THE USE OF LEAD CITRATE AT HIGH pH AS AN ELECTRON-OPAQUE STAIN IN ELECTRON MICROSCOPY

EDWARD S. REYNOLDS. From the Department of Anatomy, Harvard Medical School, Boston

Aqueous solutions of lead salts (1, 2) and saturated solutions of lead hydroxide (1) have been used as stains to enhance the electron-scattering properties of components of biological materials examined in the electron microscope. Saturated solutions of lead hydroxide (1), while staining more intensely than either lead acetate or monobasic lead acetate (1, 2), form insoluble lead carbonate upon exposure to air. The avoidance of such precipitates which contaminate surfaces of sections during staining has been the stimulus for the development of elaborate procedures for exclusion of air or carbon dioxide (3, 4).

Several modifications of Watson's lead hydroxide stain (1) have recently appeared (5–7). All utilize relatively high pH (approximately 12) and one contains small amounts of tartrate (6), a relatively weak complexing agent (8), in addition to lead. These modified lead stains are less liable to contaminate the surface of the section with precipitated stain products. The stain reported here differs from previous alkaline lead stains in that the chelating agent, citrate, is in sufficient excess to sequester all lead present.

Lead citrate, soluble in high concentrations in basic solutions, is a chelate compound with an apparent association constant (log *Ka*) between ligand and lead ion of 6.5 (9). Tissue binding sites, presumably organophosphates, and other anionic species present in biological components following fixation, dehydration, and plastic embedding apparently have a greater affinity for this cation than lead citrate inasmuch as cellular and extracellular structures in the section sequester lead from the staining solution. Alkaline lead citrate solutions are less likely to contaminate sections, as no precipitates form when droplets of fresh staining solution are exposed to air for periods of up to 30 minutes. The resultant staining of the sections is of high intensity in sections of Araldite- or Epon-embedded material. Cytoplasmic membranes, ribosomes, glycogen, and nuclear material are stained (Figs. 1 to 3).

STAIN SOLUTION: Lead citrate is prepared *by*

Key to Abbreviations

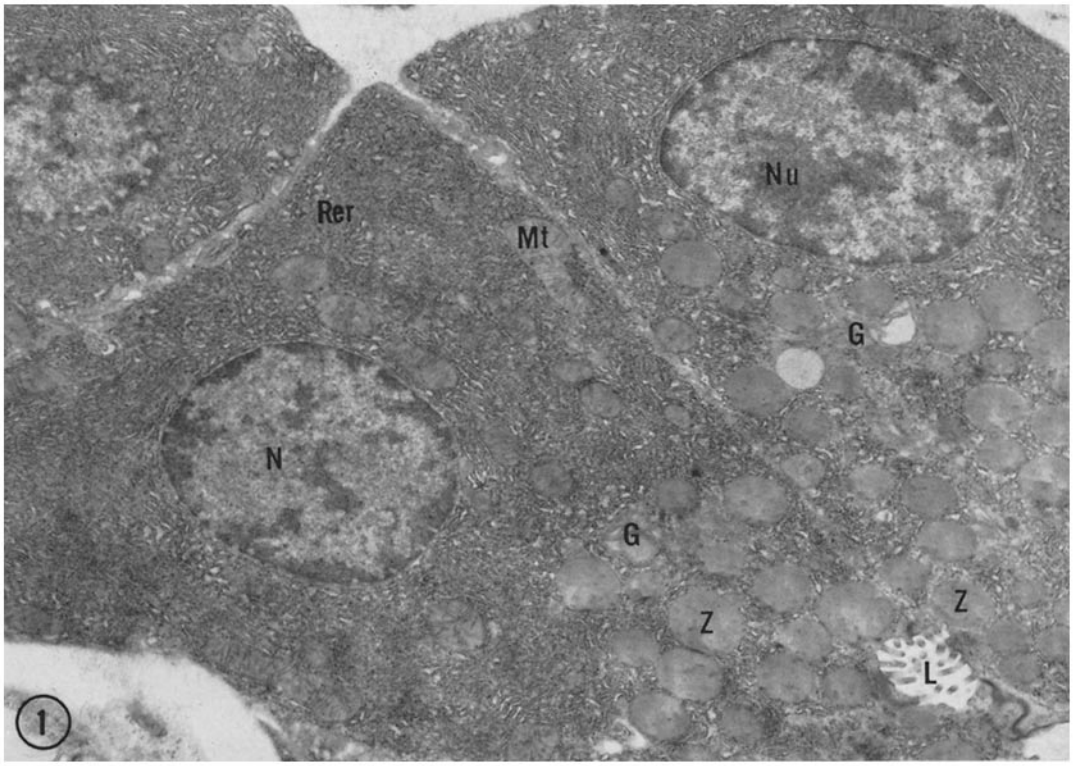
Ch, chromatin	*N*, nucleus
G, Golgi apparatus	*Np*, nuclear pore
Gly, glycogen	*Nu*, nucleolus
L, acinar lumen	*Rer*, granular endoplasmic reticulum
Mi, microbody	*Ser*, agranular endoplasmic reticulum
Mt, mitochondria	*Z*, zymogen granule

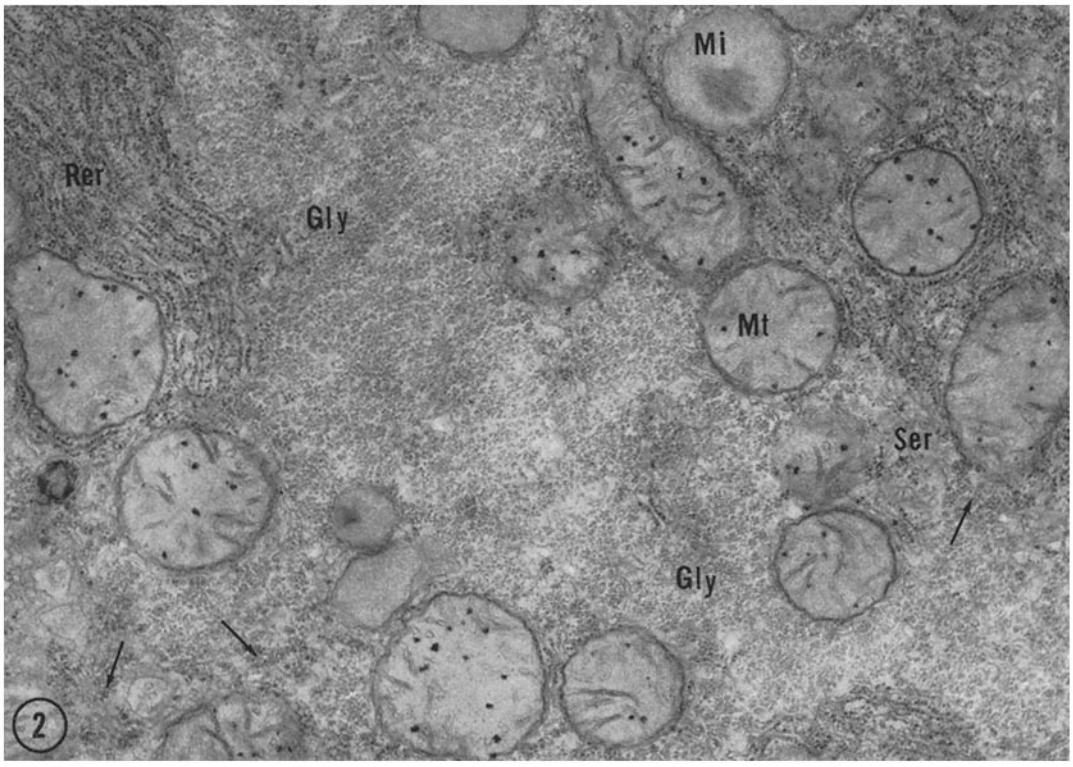
FIGURE 1

Portion of an acinus of pancreas of rat. Note the virtual absence of stain contamination. Phosphate-buffered osmium tetroxide fixation (9), Epon embedding. Stained with lead citrate 5 minutes. × 9,000.

FIGURE 2

Portion of cytoplasm of a parenchymal cell of liver of rat. For the most part in this micrograph glycogen appears as finely granular deposits of less electron opacity than ribosomes in contiguous areas. Glycogen deposits staining more intensely are indicated by arrows. In thicker sections prepared for electron microscopy, glycogen deposits stain more intensely than ribosomes. Fixation and embedding as in Fig. 1. Stained with a 1:5 dilution of lead citrate 5 minutes. × 25,000.


Rer
Mt
Nu
G
N
G
Z
Z
L
1


Mi
Rer
Gly
Mt
Ser
Gly
2

placing 1.33 gm $Pb(NO_3)_2$,[1] 1.76 gm $Na_3(C_6H_5O_7) \cdot 2H_2O$[1] and 30 ml distilled water in a 50 ml volumetric flask. The resultant suspension is shaken vigorously for 1 minute and allowed to stand with intermittent shaking in order to insure complete conversion of lead nitrate to lead citrate. After 30 minutes 8.0 ml 1 N NaOH[2] is added, the suspension diluted to 50 ml with distilled water and mixed by inversion. Lead citrate dissolves and the staining solution is ready for use. The pH of the staining solution was routinely found to be 12.0 $\pm$ 0.1. Faint turbidity, if present, is usually readily removed by centrifugation.

The staining solution, stored in glass or polyethylene bottles, is stable for a period of up to 6 months. With "aged" staining solutions it is advisable to centrifuge before use.

STAINING PROCEDURE: Grids with sections are stained by floating on single drops of staining solution. A Petri dish containing a dental wax–coated bottom is a suitable container and as many as thirty or forty grids can be accommodated at a single time. Staining times and stain concentrations vary depending upon fixation and embedment. Usually methacrylate-embedded tissue is stained for 5 to 10 minutes and that in Epon, Maraglas, and Araldite for 15 to 30 minutes with the stain as prepared. Tissues fixed in phosphate-buffered osmium tetroxide (10), or glutaraldehyde (11) stain so intensely, however, that staining times are reduced to 5 minutes and the stain solution diluted 1:5 to 1:1000 times with 0.01 N NaOH to prevent overstaining. Digestion and leaching of tissue components have not been observed within the limits of the staining times employed. Following staining, grids are washed sequentially in jets of 0.02 N NaOH and distilled water from plastic wash bottles, allowed to dry, and, if desired, coated with carbon.

Although carbon coating was necessary in sections mounted on grids coated with celloidin films, stained silver-gold or thinner sections of Epon-embedded material mounted on bare grids were routinely examined without coating. Fine granularity of staining patterns due to heating of the section in the electron beam rarely occurs, even at high magnification when the intensity of the beam is at or near cross-over. When such disturbance occurs, it is often the result of overstaining and can be corrected by reduction of staining time for that material or by dilution of the stain with 0.01 N NaOH.

MECHANISM OF STAINING: The constancy of the staining picture achieved with different alkaline lead stains (Figs. 1 to 3) (1, 2, 5, 6) indicates that the chemical mechanism of staining is similar in each instance. Indeed the pH dependency of the intensity of staining of biological materials by the general class of alkaline lead stains (1, 2, 5) is also observed with lead citrate.

Divalent lead salts in aqueous solution form "basic" salts upon addition of lead hydroxide or other alkali (8, 12). These compounds of the general type $Pb(OH)_2PbX_2$ ionize as shown in Equation 1:

$$Pb(OH)_2PbX_2 \rightleftharpoons [Pb(OH)_2Pb]^{++} + 2X^- \quad (1)$$

Such a divalent cation containing two atoms of lead could well explain the enhancement of contrast at pH 12, as twice as much lead would be bound at sites where divalent lead ions (Pb^{++}) would be bound at pH 7. The stoichiometry between lead (0.08 M), citrate (0.10 M) and hydroxyl ion concentration in this staining solution at pH 12 would tend to favor the presence of such a polymeric cation of lead (8, 12).

Progressive alkalization of the staining solution to pH 14 greatly diminishes the intensity of staining with lead citrate. Such treatment results in the conversion of cationic forms of lead present at pH 12 to anionic hydroxyplumbites (8, 12). Thus it is unlikely that these anionic forms of lead, which would tend to predominate at pH 14 under these conditions (12), could be considered responsible for staining at pH 12[3] (5–7). That cationic

[1] Chemicals of analytical reagent grade were used. Divalent lead salts of volatile acids tend to decompose with the formation of lead oxides upon storage. Lead salts other than lead nitrate are usable, but, as is also the case with lead nitrate, they should be of recent manufacture. If old lead salts are used, the final staining solution will be turbid and tend to stain poorly.

[2] 1 N NaOH was prepared by dilution of 10 N NaOH, carbonate-free (Fisher Scientific Company).

[3] Exposure to 1 N NaOH (pH 14) for short periods of time results in obliteration of most cellular details in sections. Sections exposed successively to 1 N NaOH and lead citrate at pH 12, however, "stain" because tissue-containing areas are denser than adjacent Epon. Sections exposed to lead citrate at pH 14 do not appear to stain intensively because tissue-containing areas are less dense than adjacent Epon. Glycogen, recognizable in sections at both pH's, stains far less intensively at pH 14 than at pH 12.

FIGURE 3

Portion of nucleus and cytoplasm of a parenchymal cell of liver of fasted rat. *s*-collidine-buffered osmium tetroxide fixation. Epon embedding. Stained with lead citrate 30 minutes. × 48,000.

forms of lead are responsible for staining at pH 12 is further suggested by prevention of staining in the presence of ethylenediaminetetraacetate (EDTA). This powerful chelating agent would tend to sequester only those cationic forms of lead present (8), leaving anionic forms free in solution.

Citrate apparently forms stable complexes with cationic "basic" lead salts (log *Ka* = 6.5). Thus it appears that lead citrate is capable of transferring its lead to tissue binding sites such as cysteine, (log *Ka* for lead of 12.5, reference 9) those containing orthophosphate and pyrophosphate groups (log *Ka's* 12.3 and 11.2, respectively, reference 9), and additional lead-sequestering sites formed during fixation, while lead ethylenediaminetetraacetate cannot (log *Ka* = 17.6) (9).

Glycogen aggregates which stain intensely upon alkaline lead staining in thicker sections prepared for electron microscopy appear as finely granular deposits (13) (*ca.* 300 A) staining less intensely than ribosomes in thin sections (grey interference color, Fig. 2). The abruptness of the transition from densely staining aggregates to less dense finely granular deposits in thin sections of uneven thickness suggests that the dense staining of aggregates in thicker sections is due to superimposition of lead-sequestering glycogen subunits observed in thinner sections (Fig. 2).

The glucose polymer of glycogen cannot be considered an ionic lead-binding site in the usual sense, as it is not known to form coordination complexes with metal cations. Its staining may depend upon the formation of a stable lead-glucose complex through hydrogen bonding (14).

This investigation was supported (in part) by a Public Health Service Fellowship (GSP-7309) from the Division of Medical Sciences, Public Health Service.

Received for publication, November 19, 1962.

BIBLIOGRAPHY

1. WATSON, M. L., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 727.
2. DALTON, A. J., and ZEIGEL, R. F., *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 409.
3. PEACHEY, L. D., *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 511.
4. PARSONS, D. F., and DARDEN, E. B., JR., *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 834.
5. KARNOVSKY, M. J., *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 729.
6. MILLONIG, G., *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 736.
7. LEVER, J. D., *Nature*, 1960, **187**, 810.
8. CHARBEREK, S., and MARTELL, A. E., Organic Sequestering Agents, New York, John Wiley and Sons, Inc., 1959.
9. BJERRUM, J., SCHWARZENBACH, G., and SILLEN, L. G., Stability Constants of Metal-ion Complexes: Parts I and II, London, The Chemical Society, 1957.
10. MILLONIG, G., *J. Appl. Physics.*, 1961, **32**, 1637.
11. SABATINI, D. D., BENSCH, K. G., AND BARRNETT, R. J., *Anat. Rec.*, 1962, **142**, 274.
12. REMY, H., Treatise on Inorganic Chemistry, New York, Elsevier Publishing Company, **1**, 1956.
13. DROCHMANS, P., *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 553.
14. SALTMAN, P., CHARLEY, P., and SARKAR, B., *Fed. Proc.*, 1962, **21**, 307.