applied for 7 seconds to the right parietal bone; skin closed with clips.

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20 June 1968

Ruthenium Tetroxide for Fixing and Staining Cytoplasmic Membranes

Abstract. *Rat liver and kidney were fixed for electron microscopy with ruthenium tetroxide. When compared with tissues fixed with osmium tetroxide, the membranes showed well without additional staining. The trilaminar structure of the cytoplasmic membranes was clearly seen. In width the nuclear, mitochondrial, and cytoplasmic membranes investigated resembled the cell membrane.*

Ruthenium tetroxide was first used as a stain in histology in 1887 by Ranvier (*1*); its use as a fixative also has been investigated (*2*), but its use in electron microscopy as a fixative or stain has never been reported.

Tissues of rat kidney and liver were fixed in cacodylate-buffered glutaraldehyde overnight, rinsed three times (10 minutes for each change) in isotonic sodium acetate buffer (*p*H 7.1 ± 0.1), and refixed in ruthenium tetroxide made up in sodium acetate–acetic acid buffer (*p*H 7.1 ± 0.1) adjusted to be isotonic with plasma. The optimum concentration of ruthenium tetroxide proved to be between 0.1 and 0.05 percent (weight to volume) when the tissue was fixed for 1 hour at 4°C. The tissue was rinsed in sodium acetate buffer, dehydrated in methanol, and embedded in Araldite. Sections were cut on a Huxley microtome and viewed in a Siemens Elmiskop-1B electron microscope.

A striking difference between tissues fixed with osmium tetroxide and with ruthenium tetroxide is the appearance of the cytoplasmic and nuclear membranes (Figs. 1–6). Whereas these are single, rather diffuse lines when fixed with osmium tetroxide, they appear as trilaminar structures similar in appearance to those normally seen in plasma membranes in preparations fixed with osmium tetroxide.

The widths of the membranes were measured only at the points at which both electron-dense lines appeared to be sharp and uniform. The widths reported (Table 1) are the means of at least 20 measurements taken from no less than four different cells; they were calculated by use of the magnification figures provided by the electron microscope. No external standards were used for calibration of the instrument, and all pictures were taken at the same magnification. The membrane widths of these tissues fixed with ruthenium tetroxide showed a remarkable degree of uniformity, suggesting that all have similar structure.

Table 1. Membrane widths of tissues fixed with ruthenium tetroxide.

Membrane	Width (A°)	Mean deviation (A)
Plasma, of red blood cell	81.0	10.7
Inner nuclear	77.2	5.7
Outer nuclear	78.0	8.0
Mitochondrial cristae	157.8	4.6
Of mitochondrial cristae (half-width of cristae)	78.9	
Endoplasmic reticulum (granular)	75.9	5.2
Plasma, of glomerular epithelial cell	79.2	10.4

The plasma membrane may join the endoplasmic reticulum membrane, and this in turn may fuse with the outer nuclear membrane which is continuous with the inner nuclear membrane in the region of the nuclear pore (Fig. 3). These observations agree with the suggestion that all these types of membrane are similar in structure. In one instance the plasma membrane was seen to be continuous with the outer nuclear membrane, leaving the inner nuclear membrane apparently exposed to the extracellular space.

The plasma membrane of many types of cell shows a more complex outer structure (Fig. 2) presenting an electron-dense coating in addition to the electron-dense layers normally seen in preparations fixed with osmium tetroxide and potassium permanganate. In some types of cell, this additional layer

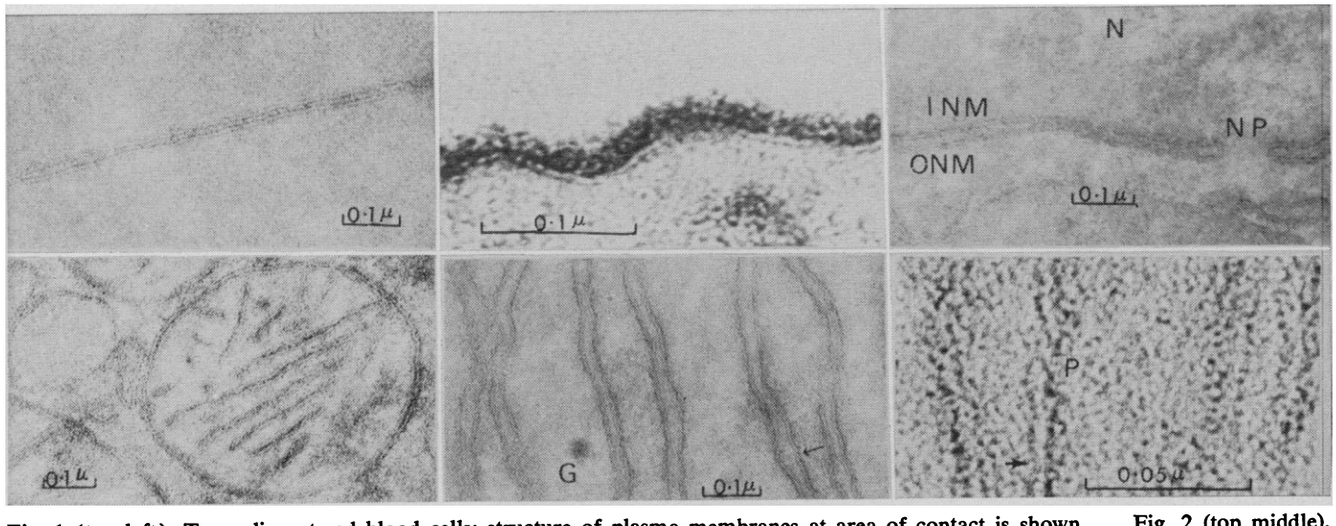

Fig. 1 (top left). Two adjacent red blood cells; structure of plasma membranes at area of contact is shown. Fig. 2 (top middle). Outer plasma membrane of glomerular epithelial cell; outer coating is shown. Fig. 3 (top right). Section through edge of nucleus of epithelial cell of glomerulus; *N*, nucleus; *INM*, inner nuclear membrane; *ONM*, outer nuclear membrane; *NP*, nuclear pore. Fig. 4 (bottom left). Mitochondria of liver cell. Fig. 5 (bottom middle). Endoplasmic reticulum of liver cell; *G*, densely stained glycogen particle. Arrow indicates membrane pore. Fig. 6 (bottom right). Enlargement from Fig. 5; *P*, membrane pore (Fig. 5). Arrow shows region in one of the electron-dense lines resolved into trilaminar structure.

makes measurement of the width of the normally visible triple structure impossible, being closely apposed to the surface. However, when the membranes were distinct, their widths either conformed to those we report or were slightly greater.

The characteristic five-layered structure of mitochondrial cristae has been demonstrated by use of material fixed with potassium permanganate (*3*), as well as by freeze-substitution with a 2 percent solution of osmium tetroxide in acetone (*4*). The mitochondrial cristae, of tissues prepared by these two methods, show electron-microscopically three dark lines of similar density, of which the middle line is approximately 40 percent wider than the two outer ones. The mitochondrial cristae as seen in tissues processed with tetroxide (Fig. 4) show similar structure, but the middle electron-dense line is much wider relative to the outer lines and stains more deeply.

The membranes of the endoplasmic reticulum show occasional pores which may be the sites through which proteins and other macromolecules are transported. The electron-dense regions of the membranes may show, at high resolution, a trilaminar structure (Fig. 6) characterized by two electron-dense bands enclosing a slightly wider, less dense region.

Attempting to find a chemical basis for these differences between osmium and ruthenium tetroxides, we studied the reactivity of ruthenium tetroxide with tissue components. Using an ether extract of rat liver, separated chromatographically on silica gel, we found that ruthenium tetroxide reacts strongly with some of the more polar lipids that show no reaction with osmium tetroxide. Ruthenium tetroxide also reacts strongly with protein, glycogen, and the common monosaccharides, only slightly with mucopolysaccharides, and not strongly with the basement membranes in the tissues examined.

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Ecdysones and Analogs: Effects on Development and Reproduction of Insects

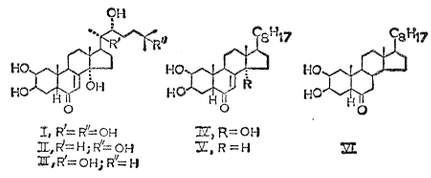
Abstract. *Ingestion of certain synthetic ecdysone analogs inhibited larval growth and development in several species of insects, whereas 20-hydroxyecdysone was inactive or considerably less active. Natural 20-hydroxyecdysone and ponasterone A, and a synthetic ecdysone analog inhibited ovarian maturation and egg production in the adult housefly. These effects appeared to be related to hormonal activity.*

The occurrence of steroids, with structures and biological activities similar to or identical with those of the molting hormones of insects, in various plants (*1*) poses a question of the roles of these substances in plants. It is not known whether these phytoecdysones are end products of sterol matabolism, analogous to the bile alcohols or acids of vertebrates, as suggested by the A/B ring *cis* configuration, or have a physiological or biochemical function in plant growth and development, as in insects. Of equal interest is their possible effect on insect host–plant relations. Desert locusts *Schistocerca gregaria* (Forskal) fed on moistened autumnal bracken-fern pinnae, plus wheat seedlings, took longer to develop and were smaller than locusts fed on wheat seedlings alone (*2*); it was suggested (*2*) that the effects reflected only dietary deficiency, and that it is unlikely that the plant ecdysones are a defense against insects.

We do not consider that this report (*2*) has answered the primary question of whether the ecdysones can act as plant protectants, or as regulators of insect host–plant interactions, because (i) although bracken contains ecdysones, including α-ecdysone and 20-hydroxyecdysone (*3*), at titers greater than the best insect sources, this fern is still a poorer source than are other plants (*1*); and (ii) testing for the effect of a minor component, by feeding the entire plant, is complicated by many factors—acceptance and ingestion of the plant by the insect, the overall nutritional value of the plant, and the presence of more than one toxic component.

We believe that this question can best be answered by determination of whether ecdysones and related steroids interfere with the normal development of insects when added to diets that otherwise support optimum growth and reproduction. We have therefore examined the effects of ecdysones and synthetic analogs by feeding them in nutritionally adequate diets.

Of the natural ecdysones, 20-hydroxyecdysone (I) was the most extensively studied, but α-ecdysone (II) and ponasterone A (III) also were tested in certain insects.



The synthetic steroids included three ecdysone analogs [Δ^7-5β-cholestene-2β,3β,14α-triol-6-one (IV), Δ^7-5β-cholestene-2β,3β-diol-6-one (V), and 5β-cholestane-2β,3β,diol-6-one (VI)] and their corresponding 5α-isomers (*4*). The test steroids were incorporated into the diets (*5*) by coating them on the dry components with volatile organic solvents. Five species of laboratory-reared insects were used: the tobacco hornworm *Manduca sexta* (Johannson), the flour beetle *Tribolium confusum* Jaquelin (duVal), the housefly *Musca domestica* (L.), the German cockroach *Blattella germanica* (L.), and the firebrat *Thermobia domestica* (Packard). Newly hatched larvae, or nymphs, and newly emerged adults were employed in the growth and reproduction tests, respectively.

Larvae of the housefly were very susceptible to the action of the hormone analogs: when added to an aseptic semidefined diet, compound IV severely inhibited development at concentrations as low as 37.5 parts per million (ppm) (Table 1). Removal of the 14α-hydroxyl group resulted in a compound (V) one-fourth as active in the housefly, but saturation of the Δ^7 double bond in V, to form VI, did not further reduce the activity. Ponasterone A was approximately one-fourth as active as compound IV, whereas 20-hydroxyecdysone was inactive at the highest concentration tested.

The various species showed considerable specificity in susceptibility to compound IV (Table 2). With the German cockroach and the flour beetle, removal of the 14α hydroxyl from IV, to form the diol (V), resulted in a compound about one-tenth as active. These two insects differed from the housefly in that further modification of compound V by saturation of the Δ^7 double bond, to form compound VI, further decreased activity so as to make the latter compound essentially inactive for both insects in our assay systems. The 20-