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An Outline of the Use of Horseradish Peroxidase in Neuroanatomical Tract-Tracing

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Quite possibly, the advent of horseradish peroxidase (HRP) histochemistry has stimulated more research and provided more data in contemporary neuroscience than any other experimental methodology. The goal of this brief overview is to familiarize the reader with the basics of the HRP technique as it is applied to neuro-anatomical tract-tracing and to convince those investigators who have not used HRP methodology in neuroanatomical experiments to strongly consider doing so.

The proper utilization of HRP in tract-tracing experiments begins with an appreciation of the fundamental phenomena involving its uptake and transport within the nervous system. When HRP is introduced into (or onto) a restricted locale of the nervous system, it is taken up by virtually every neural element in the vicinity. Somata, dendrites, axon terminals and cut axons all actively take up the HRP; only intact axons take up little of the substance from the extracellular space. Once inside a neuron, the HRP is actively transported to all other parts of that neuron.

In neuroanatomical tract-tracing, the HRP technique takes advantage of these uptake and transport phenomena to identify neural elements which are labeled with the enzyme. For example, a typical HRP experiment might begin with the injection of a small volume of HRP solution into the cortex of a mammal. After a short survival period, the animal would be sacrificed, perfused with fixatives and the brain removed. When the thalamus is then sectioned and reacted to reveal HRP, retrogradely filled somata of thalamocortical neurons could be identified microscopically along with zones of dust-like precipitate marking the terminal fields of anterogradely labeled cortico-fugal axons. While such an experiment proceeds quite straightforwardly, numerous choices must be made at each step of the procedure, each choice depending on considerations of practicality, cost, equipment available, and most importantly, the objectives of the research.

The first choice which the investigator must make concerns the form of the enzyme. HRP is available in two principal formseither as free HRP or as a conjugate of HRP with another protein. Although the use of several conjugates has been reported, the most frequently used combination is HRP conjugated to wheat germ agglutinin (HRP-WGA). Uptake of HRP-WGA by axon terminals is apparently more sensitive than uptake of free HRP and thus, the conjugate is used to retrogradely label somata which send relatively small projections to the area of interest. On the other hand, free HRP is typically used to label the parent somata of more densely terminating axons, to label cut axons or in studies which seek to maximize anterograde transport. Although there is a sizeable price difference between free HRP and HRP-WGA or other conjugates, this price difference is largely offset by the relative concentrations of the two forms as they are used: free HRP is usually applied as a 10-30% solution while a 0.1-1% solution of HRP-WGA is typically used. Also, for a given volume, the size of the injection site varies directly with concentration of the HRP. Thus, use of HRP-WGA allows a much lower concentration and hence, a smaller injection sitea concern where limiting the spread of the HRP is important.

Having chosen the form of HRP, the next decision is how best to apply the HRP to, or onto the area of interest. Solid HRP crystals can be applied to the surface of the brain, for example, where they become dissolved in extracellular fluid. Alternatively, small pieces of gel containing HRP can be implanted in the CNS where the HRP is then slowly released into the tissue, presumably enhancing prolonged uptake of HRP. However, an aqueous solution of HRP remains the most common and, unless experimental constraints dictate otherwise, convenient form in which to apply HRP.

The next step is to determine the means of delivery of the enzyme. If either a solid or gel form of HRP is used, a topical application of HRP, for example to the surface of the brain or to the end of cut nerve, is most appropriate. However, the most

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Editor's Column

There are many things happening which will influence the conduct and directions of research in the near and far future: The debates about animal rights and human welfare will certainly have an impact on our ability to carry out the programs we feel important; the ways in which the congress and administration meet (or fail to meet) the growing federal debt will influence the funding available for research; the rapidly changing demographics are altering the need and advisability for some types of training programs on which we have traditionally relied for graduate student support for research. These and other issues must be addressed by many people and the best means found to resolve differences of opinion and policy. Such issues can be ignored by the neuroscience community only at the risk of having its interests ignored in the decision making process as the issues are resolved. We hope to publish articles germain to some of these issues in the Carrier. If you have comments on these or other such issues or wish to write an article for the Carrier, please contact me at the address or phone given below.

One of the questions which we have been asked about the Kopf line of stereotaxic instruments is what happens when one of the carriers or frames is dropped or badly treated? The Kopf engineers answer that the materials which are used for the construction of the Kopf stereotaxic instruments are of the highest quality and will withstand a lot of abuse. However, severe stress may cause a loss of the very precise alignment of the instruments and necessitate a factory "tuneup" of your frame or carrier. This can usually be carried out quickly and relatively inexpensively. If you suspect that your instrument has become misaligned, it is best to contact the Kopf factory for instructions and an estimate.

We had the occasion to attend the recent FASEB meetings in St. Louis. The opportunity of spending time in the Kopf exhibit proved to be very enjoyable as I met a large number of you who receive and read the Carrier. I hope that we have the opportunity in the future to meet more of you and to get your comments about the Carrier. The article in this issue by Lex Towns is on the use of horseradish peroxidase in tract-tracing. For those who have used the technique, the article should give some technique hints; for those who have not used it, the article should provide a good introduction to the methods and pitfalls of the technique and allow an assessment of its usefulness for your, particular application.

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HRP *Continued from Page 1*
prevalent application technique involves injection of an aqueous solution of HRP into the CNS. Here the most common approach is to use a micro-syringe, either directly or connected via fluid-filled microtubing to a micropipette, to pressure inject nanoliter quantities of HRP solution. By fitting the microsyringe, or the micropipette to which it is connected, to an electrode carrier, standard stereo-taxic techniques can be used to inject the HRP into deep brain structures. In addition to pressure injections, HRP can be injected iontophoretically by passing small amounts of DC current through a micropipette containing the HRP solution. With iontophoresis, smaller, more precisely localized injections can be obtained than with a micro-syringe. Thus, whenever meaningful interpretation of the data demands that the site of injection be very precisely localized, iontophoretic injection of the HRP should be considered.

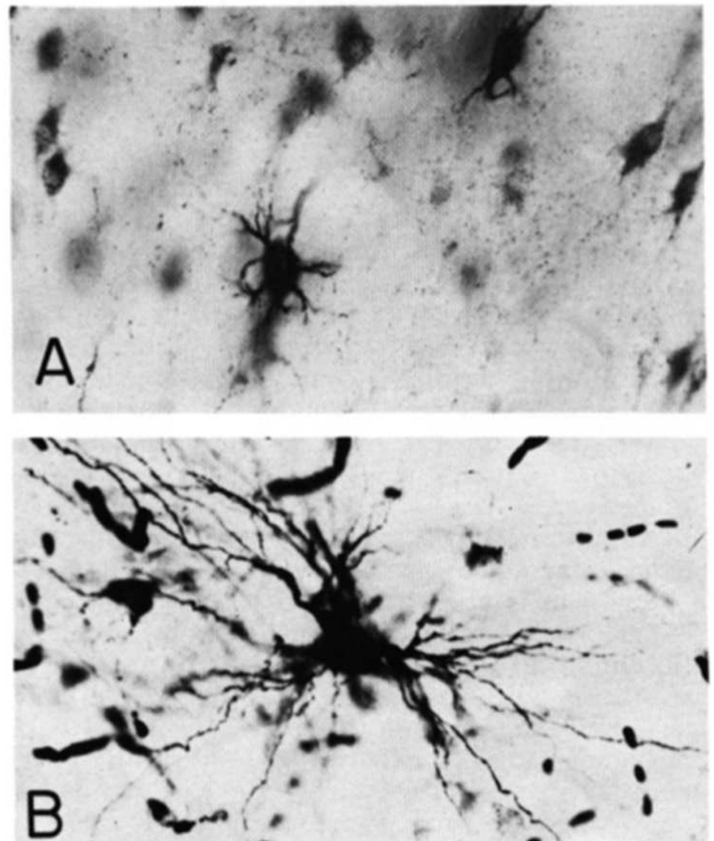

Figure 1 *illustrates several neurons which have been retrogradely labeled with HRP. A. Two densely labeled cells and numerous lightly labeled neurons are seen in this tissue which has been reacted by the TMB method. These neurons of the monkey thalamic intralaminar nucleus were retrogradely filled after injection of HRP in the visual cortex. B. A densely filled neuron which has been reacted by the DAB method is easily seen. This exquisitely labeled cell of the rabbit dorsal lateral geniculate nucleus was retrogradely labeled through its cut axon after HRP was injected in the internal capsule.*

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Visits From Representatives

Mr, J, David Kopf showing Mr. Sushit Bilaney the control panel of the Brown and Sharpe 1000 V.C. This computer controlled machining center enables us to manufacture parts that could not be made without the aid of computer controlled machines. The Kopf factory presently has two of these machining centers in operation. More details on the factory will be given in another issue of the Carrier.

Right to left J. David Kopf, Sushil Bilaney and Dan Nichols outside the Kopf factory.

During the past year, the following representatives have visited the factory to learn more about the use and care of our instruments:

Douglas Clark, Clark Electromedical Instruments, P.O. Box 8, Pangbourne, Reading RG8 7HU, England. Mr. Clark's territory is the U.K.

Teresa Batter, Schueler and Company, P.O. Box 246, Williston Park, New York 11596. They have offices in South America, Asia, the Mideast and Europe.

Sushil Bilaney, Bilaney Consultants GmbH, Furstenwall 1, 4000 Dusseldorf, Federal Republic of Germany. His territory includes Germany, Austria, Switzerland, Belgium and the Netherlands.

MODEL 750 *Continued from Page 4*

number. Push **E** pad to enter, switch to **RUN** and you are ready to go. Pushing the **START** button initiates the pull of up to 100 different pipettes.

VERSATILE:

The 750 can produce pipettes with large tips (5-6 microns) as well as extremely fine tips (less than .05 microns), and patch clam micropipettes. Pipette glass diameters of 0.4mm to 3.0mm, thin or heavy wall may be held and pulled. Unique heater control permits the use of glass with a high melting point.

The vertical design and super accurate ball bearing slide mechanism allow for straight, concentric shapes to be pulled.

SPECIFICATIONS:

H"Dx9-l/2"Wxl4-l/2"H
30 lbs, 9 oz.
105-130 Vac @ 3 amps, or 220-240 V @ 1.5 amps,
50-60 Hz. via rear panel switch plus one fuse change.

STANDARD ACCESSORIES:

Set of platinum rhodium filaments
Forceps
Aus Jena Loops
Filament wrench
Instruction Booklet

ANNOUNCEMENTS
New catalogs are now available.

If you would like to receive one please send us your correct mailing address.

HRP *Continued from Page 2*

After placing the HRP, the animal survives for some period of time to allow uptake and transport of the HRP. For most studies in the mammalian CNS, survival periods of 24-72 hours seem appropriate. However, when particularly long or short pathways are being studied these limits may not be appropriate. In any event, optimal survival times should be determined empirically.

Perfusion and fixation are a crucial part of the HRP experiment. The perfusion should start with a vascular rinse of saline or buffer in order to clear the vascular bed of blood because blood cells have a high endogenous enzyme content which can contribute to excessive background activity in the reacted tissue. Fixation must accomplish two somewhat contradictory goals; first, fixation should be long enough to accomplish good structural preservation of the tissue, but second, it should be as brief as possible to avoid unnecessary suppression of enzymatic activity. While the effect of a number of variables of fixation has been assessed, most investigators use a paraformalde-hyde-glutaraldehyde combination in phosphate buffer at roughly physiological pH (7.4) applied by standard vascular perfusion techniques. In order to minimize degradation of HRP, a vascular rinse of buffer (or buffer-sucrose for material to be frozen for sectioning) to rinse out excess fixative should conclude the

HRP *Continued from Page 3*
perfusion.

For most light-microscopic studies of HRP transport, frozen sections of tissue are the most practical. Alternatively, fixed, unfrozen tissue can be cut on the Vibratome if preservation of ultra-structure is of concern. In no case are sectioning procedures which require tissue dehydration (i.e., paraffin embedding) to be used as the dehydration steps denature the enzyme and render it unreactive.

Having produced fixed, sectioned neural tissue which contains HRP, we now come to the central protocol of the HRP procedurethe histochemical reaction to reveal HRP. When the sections containing the HRP are placed in an incubation solution of hydrogen peroxide and a chromogen (to be specified below), HRP and H202 rapidly form a complex and this complex [HRP-H202] rapidly oxidizes the chromogen to form a stable, insoluble, light-dense, colored precipitate. The result of this histochemical procedure is that wherever there is HRP in the tissue, a visible reaction product will be formed.

The most commonly used chromogens are derivatives of benzidine: benzidine dihydrochloride (BDHC), diarninobenzidine (DAB), o-tolidine, o-dianisidine, and tetramethylbenzidine (TMB). Of these, the TMB protocol of Mesalum1 offers some distinct advantages in sensitivity and ease of use and is probably the most widely applied method. However, BDHC and DAB are both frequently used and, though less sensitive than TMB, form a smaller, less bulky reaction product; an advantage when visualizing the fine anatomy of dendrites, axons or axon terminals. Because all of these chromogens are benzidine derivatives, they are potentially carcinogenic and, although the carcinogenicity of TMB has been denied, all contact with any of these chromogens should be eliminated.

Following reaction and stabilization of the HRP, the tissue can be mounted on slides and stained. Usually a neutral red stain is used because it does not interfere with the visualization of the reaction product which is typically blue or brown. The slide mounted, stained tissue can then be dehydrated, cleared and coverslipped by usual methods.

The HRP-label in the tissue can be visualized by either light-or darkfield microscopy. Figure 1 illustrates the typical appearance of labeled cells as revealed by the TMB and DAB protocols. Before proceeding to data interpretation and analysis, the investigator should become familiar with the appearance of HRP label as illustrated in the numerous published micrographs of HRP material. Better still, any opportunity to study reacted tissue from a colleague's investigation should be used to good advantage.

Obviously, this short outline of the HRP technique hardly does justice to the potential for its utilization. Interested readers should consult the enormously helpful and generously detailed references listed below[1,2] for a more thorough description of the HRP technique. In addition, there are numerous, recent extrapolations of the basic HRP technique which go well beyond the standard tract-tracing approach reviewed here. For example, the HRP protocols have been modified for electron microscopy, thus allowing an ultrastructural description of afferent/efferent synaptic relationships. In anothermajortechnical advance, recording micropipettes filled with HRP solution have been used to impale somata or axons; after electrophysiological recordings are made, HRP is injected electrophoretically into the cell[3]. Such a sophisticated technique allows a single neural element to be described functionally and, after the tissue is reacted, anatomically as well. These kinds of applications along with continuing developments (for example, the use of HRP in transsynaptic studies[4]) ensures that the horseradish peroxidase technique will long continue to be an important tool in n euro scientific research.

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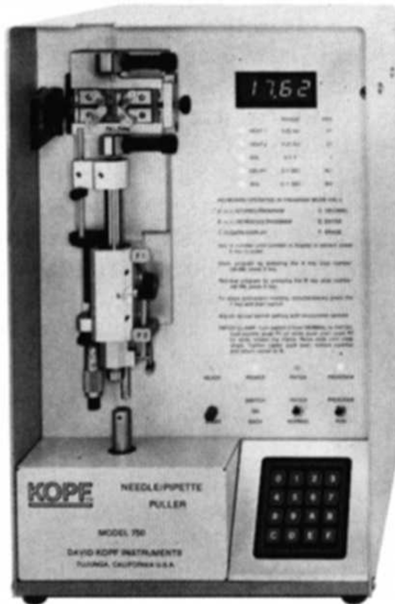
NEW
Kopf Model 750
Microprocessor Controlled Needle/Pipette Puller



The new microprocessor controlled Model 750 Vertical Needle/Pipette Puller.

PROGRAMMABLE REPEATABILITY

You may easily program and store up to 100 different pipette configurations for instant recall at the push of a button. Programs will remain in memory until erased. The puller may be unplugged without disturbing the stored programs. You can modify any parameter for quick, easy experimentation without disturbing the programs in the memory.

PRECISE CONTROL:

By setting or adjusting values of the pulling sequence you can vary the length, taper and tip to achieve an endless variety of shapes and sizes, shapes you cannot duplicate with other pipette pullers. You can adjust the proximity of the heater filaments to the pipette (Pat. Pend.), altering the shape of the pipette. Changing filament temperature or solenoid force will alter shank length. The micrometer positioned optical switch controls the amount of gravity fall and the solenoid pulse delay time will vary tip configuration.

CLOSED LOOP TEMPERATURE CONTROL

(Pat. Pend.):

The temperature control is divided into 2000 units. Heater and temperature resolution is 0.01 arbitrary units (AU) which is comparatively ten times finer than any other pipette puller available. This assures precise control of all heater temperature settings.

ELIMINATION OF HEAT:

Residual heat in the filaments can adversely affect pipette shape. The Model 750 eliminates this problem in four unique ways:

1. When on, the filaments expand toward the pipette, the minute they go off, they contract by as much as 1.5mm on each side of the glass.
2. The filaments have an extremely low thermal mass and are held by large binding posts. They cool instantly.
3. Vertical design: All residual heat rises away from the pipette.
4. Actual tip of the top pipette is pulled below heater area where there is no residual heat. The combined effect of these phenomena eliminates any need for air jet cooling.

UNIQUE FILAMENT (Pat. Pend.):

Filament contraction provides a wider gap to facilitate pipette loading. The adjustable filaments accommodate different size pipettes and allow you to change the proximity of the filaments to the glass. A visual filter allows precise adjustment of the filaments with the heater on and also protects the filaments from drafts.

USER FRIENDLY:

Easier to use than a hand held calculator. The program menu and digital display guides you through programming, storage and retrieval of programs. Operation is automatic at the push of a button.

A pipette is clamped to the fixed top clamp through the filaments to the lower adjustable clamp on the vertical slide. Starting at the top of the menu, parameter values are entered by the touch pad control keyboard and appear on the digital display. Select value, push **E** to enter and the program will automatically index to the next parameter until the program is complete. The mode is then switched from **PROGRAM** to **RUN**. When switched from **PROGRAM** to **RUN**, the **READY** light comes on. Pushing **START** energizes the heater filaments and the rest is automatic. Initial pull starts by gravity after the pipette becomes viscous. At a pre-set distance of gravity fall, the micrometer adjustable optical switch is triggered, the filaments turn off, the delay for cooling, if any takes place, and the solenoid is then pulsed for the final pull.

PATCH CLAMP TECHNOLOGY:

Patch Clamp Technology is a two step procedure and is accomplished in the **PATCH** mode with **PI** depressed. As before, all parameter values, including those for the second pull, are programmed using the program menu, keyboard and digital display. After the first pull, **P2** is depressed, the vertical slide is repositioned to center the pipette, and the **START** button is pushed to initiate the second pull. Both steps can be accomplished in seconds.

PUSH BUTTON PIPETTES:

For programs in memory, simply push the B pad to retrieve the program followed by the program

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