Brief Notes

s-Collidine as a Basis for Buffering Fixatives.*

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In 1952 Palade introduced the use of Michaelis' veronal-acetate buffer for regulating the pH of osmium tetroxide–fixing solutions for electron microscopy (1, 2). This buffer has enjoyed wide popularity and is regarded as satisfactory by many cytologists.

The veronal-acetate buffer suffers from several limitations, however. It is not particularly stable, since it supports the growth of mould or bacteria. Hence it is not practical to make up large amounts in bulk nor to store it over a considerable period of time. It is not possible to make up a solution much more concentrated than that recommended by Palade, which approaches saturation for veronal (diethyl barbituric acid). The veronal-acetate mixture was developed by Michaelis (3) to meet the need for a relatively non-toxic buffer system capable of broad pH range and constant ionic strength. For this it works very well. It is actually two buffer systems in one; *i.e.*, sodium acetate-HCl and sodium veronal-HCl. The former is most efficient between pH 4.2 and 5.2 (pK for acetic acid is 4.76), but is nearly inoperative at pH 7 to 8. The veronal-HCl system is most efficient between pH 7.5–8.5 (pK for veronal is 7.96 (4)). As recommended by Palade, osmium tetroxide is most commonly used at pH 7.2–7.5. At these pH's the veronal-HCl system is operating at the acid edge of its range, where it is not very efficient in suppressing changes toward further acidity (2). Thus the combined veronal-acetate buffer system is not particularly effective in the pH region between its two buffer systems, namely between pH 5.2 to 7.5. This has been confirmed by titration curves.

Even though our experience with Palade's buffered osmic fixative has been satisfactory, we undertook a search for a medium which might offer improvements from the point of view of stability and buffering capacity. Our attention was focused on *s*-collidine (γ-collidine or 2,4,6-trimethylpyridine), which has a pK of about 7.4 (7.37 in 0.05 M solution at 22°C. by average of three titrations). When half neutralized with a strong acid such as HCl, the resulting solution exerts its maximum buffering capacity in the neighborhood of pH 7.4. Such buffers appear to be stable indefinitely at room temperature. The ingredients of the buffer appear neither to react with nor to complex strongly with osmium tetroxide, and the buffer is stable in the presence of permanganate (5). Addition of osmium tetroxide solution does not alter the pH any more than does an equivalent amount of water and the resulting collidine-buffered osmium tetroxide solutions are stable for many days. Isotonic or hypertonic fixing solutions can be prepared without other solutes, as the collidine is quite soluble in water.

We have been using collidine-buffered osmium tetroxide solutions as a fixative for electron microscopy for about 3 years. It has not entirely displaced veronal-acetate buffers in our laboratory, but it seems to have made for itself a useful place by virtue of greater stability and convenience.

Blocks of tissue fixed in collidine-buffered osmium tetroxide yield results very similar to those prepared with Palade's fixative. The quality of the electron micrographs obtained after collidine seems to be at least as satisfactory as those based on Palade's fluid, and there is some suggestion that occasionally it gives slightly superior results. However, the main advantages of the *s*-collidine buffer reside in its superior stability and convenience. The only drawback relates to difficulty in obtaining *s*-collidine of adequate purity.[1]

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[1] All samples of "pure" *s*-collidine which we have purchased (from Eastman Kodak (4815) and Matheson Coleman and Bell) have been contaminated with less completely methylated pyridines (lutidines, picolines, and pyridine). These give yellowish crystalline or oily complexes with osmium tetroxide and yield inferior fixing solutions. A test for the presence of these impurities is to make up 0.2 M buffer with the *s*-collidine and then to mix the buffer with an equal volume of aqueous 4 per cent or 5 per cent osmium tetroxide solution. If the collidine is of sufficient purity, the resulting solution remains absolutely clear. Contaminated collidine yields a cloudy solution.

It may be that other suppliers of organic chemicals

It has become our practice to prepare stock solutions of *s*-collidine buffer by dissolving 2.67 ml. (20 m.eq.) of pure *s*-collidine in about 50 ml. of distilled water, adding 9.0 ml. of 1.0 N HCl (9 m.eq.) and diluting to 100 ml. with distilled water. This produces 100 ml. of 0.2 M *s*-collidine buffer of 7.40–7.45. This may be mixed in any proportion with stock solutions of osmium tetroxide to give the desired concentration of fixative and buffer. We commonly employ 1 ml. of the stock buffer with 2 ml. of 2 per cent osmium tetroxide, giving a final concentration of 1.33 per cent osmium tetroxide in 0.067 M buffer, or ½ ml. stock buffer with 1 ml. of 4 per cent osmium tetroxide to give 2.67 per cent osmium tetroxide in 0.067 M buffer. The pH of the buffer rises somewhat on dilution with water. An example for one batch of buffer was:[2]

0.20 M – 7.44
0.10 M – 7.49
0.05 M – 7.50
0.025 M – 7.50

The initial pH of the buffer also may be adjusted according to the volume of 1.0 N HCl added. For 2.67 ml. of *s*-collidine in a final volume of 100 ml., the pH of the 0.20 M buffer varied as follows as HCl was added:

5.0 ml. 1.0 N HCl – pH 7.74
6.0 ml. 1.0 N HCl – pH 7.67
7.0 ml. 1.0 N HCl – pH 7.59
8.0 ml. 1.0 N HCl – pH 7.50
9.0 ml. 1.0 N HCl – pH 7.41
10.0 ml. 1.0 N HCl – pH 7.33

SUMMARY

A buffer system comprised of *s*-collidine (2,4,6-trimethylpyridine) and HCl is satisfactory for buffering solutions of osmium tetroxide or potassium permanganate used as tissue fixatives for electron microscopy. The buffer is more stable and convenient to use than Michaelis' veronal-acetate solution, and has a more powerful buffering action at pH 7.4.

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can provide material of consistent and adequate purity. However, we decided to work out a purification procedure adaptable to impure material. Distillation does not suffice, nor does extraction with charcoal. We adapted a method for purifying a related compound (2,6-lutidine) published by Biddiscombe *et al.* (6). This provides *s*-collidine of satisfactory quality. 100 gm. of commercial *s*-collidine was placed in a 1 l. flask equipped with a reflux condenser. 100 gm. of reagent grade urea and 85 ml. water were added and the mixture refluxed for 30 minutes. Two layers formed. The mixture was cooled slightly, transferred to a covered beaker, and quickly cooled with stirring, to induce crystallization of a urea complex of *s*-collidine. The crystals were filtered free of mother liquor in a Buchner funnel. The damp crystals were transferred to the beaker again, 50 ml. water added, and the mixture heated until all solids dissolved. The material was recrystallized and gathered by filtration in a Buckner funnel. A third crystallization was used if contamination was very serious. The crystals were suspended in 220 ml. water, transferred to the 1 l. reflux flask, 175 gm. of KOH added, and the mixture refluxed for 6 hours. This procedure hydrolyzed the urea, releasing the *s*-collidine and ammonia gas. The *s*-collidine was separated from the aqueous medium in a separatory funnel. It was then dried over NaOH and distilled. The sample distilling at 170–171°C. was collected as the purified product, with about a 50 per cent yield.

[2] All pH measurements were made with a standard glass electrode in a Leeds and Northrup pH meter (Model 7664) using Beckman 3501 pH 7.0 buffer as a reference standard. Readings were taken after 1 minute to allow for slight drift of 0.06–0.07 pH unit upon contact with the *s*-collidine solution.

An Improved Grid-Holder for the Siemens Electron Microscope. BY P. F. ELBERS. (*From Centrum voor Electronenmicroscopie der Rijksuniversiteit Utrecht, Netherlands.*)*

Handling standard Siemens grid-holders involves several inconvenient operations. These manipulations may, moreover, so disturb the specimen being studied that observations made in the electron microscope are invalidated. For example, to mount the grid, the cap must be removed and re-

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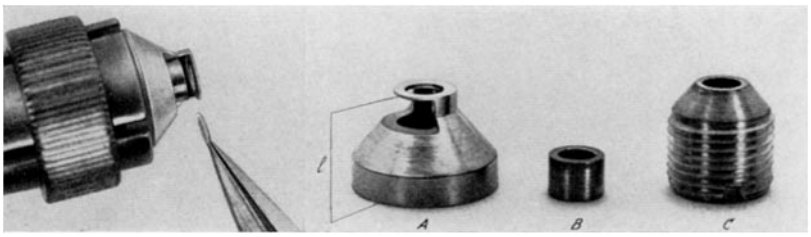

TEXT-FIG. 1. The insertion of a grid in the holder (left), and the three parts of the holder (right).

placed with the fingers. Contamination may result. Even when the tweezers are held in a steady hand, it is difficult to insert the grid in the cap without touching the cap wall. Grids are often bent or wrinkled in this manner, and supporting membranes similarly distorted or actually ruptured. Good thermal contact between grid and grid-holder cannot always be achieved—and this may lead to thermal drift in the specimen. Finally grids are not easily removed from the standard holders without damaging or destroying the specimen.

These problems can be avoided to some extent by using the variant design of grid-holder shown in the accompanying photographs. Piece (*A*) determines the specimen's position in the electron microscope. The distance (*l*) can be corrected by grinding down the bottom face of (*A*). The top ring of (*A*) is adequately rigid. Inserted into (*A*), piece (*B*) prevents distortion of the grid when (*C*) s screwed in (*A*).

To insert the grid, the holder can be taken up in a chuck, as shown in the photograph. This chuck contains a screwdriver device for loosening and tightening (*C*). Another chuck is used to insert the holder in the microscope.

This grid-holder with its auxiliary parts offers the following advantages:

(*a*) The grid can be inserted in it without touching the holder with the fingers.

(*b*) The grid can be inserted from the side, directly onto its seat.

(*c*) Small parts of the holder which touch the grid do not need to be disassembled or handled and are, therefore, not so subject to contamination.

(*d*) Screwing down of insert (*B*) ensures good thermal contact.

(*e*) Removing the grid from the holder is a simple operation.

On request, the designer will forward a scale drawing of the parts of the chucks and holder (for 3 mm. grids).