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Graham R C & Karnovsky M J. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291-302, 1966.
[Department of Pathology, Harvard Medical School, Boston, MA]

This paper presented the use of 3,3'-diaminobenzidine as an electron donor in cytochemical peroxidase reactions at the light and electron microscopic levels. The paper also presented the use of horseradish peroxidase as a macromolecular tracer at the ultrastructural level. [The *SCI*® indicates that this paper has been cited in over 6,170 publications, making it the most-cited paper ever published in this journal.]

An Electron Donor and a New Macromolecular Tracer

Morris J. Karnovsky
Department of Pathology
Harvard Medical School
Boston, MA 02115

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In 1965 Lord Florey delivered the prestigious Dunham Lectures at Harvard Medical School. As a junior member of the faculty, I acted as his chauffeur, which allowed for periods of stimulating discussion. We were mutually intrigued by the possibility of localizing the ultrastructural equivalent in the microcirculation of the small pores that subserve the passage of molecules smaller than albumin across the endothelium, according to the classic physiological studies of John Pappenheimer. As Lord Florey discussed in one lecture, the ultrastructural tracers then available were too large. In a diversion, he leaned over the lectern, fixed me sternly with his eyes, and said that perhaps I "would provide the answers"! Sitting in the front row, I felt as small as a particle minute enough to pass through the legendary pores!

A possible approach would be to administer intravenously an enzyme of small size as a tracer and to detect its localization in tissues through an enzymatic reaction yielding an insoluble electron-opaque precipitate. The amplification of the enzymatic reaction should generate much reaction product and enhanced sensitivity. Werner Straus[1] had originated the use of intravenously injected horseradish peroxidase (HRP) as a macromolecular tracer at the light microscopic level. HRP seemed to have the requisite properties. The problem was to design an electron donor for the peroxidase reaction, which, unlike the classical electron donors, would yield an electron-opaque reaction product. Study of several potential electron donors led me to the idea that a compound such as 3,3'-diaminobenzidine (DAB) might yield an insoluble polymer, which would reduce osmium tetroxide to give an electron-opaque precipitate. Test-tube experiments confirmed this, and the technique was applied to tissues from mice injected intravenously with HRP. The results by electron microscopy were stunning: there, in exquisite detail, were images of pinocytosis and macromolecular transport at a resolution never seen before, and my presentation of the technique at the Annual Meeting of the American Society of Cell Biology (1965)[2] was most exciting—at least to me.

Somewhat prior to this, Richard C. Graham, Jr., came to my laboratory as a postdoctoral fellow. In addition to developing several original cytochemical techniques, he thoroughly explored the optimum conditions for performing the DAB-HRP reaction. We decided that our first DAB paper would present the technique in detail and its application to protein reabsorption in renal tubules.

In later studies on the ultrastructural bases for microcirculatory permeability, I came to the conclusion that incomplete "tight" junctions were the site of the small pores in endothelium.[3] The localization of the blood-brain barrier to the endothelium[4] followed shortly. Since then, DAB and HRP have become widely used reagents in biological research.[5]

1. **Straus W.** Segregation of an intravenously injected protein by "droplets" of the cells of rat kidneys. *J. Biophys. Biochem. Cytol.* 3:1037-40, 1957. (Cited 80 times.)
2. **Karnovsky M J.** Vesicular transport of exogenous peroxidase across capillary endothelium into the T system of muscle. *J. Cell Biol.* 27:49A-50A, 1965. (Cited 150 times.)
3. ———. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* 35:213-36, 1967. (Cited 1,355 times.)
4. **Reese T S & Karnovsky M J.** Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* 34:207-17, 1967. (Cited 985 times.)
5. **Robinson J M & Karnovsky M J.** The cytochemistry of oxidase. (Pearse A G E & Stoward P J, eds.) *Histochemistry. Volume 3.* Edinburgh, Scotland: Churchill Livingstone. (In press.)

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