

## SIMPLE METHODS FOR "STAINING WITH LEAD" AT HIGH pH IN ELECTRON MICROSCOPY

MORRIS J. KARNOVSKY. From the Department of Pathology, Harvard Medical School, Boston, and the Institute of Histology and Embryology, School of Medicine, Geneva

The lead hydroxide stain of Watson (1958) used for increasing contrast in thin sections for electron microscopy has found acceptance in many laboratories. However, this stain has an unfortunate tendency to form precipitates (probably of lead carbonate (5)) on exposure to the air, thus contaminating the sections and irritating the observer. This drawback has led to the development of several modifications (2, 3) of the original method of staining and the use of ingenious devices (4, 5) for preventing exposure to air and consequent precipitate formation. We offer the following alternative methods which, we believe, are simpler to perform than those hitherto described. They have the additional advantages mentioned below.

The methods are based on the observation that highly alkaline solutions of lead salts ( $\text{pH} > 11.5$ ) yield relatively stable solutions which stain rapidly and intensely, thus obviating the hazard of precipitation to a marked degree. The methods have these additional advantages: the staining solutions are easily and rapidly prepared, are simply stored, and are stable for long periods of time. Furthermore, they can be efficiently used, many grids being treated simultaneously, without excessive precautions being taken against lead carbonate precipitation. Finally, "difficult" material, embedded in media which characteristically yield rather low contrast, such as epoxide resins, can be rapidly and easily stained. "Clean" preparations, of high contrast, are routinely obtained. As will be discussed later, it is thought that in these highly alkaline staining solutions lead is present as an hydroxide complex anion (plumbite ion) and that this anion is responsible for the staining.

The methods of preparation are based on this hypothesis.

Two methods for preparing the staining solutions have been found useful:

### *Method A*

Lead monoxide is dissolved in hot NaOH to produce the plumbite ion.

To 15 to 20 ml of 1 N NaOH in a flask is added lead monoxide (PbO) in excess, and the mixture is gently boiled for 15 minutes. The flask is rapidly cooled, and the solution filtered. The filtrate can be kept as a stock solution in a closed container for months.

The final staining solution is prepared by diluting the stock solution 1/50, 1/100 or 1/200 with distilled water, the degree of dilution depending on the rate of staining required. In general, the 1/50 or 1/100 dilutions are most useful, but on occasion higher (1/200) or lower (1/20) dilutions have been used. The diluted stains can also be kept as a stock for several months. An aliquot should be placed in a 12 ml centrifuge tube, and the tube stoppered. This solution is for immediate use. As the stain is used the solution should be replenished from the stock bottles.

### *Method B*

Lead monoxide is dissolved in sodium cacodylate solution and the solution is then alkalisied.

(a) To 10 to 15 ml of 10 per cent sodium cacodylate in water lead monoxide is added in excess, and the mixture is gently boiled for 15 minutes. After cooling the mixture is filtered.

(b) Alternatively, and more simply, the boiling of the mixture may be omitted, yielding a somewhat less concentrated but nevertheless entirely satisfactory stain for most purposes. In this instance, lead monoxide is added in excess to 10 to 15 ml of sodium cacodylate, the container is stoppered, and the mixture is frequently and thoroughly shaken over a period of 10 to 15 minutes. The mixture is then filtered.

An aliquot of 2 ml of the filtrate from either (a) or (b) is placed in a 10 ml graduate and is diluted to 10 ml with 10 per cent sodium cacodylate, with stirring. (The filtrate cannot be kept as a stock solution because it is unstable.) 1 N NaOH is added drop by drop with *thorough* stirring (for at least 15 seconds) between each drop. A faint cloudiness will form as the drops are added and will then slowly disappear with stirring after the final drop has been added. It is important not to over titrate. Usually about six drops of 1 N NaOH are sufficient. When the solution is clear it is decanted into a 12 ml centrifuge tube, and the tube is stoppered. It can be kept thus for several months.

#### *Staining Procedure*

Staining is performed in a simple staining chamber, which accommodates numerous grids. The lid of a glass Petri dish is partially filled with melted dental wax, which is then allowed to cool. This is the staining surface. A small, shallow glass container containing NaOH pellets is placed on the wax at one side of the dish. The base of the Petri dish is utilised as a lid, which can be pressed gently into the wax to seal off the chamber.

The staining solution is then centrifuged (*not filtered*) for a few minutes. This procedure is repeated before each batch of grids is stained. With the aid of a clean Pasteur pipette and a rubber bulb a small quantity of stain is withdrawn from below the surface, and small drops (one for each grid) are placed on the wax. The drops should be of such a size that the grids float on the top of the dome of the drop and do not slide down to the sides. The lid of the chamber is gently pressed into the wax to form a seal. Staining times vary from 1 minute to 30 minutes, depending on the material, embedding medium, desired intensity of staining, etc. Staining should not be undertaken on glass surfaces as drops suitable in size and shape are not easily obtained.

At the end of the staining period the grids are seized at their edge with a forceps and are washed

in three successive beakers of distilled water by dipping them rapidly in and out, breaking the surface of the water (1, 2). They are then dried on filter paper.

After use the drops are flushed off the wax surface in a jet of cold water and, after drying, the chamber is used again.

#### RESULTS

Osmium tetroxide-fixed material embedded in methacrylate or Vestopal W stains strongly within a few minutes (1 to 10 minutes, depending on the material and degree of increased contrast required). Epon-embedded material usually requires somewhat longer (10 to 30 minutes). Because the pH of the solutions is high (11.9 to 12.3, uncorrected for alkali-ion effect), it is probably advisable to keep the staining period as short as possible, as destaining and extraction may occur with prolonged immersion. Within the staining times indicated, overt digestion of the embedding plastic or of the tissue has not been observed. However, with the more concentrated solutions prepared by Method A, destaining of Vestopal-W-embedded material has been observed after 30 minutes, whereas with stains prepared according to Method B no such effect was noted. On the contrary, the intensity of staining increased progressively with time.

The complex factors involved in assessing the effect of length of staining time have been discussed by Watson (1), and the above-mentioned staining times are merely intended as a rough guide.

On the basis of a limited variety of tissues examined, it can be stated that the results obtained are virtually indistinguishable from those obtained with Watson's lead hydroxide technique (Fig. 1). There may be minor differences, but this question has not been investigated to date.

#### *Mechanism of Staining*

The question arises as to the mechanism involved in the staining process. Dalton and Zeigel (2) found that with various lead salts the intensity of staining increased as the pH of the solutions increased. They proposed that with increasing pH levels phosphate, sulfhydryl and carboxyl groups in the tissue became more completely ionised, thus binding  $Pb^{++}$  ions. Thus lead hy-

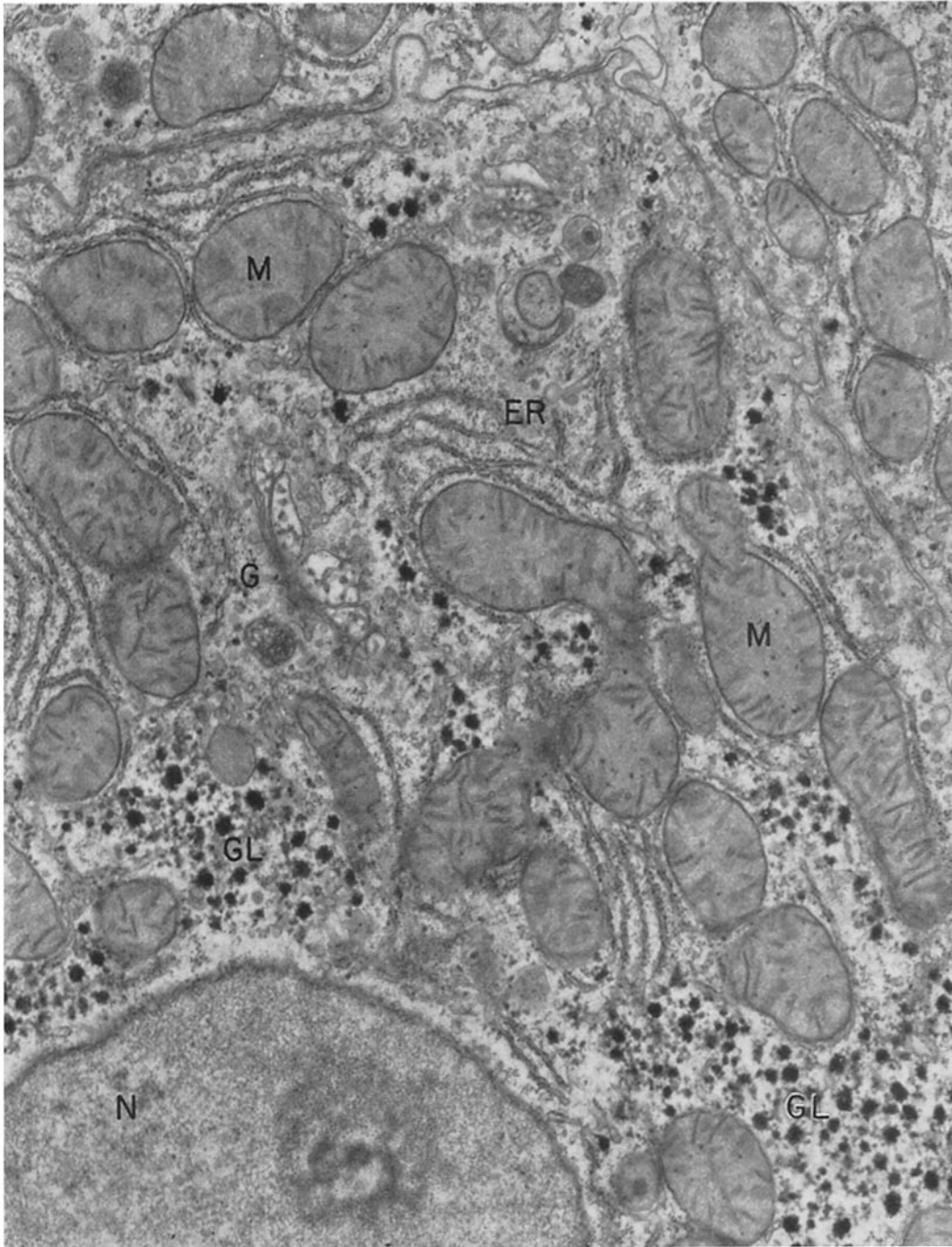


FIGURE 1

Osmium-tetroxide-fixed, Vestopal-W-embedded section of rat liver, stained with highly alkaline solution of lead, prepared according to Method A. Stain prepared according to Method B gives similar results. *GL*, glycogen; *ER*, endoplasmic reticulum; *G*, Golgi apparatus; *M*, mitochondria; *N*, nucleus.  $\times 30,000$ .

dioxide (pH 8.15) stained more intensely than monobasic lead acetate (pH 7.0).

Whilst such a mechanism may well be operative for staining solutions of relatively low pH, we suggest that it is not the basis for the staining we obtain with our method, where the pH of the stains is above 11.5. At such high pH levels, lead is present as plumbite ion,  $\text{Pb}(\text{OH})_3^-$  or  $\text{Pb}(\text{OH})_4^{=}$  (6, 7). Lead monoxide, like lead hydroxide, is amphoteric and acts as an acid anhydride, forming plumbite ion on treatment with base (6, 7).

It is suggested that the plumbite ion is responsible for the staining we obtain. Lever (3), in discussing the preparation of his staining solution, which is basic, mentions the formation of plumbite but does not ascribe a role to it.

Because it is unlikely that positively charged groups are available in the tissue for ionic binding of the negatively charged plumbite ion at high pH levels, another mechanism is suggested, namely hydrogen bonding of the plumbite ion to groups in the tissues.

In support of this hypothesis is the observation that complete absence of staining is found in sections first stained and then treated for 5 to 10 minutes with 8 M urea, whereas if control sections are first treated with urea, and then stained, no decrease in staining intensity is observed.

If hydroxylated compounds (*e.g.* sucrose, polyvinyl alcohol, gum acacia) are added to the staining solutions, the rate of staining is much decreased, or staining is abolished, and, incidentally, the rate of precipitation of lead carbonate on exposure to air is much decreased.

The staining component is also removed from the staining solutions by filtration through filter paper. This adsorption of the staining component on the paper becomes progressively less significant as the quantity of stain filtered increases. Therefore, concentrated solutions, or large volumes of dilute stain, can be filtered with relative impunity, but the final dilute staining solutions should not be filtered, as considerable loss of staining component occurs.

Staining at high pH is much more rapid and intense than staining at lower pH. The lead-containing cacodylate solutions, before treatment

with 1 N NaOH, have pH values between 8.0 and 9.0, stain slowly and weakly, and are unstable. The alkalisied solutions (pH 11.9 to 12.3) stain rapidly, intensely, and are stable.

In our experience, the stain prepared according to Method B is more stable than that prepared in Method A and is used routinely in our laboratory. There is less danger of extraction and destaining with long staining times. On the other hand, it is easier to prepare the stain by Method A, and the stock solution, from which the final dilutions are made, can be stored for long periods of time. Stain prepared by Method A has been used routinely by several workers on Epon-embedded materials, with excellent results. In general, however, the cacodylate-lead stain is to be preferred. The exact mechanism whereby the cacodylate stabilises the staining solution is at present unknown.

The author is indebted to Dr. Jean Paul Revel for much useful advice. The author also thanks Drs. Don W. Fawcett, Susumo Ito, Jean Paul Revel, Ch. Rouiller, and G. Simon for using one or another of the stains finally evolved, and for their helpful opinions. The skilled technical assistance of Miss Katharina Hug is gratefully acknowledged.

This work was supported in part by training grant 2G-113 and grant C-2451 from the National Institutes of Health, United States Public Health Service.

Received for publication, July 24, 1961.

#### REFERENCES

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. LEVER, J. D., *Nature*, 1960, 186, 810.
4. PEACHEY, L. D., *J. Biophysic. and Biochem. Cytol.*, 1959, 5, 511.
5. PARSONS, D. F., and DARDEN, E. B., JR., *J. Biophysic. and Biochem. Cytol.*, 1960, 8, 834.
6. MELLOR, J. W., *Mellor's Comprehensive Treatise on Inorganic and Theoretical Chemistry*, New York, Longmans, Green and Co., 1957, 7, 665.
7. PAULING, L., *General Chemistry*, San Francisco, W. H. Freeman and Co., 1953, 480, 566.