pH value of more than 4.0 (MURAKAMI et al., 1986). These abundant anionic and cationic groups in the distal convolution may act as a buffer for the protection of the tubular cells from acid urine.

Formaldehyde or glutaraldehyde mainly reacts with non-ionized amino groups to fix tissues, and leaves the ionized amino groups intact (PUCHTLER and MELOAN, 1985). Thus, it is believed that these intact or ionized amino groups, including diamines and other amines, react with our anionic iron colloid. For light microscopy, counterstaining with eosin or aluminium-nuclear fast red complex is recommended since it contrasts with the Prussian blue reaction. Eosin is a representative anionic dye (Seki, 1961; ROMEIS, 1968) and stains those tissue cationic sites which are left unstained with our polyanionic iron colloid. The aluminium-nuclear fast red complex is cationic (Seki, 1961; ROMEIS, 1968) and stains the tissue anionic sites. In electron microscopy, the omission of the osmium counterstaining contrasted more clearly with the colloidal granules in the tissues. However, the osmium counterstaining is useful since it stains those tissue cationic sites (BEHRMAN, 1984), which were left unstained with our anionic colloid. Anionized ferricyanide can be used as a counterstaining medium or as a substitute for osmic acid. Furthermore, it should be added that cationized ferric thiocyanate is available for the staining of tissue anionic sites for electron microscopy. The details of the ferricyanide and ferric thiocyanate stainings will be reported elsewhere.

A column chromatographic affinity test showed that the staining intensity of our anionic colloid with ion-exchange resin particles increased as the pH values were decreased. In the tissue staining, however, no such marked differences in the staining intensity were noted at pH values of 4.0 and 7.0. This may be caused by the limited ionization of aldehyde-fixed amino groups at pH value 4.0. Lysine and arginine or their derivatives, including histone, are strongly basic. These strongly basic substances are thought to allow a diffuse and intense staining of the nuclei and macula densa of the distal convolution at pH value 9.0. Transmission electron microscopy of the detailed reactive sites in these stainings was omitted. As described above, the glomerulus and brush border of the proximal convolution were hardly stained at pH value 9.0. This may show that the cationic sites in these structures are weak and barely ionized as cations at pH value 9.0.

Our anionic colloid can also be used widely for the detection of anionic sites.
in other tissues or intracellular structures. For example, mast cells or their granules containing histamine are distinctly and selectively stained at pH values 4.0–9.0 (Fig. 6).

Our anionic iron colloid is finer and more permeable than other anionic iron colloids (Gasic et al., 1968; Seno et al., 1983b). However, the blocks for transmission electron microscopy should be sliced into very thin pieces for staining even when our colloid is used, as it permeates into tissues only 0.1–0.2 mm in 4 hrs at room temperature.

Our preliminary experiments in this study have shown that a much finer and more permeable colloid (0.5–1.0 nm in granular size) is produced when hydrazine hydrate (100%) is used as a substitute for ammonia in the preparation of the original mixture. This hydrazine-induced anionic colloid is available for staining at a slightly lower pH range of 2.0–8.0. Our preliminary experiments have also shown that a substitution of ammonia and cacodylic acid for sodium cacodylate (0.1 M) and that of ammonium thiocyanate for sodium thiocyanate (1 M) produce a coarser colloid (3.0–5.0 nm in granular size). This colloid is available for staining at pH values 4.0–9.0 though its tissue permeability and stainability are markedly decreased.

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