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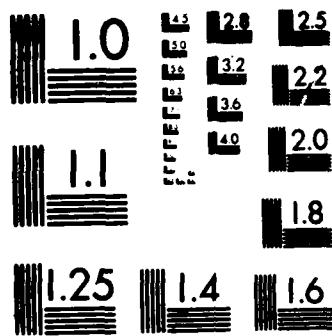
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LABELED ACETYLCHOLINESTERASE

ANNUAL REPORT

Gilbert R. Hillman

November 30, 1984

Supported by

University of Texas Medical Branch
Department of Pharmacology and Toxicology
Galveston, Texas 77550

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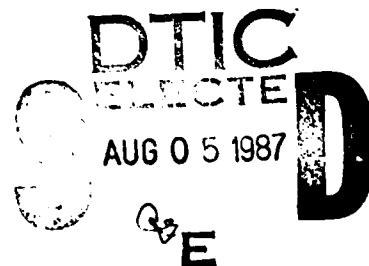
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Methods were developed for measuring acetylcholinesterase in tissue sections, using histochemical staining and computer image analysis. The staining procedure was standardized, and computer techniques were established for reproducible quantitation of the staining. Using this method, the staining was calibrated using artificial tissues with known enzyme levels. Studies of the effects of various staining periods indicated that 45 minutes was optimal for nonsaturated staining. The system was then used in studies of the inhibition of the enzyme by some carbamate and organophosphate compounds. Brain, heart, muscle, and several organs were examined; inhibition was seen in muscle and brain. We have begun the phase of the study in which rats will be treated in vivo with organophosphates and the distribution of enzyme described.

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Summary

The overall purpose of the study is to explore the use of computer image analysis in conjunction with histochemical techniques to describe the distribution of acetylcholinesterase (AChE) activity in nervous and muscular tissue in rats treated with organophosphates (OPs). The objective of the first year of work on this project was to establish the methods to be used for the remaining 2 years. We began by adopting a version of the AChE staining method as modified by Hanker, which gave staining that had density and contrast properties consistent with the optical properties of our video system. The staining procedure was adjusted and repeated until it gave reproducible results using normal tissues. We then wrote computer programs for analyzing images of the stained tissues. These programs provide a numeric quantity which represents the degree of staining in a tissue section. These techniques were then used in pilot experiments in which several experimental parameters were varied and the staining measured. The staining was calibrated by experiments in which brain tissue was homogenized and AChE measured by biochemical methods; the same homogenates were then frozen, sectioned, and stained as if they were tissues. Very high correlation was seen between the two methods. Other experiments examined the effects of varying the incubation period for staining, and of inhibiting AChE by addition of carbamates or OPs to the staining reaction. Limited experiments using in vivo inhibition of AChE in rats by diisopropylfluorophosphonate (DFP) showed strong inhibition in stained brain section. The results of these studies indicate that the determination and mapping of AChE by quantitative histochemistry is feasible and reliable. We are now in a position to apply these methods to studies of animals treated with soman.

Foreword

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION

Studies of acetylcholinesterase (AChE) levels in nervous tissue of animals indicate that poisoning with soman or other organophosphates results in disappearance of enzyme activity, which returns with time. However, it would not be possible to know, if enzyme levels are determined by homogenization and chemical assay, whether the enzyme levels disappear and reappear uniformly over all enzyme-containing tissue regions. Since cases have been reported in which exposure to organophosphates resulted in very persistent neurological deficits, it would be of interest to know whether there are brain loci at which there is an equally persistent loss of enzyme activity. If such a location cannot be found, one might suspect that some of the toxic effects of soman may be due to some mechanism other than cholinesterase inhibition, or at least that short-term AChE inhibition leads to some form of permanent damage. Such findings might be extremely helpful in developing means for protecting individuals who are at risk for soman exposure against permanent injury should exposure occur.

Our intention for the first year of the project was to demonstrate that we could use computer image analysis techniques to quantitate and localize the staining of AChE in brain and muscle sections. Therefore, this year much of our effort was dedicated to methods development. At the beginning of the project, the imaging computer system had recently been installed and software was on hand which permitted basic image reading and display operations. However, we did not have programs for convenient picture manipulation, and we had not determined what approaches would be most productive for analysis of staining in images. Progress was made in several directions during the first year.

METHODS

Histological Techniques

The classical Koelle-Friedenwald AChE staining technique, as modified by Karnovsky and further modified by Hanker (1), was set up and tested. In this method, the rat is anesthetized with ether and the brain is removed and placed in normal saline solution. No dissection of the brain is necessary, as brain structure can be distinguished by eye in the frozen sections. We freeze the brain at -10 C and cut it into sections 12um thick, using the IEC cryostat. The sections are transferred to warm slides and kept frozen until ready to stain. AChE activity appears to decrease slowly while sections are stored frozen. After a week of storage, there is a noticeable reduction in staining compared to fresh sections. If storage time is limited to less than 2 days, however, there is no detectable loss of enzyme activity, and in our experiments we always stain the sections within 1 day of sacrifice. Staining is performed according to the method of Karnovsky (2), as modified by Hanker (1). The stain is prepared by adding 24mg acetylthiocholine (ATCh) iodide to 32ml 0.06N sodium acetate. To this solution we then add 1ml acetic acid (0.1N), 2.4ml sodium citrate (0.1M), 5ml copper sulfate (0.03M), 2ml of inhibitor solution (or water), and 5ml potassium ferricyanide (0.005M). The slides are incubated in the stain for 45 minutes and then rinsed. They are then dipped in a 0.2% solution of thiocarbohydrazide (Sigma T-2137) for 10 seconds and rinsed under running water for 10 minutes. The staining is enhanced by incubating the sections with a 0.2% solution of osmium tetroxide for 5 minutes. We rinse the slides once again, and fix them with cold 10% neutral buffered formalin. Fairly extensive trial and error resulted in a reproducible procedure which produces slides that fall within a contrast and density range compatible with the video imaging equipment. The staining technique is now used routinely with frozen unfixed sections. While the Hanker technique specifies perfusion fixation of the animals, we found that this reduced the apparent levels of enzyme, and gave no advantage at the gross anatomical level we were using.

Computer Programs

Prior to the beginning of the project, our computer image analysis system had been put in place and was operational. This system consists of a Hamamatsu C-1000-01 video camera connected to a Grinnell 274 image analysis system having an 8-bit video digitizer. The Grinnell system is interfaced to a Masscomp 560 supermicrocomputer, which runs the UNIX operating

system.

This hardware combination functioned well but no software was available for it; even Grinnell's software library did not run on our machine. It was therefore necessary for us to write all software "from scratch". We did this using the C programming language. Prior to the beginning of the project we had written a few utility programs for capturing images. Many more programs were needed for effective acquisition, manipulation, and analysis of histological images.

During the year much effort has gone into developing computer programs that facilitate working with pictures in a context of analytical histochemistry. These programs may be divided into two classes: utility programs, which perform functions that might be useful in many projects, and application programs, designed for a specific analysis.

Some of the utility programs include programs for acquiring images and storing them on disk or on tapes; cropping images to exclude portions not of interest or to block out extraneous objects in the picture; determining by cursor-joystick interaction the gray level at any point in a picture; and showing, by gray scale manipulation, those regions of a picture falling within a specified gray level range. Other programs display images with modified contrast or density, store modified images, and construct three-dimensional graphics of either the contour-landscape type or the animated revolving type. Programs were also written for computation and graphic display of gray level histograms. The computation can be done either in software or in hardware, using the Grinnell image analyzer board.

The only specific application program needed so far for this project is the one that computes staining density from the histogram, as described in a later section. This was one of the first programs written.

Calibration of Video System

A system was devised for calibrating the video camera setup. A density standard, consisting of a step density wedge made of variably fogged film, was adopted as a density standard. The video system was adjusted each day to produce the same gray levels for the four densities of the standard. In this way the setup of the video system could be reproduced from day to day. We found that the apparent f/stop settings of the camera lens were not highly reproducible: Very small movements of the f/stop ring produced large variations in measured gray levels, and there was considerable mechanical

backlash in the aperture linkage in the lens. Repeatable camera setup can be achieved only by a technique which measures the apparent brightness of a known object and adjusting the camera until the measured brightness conforms to a previously determined standard.

Analytical Method

An analytical method was developed for determining the degree of staining from the digitized images of brain slices. The analysis is based on the shape and area of the gray level histogram. Many algorithms were tested. The one adopted operates as follows: The video camera is calibrated as described above. A tissue section is stained as described above. The microscope slide with the section mounted on it is placed on a light box under the video camera. An image is digitized, and the gray level histogram is computed, either by the Grinnell hardware or by software in the host computer. The histogram is reversed so that portions of the image having more staining (i.e., darker parts of the image) are represented as having higher gray levels, so that larger numbers will correspond to the intuitive direction of increased staining.

A typical histogram is shown in Fig. 1. The histogram is computed by counting how many pixels in the entire image have each of the 255 possible gray levels (brightnesses). Since there are 245,760 pixels per image, the histogram is a bar graph with 255 bars having heights up to several thousand pixels. Since there are so many bars, the histogram is usually plotted as a line graph with the tops of the bars connected, rather than as a bar graph, as is usual with histograms.

In the experiment of Fig. 1, a section of brain including the caudate nucleus, which has a high AChE content, was stained normally (left) and an adjacent section was cut and the slide immersed in 0.1 mM echothiophate before it was stained. Echothiophate is a polar organophosphate used in ophthalmology; we selected it for this experiment because it should irreversibly inhibit AChE in a tissue section (which would be permeable to externally-applied drugs), but would be safe and convenient for use as it is nonvolatile and is not absorbed through the skin. The drug did effectively inhibit AChE activity in our sections.
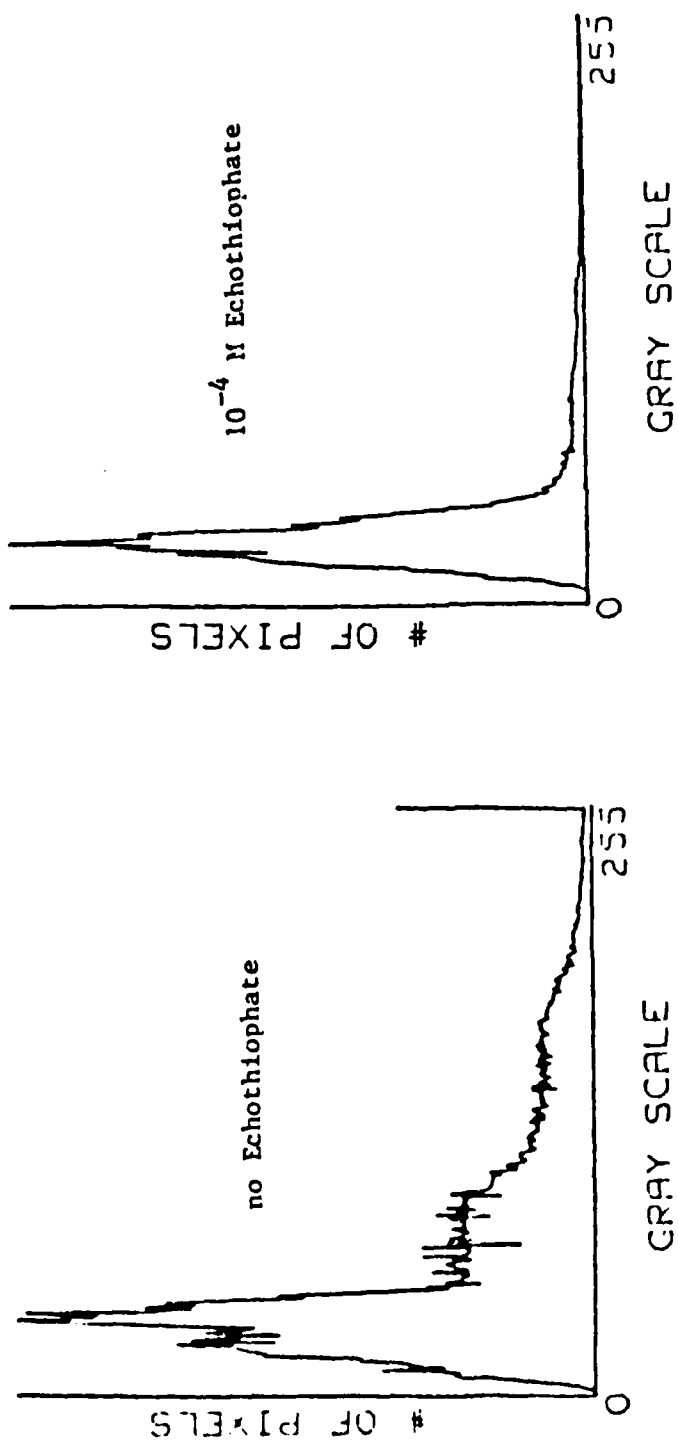
Fig. 1. Gray level histogram of stained brain section

The program that analyzes the histogram first searches, starting at gray level 10 of the inverted histogram, for a peak. This peak corresponds to the population of pixels constituting the bulk of unstained brain tissue. Gray levels below 10, most of which are zero, correspond to the background of the image outside the brain slice. Once a peak has been found, the program simply sums all pixels more densely stained than the peak, and computes the mean and total gray levels of these pixels. This number is used as the measure of total staining of the tissue slice.

Calibration of Staining

Experiments were done to relate our observed degree of staining to actual AChE levels. The approach to this problem was to prepare standard artificial tissues whose enzyme levels could be measured biochemically, and measure the same tissues by staining. If tissues having a range of enzyme levels were used, a monotonic relationship should be seen between enzyme content and staining. A linear relationship would be expected ideally, but a nonlinear relationship would be acceptable as long as it is repeatable. Once this relationship was known, the staining would be calibrated. We had originally planned to use erythrocyte ghosts as the tissue for this purpose. We found, however, that when centrifuged, the erythrocytes formed a pellet having highly heterogeneous density, and it was difficult to quantitate the staining in a simple way. At the suggestion of a colleague (Dr. Anthony Altar, personal communication) we switched to brain paste. This preparation was made by homogenizing 3g. of rat brain in an equal amount of cold phosphate buffer at pH 7.4, using a glass hand homogenizer. This material could be divided and assayed biochemically, and could also be frozen and sectioned as a tissue. A single batch of brain paste was subdivided and portions of it treated with different concentrations of echothiophate to produce different cholinesterase staining levels for activity. The brain preparations were chemically assayed using the Ellman assay, in which the hydrolysis of acetylthiocholine is coupled to bis-dithionitrobenzoate DTNB, a color reagent for thiol groups. The reaction is followed in a spectrophotometer (Gilford Model 250 recording spectrophotometer) at 412 nm. Fresh brain paste preparations were used, without freezing.

The same brain paste preparations were also quickly frozen as pellets on a cryostat chuck and sectioned and stained in the same way as other tissues. The gray level histogram of such a section (Fig. 2) shows a single normally-distributed peak of density.

When analyzed, these materials gave a reasonably linear relationship between biochemical and histochemical determination of enzyme activity. These findings are illustrated in Fig. 3. We can therefore relate measured staining to biochemically determined enzyme activity levels. The abscissa scale is the mean value of the gray levels of all pixels which the computer identifies as "stained", on the basis of their density. When the enzyme activity is greater the gray level has a lower numeric value, since a high gray level corresponds to greater brightness in the image. This is a relatively "raw" form of the data. Each point in the graph corresponds to a single measurement, and data were not obtained in the central region of the graph because the steep sigmoid nature of the echothiophate dose response curve makes it somewhat difficult to obtain data that fall at intermediate inhibition levels. This graph represents preliminary results, which will be enlarged upon during the second year of the project.
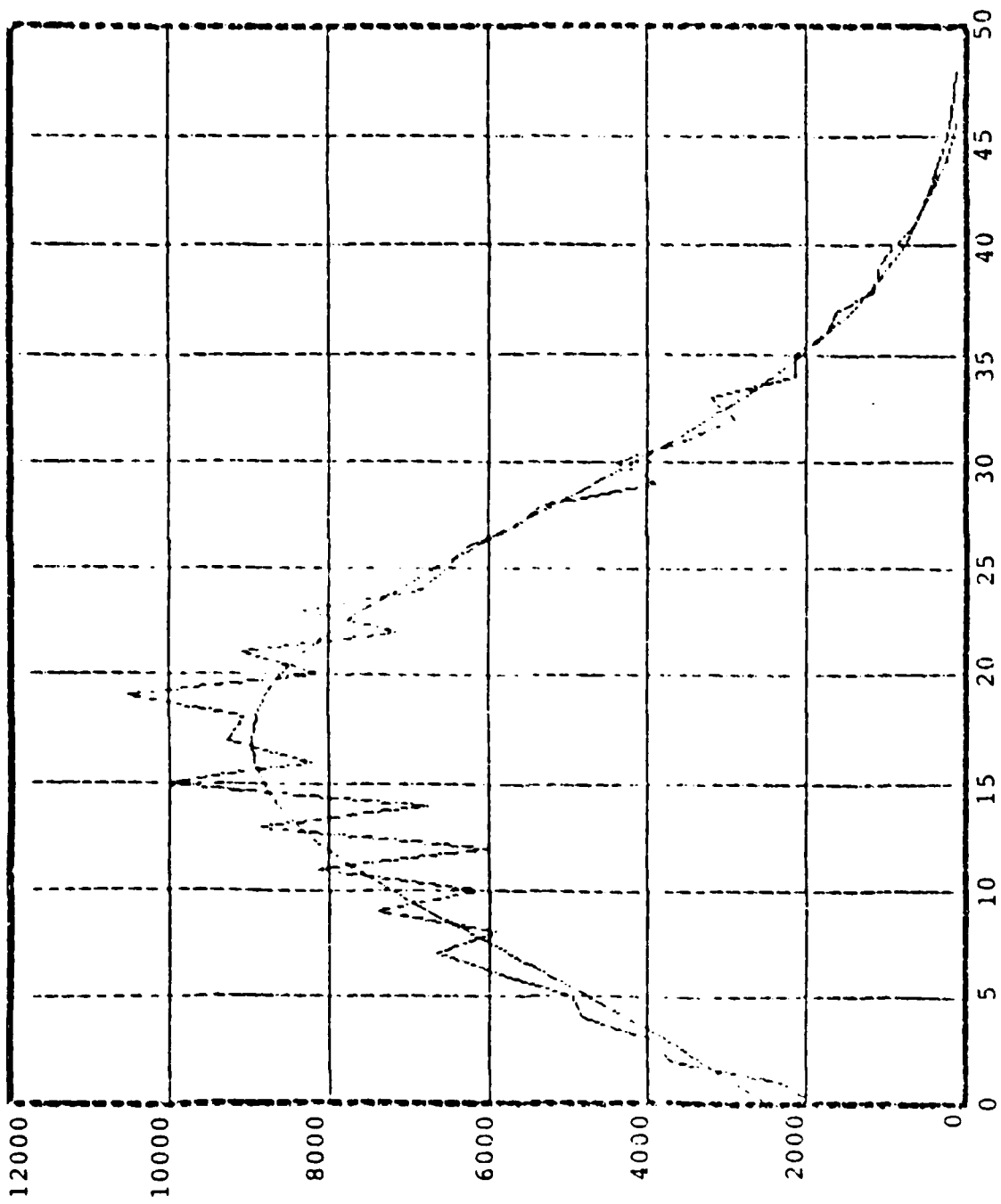
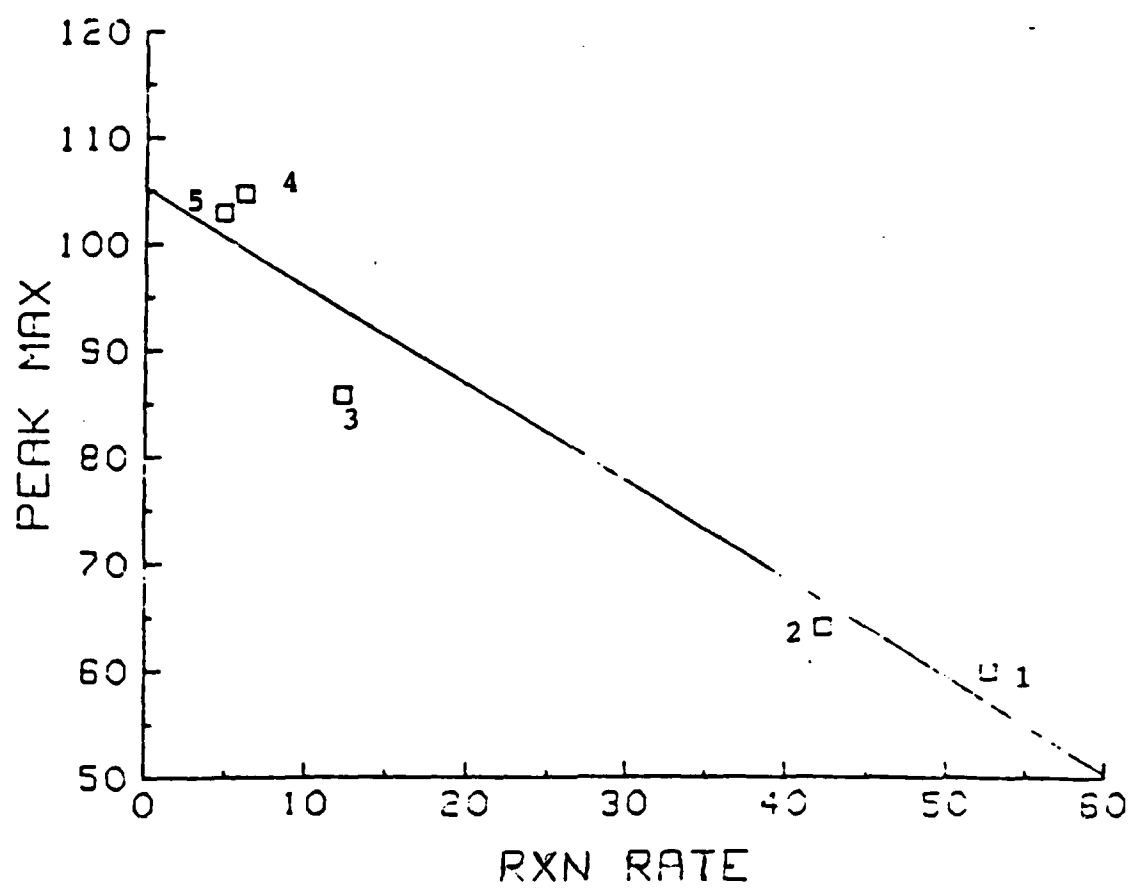Fig. 2. Grey level histogram of sectioned, stained brain paste

Fig. 3. Calibration of histochemical measurement of AChE

RESULTS

Incubation Time Studies

Experiments were done in which the length of time of incubation of the sections with ATCh was varied; the intention was to produce a series of slides with gradations of staining. Slides were prepared with brain slices including the caudate nuclear region, which has a substantial amount of AChE. These slides were stained under standard conditions, except that the time of incubation in the ATCh solution was varied over a range from 45 minutes to 2 hours. These experiments were successful, producing images which covered a range of densities. The computer quantitation of these images produced a smooth curve dependent upon incubation time, showing saturation after an incubation period of about 2 hours. Typical results are shown in Fig. 4. The saturation probably indicates that the optical density of the stained regions was so great that negligible light was able to penetrate it. Under these circumstances further staining would produce no increase in apparent density. This experiment gave confirmation of a correlation between computer quantitation and qualitative judgment of slide density. If maximum density has been achieved, it is impossible to detect small differences in staining. Therefore, we concluded that the 2-hour staining time was excessive; we wished to use a staining time that produced submaximal staining for normal tissues, so that changes in staining, either positive or negative, would be apparent. On this principle, we set 45 minutes as a standard incubation time for future experiments. While the 45-minute time was entirely satisfactory, the choice of this time was partially a matter of convenience, minimizing the incubation period. Satisfactory results could be obtained with any of the times studied except for the longest ones, at which saturation of staining occurred. We conclude that the staining time is not highly critical, though whatever time is chosen must be used consistently.
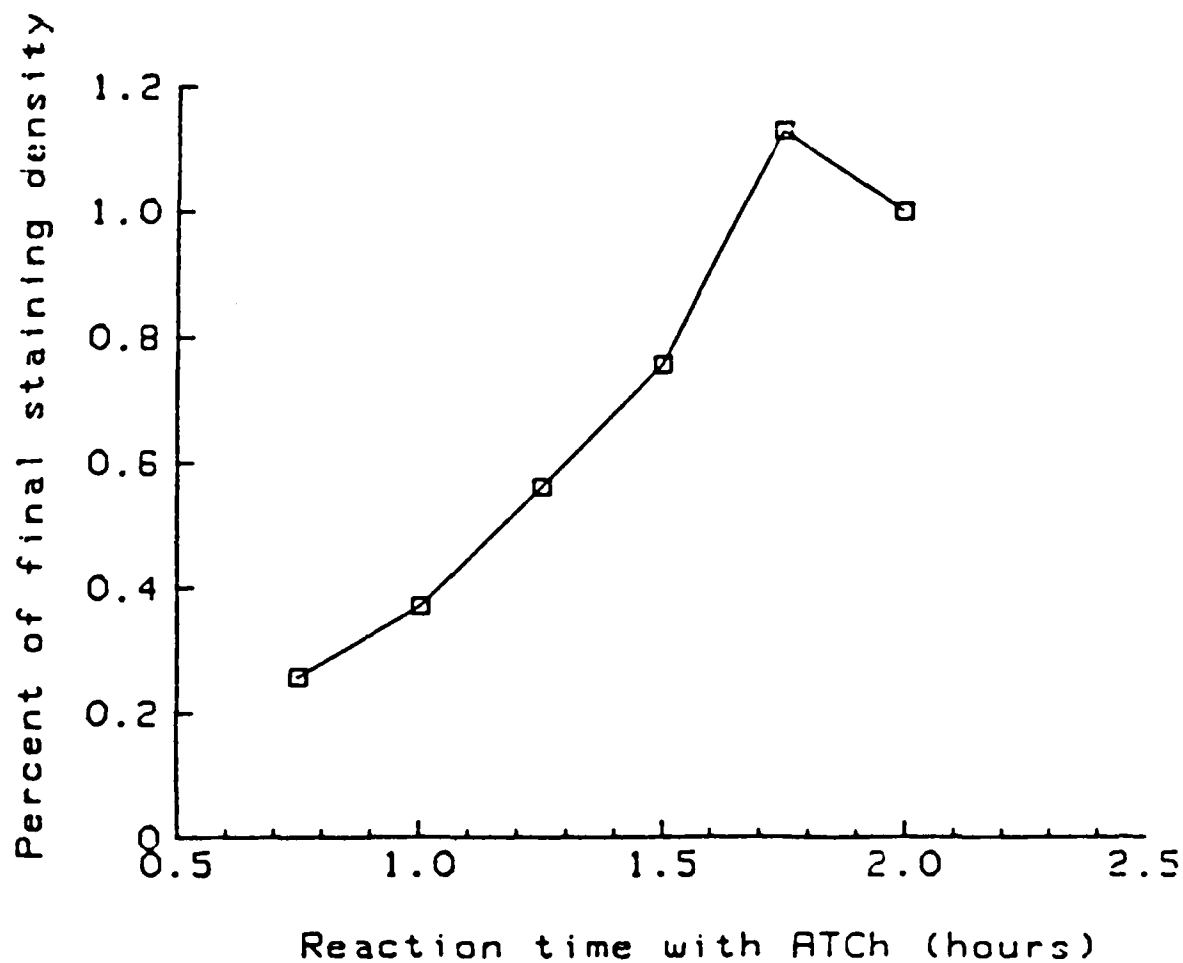
Fig. 4. Time course of staining of brain slices

Enzyme Inhibition In Vitro

Another series of experiments involved the inhibition of the enzyme with eserine (physostigmine), applied to the slide-mounted sections. Brain sections were used, with eserine added to the ATCh incubation medium. The incubation time in ATCh-plus-eserine was varied from 45 to 90 minutes. Inhibition occurred as one would expect, and computer quantitation gave results in agreement with the visual appearance of the slides. Results of such an experiment are shown in Fig. 5.
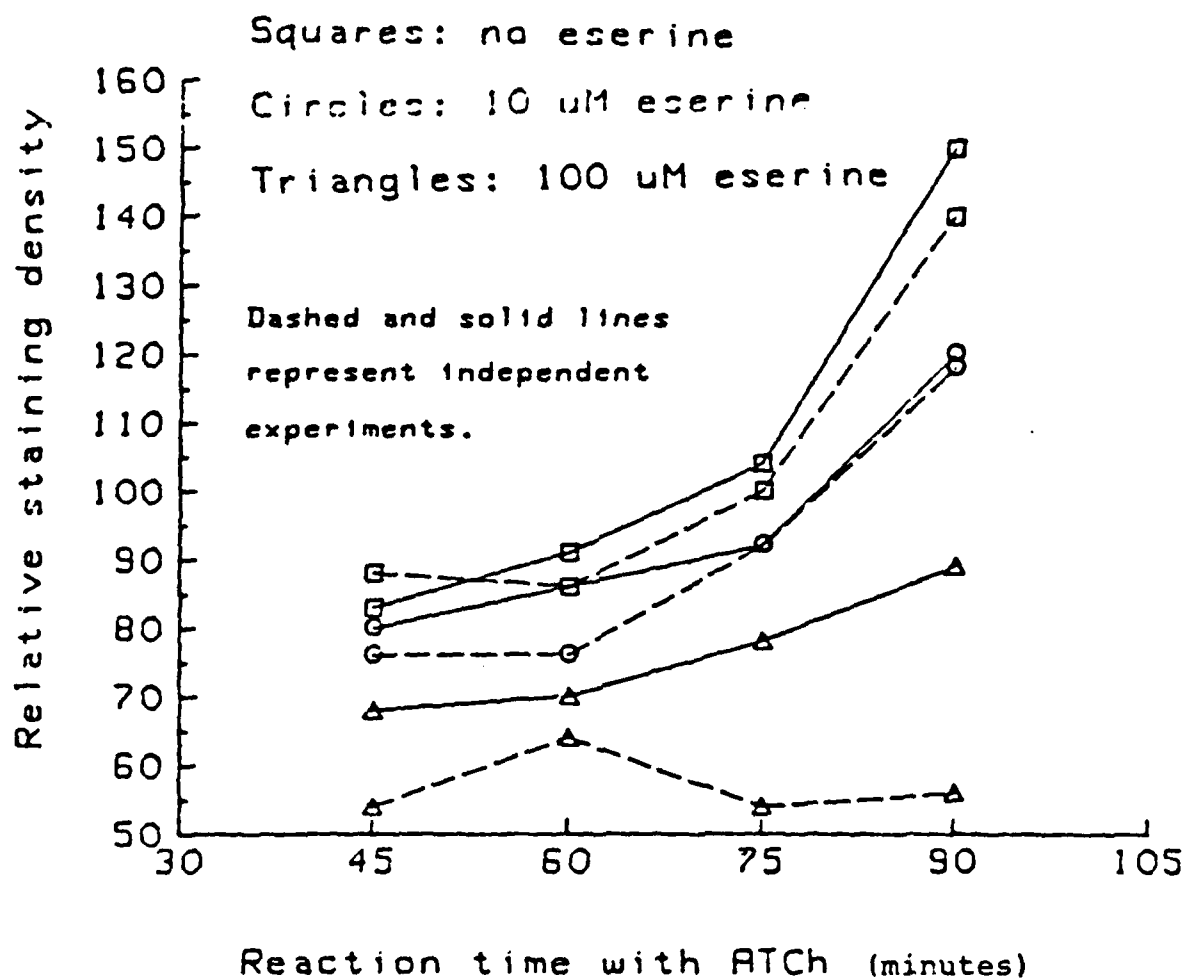
BRAIN

Fig. 5. Inhibition of AChE staining by eserine

We found that the enzyme activity _in situ_ in brain slices was less sensitive to eserine than enzyme in solution. Whole brain homogenates were prepared by hand homogenizing rat brain with 3 times the brain weight of sodium phosphate buffer at pH 7.0. Samples of the homogenate were incubated with eserine at various concentrations from 10^{-6} M up. At concentrations above 10^{-5} M, which were required for partial inhibition of brain slice staining, the activity of the homogenate was completely inhibited, as determined using the Ellman ATCh spectrophotometric assay. There was strong inhibition of the homogenates at 10^{-6} M, but extensive studies were not conducted of the interactions between eserine and brain homogenates, once it was established that the homogenates were not inhibited at the same concentrations as the slices. We had hoped to correlate the degree of inhibition on slides with that determined in solutions by spectrophotometric methods, but the biochemical experiments indicated inhibition at lower levels of eserine than those required to inhibit staining. Nevertheless, a clear monotonic relationship between eserine levels and staining was obtained. This relationship is shown in Fig. 6. Fig. 6 shows one experiment with one measurement per point. This experiment was preceded by preliminary experiments giving similar results.
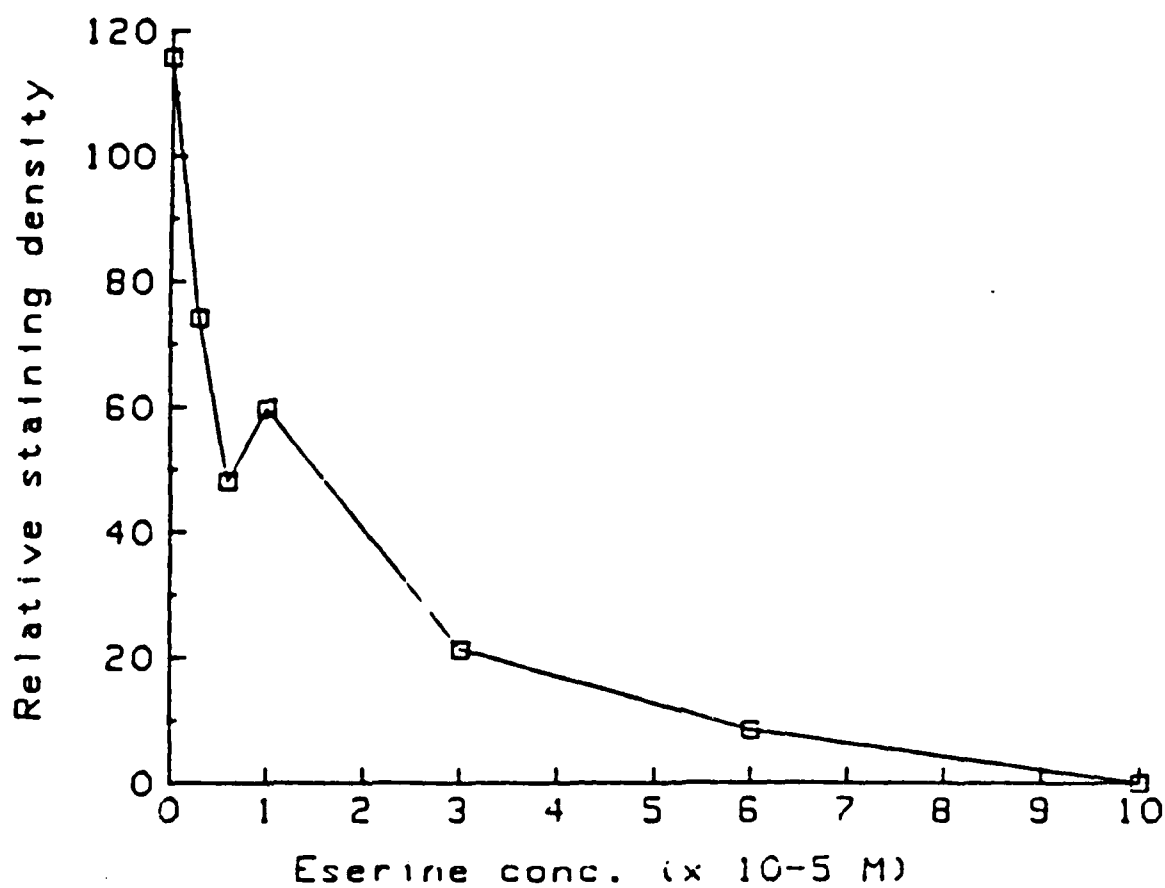
Fig. 6. Dependence of eserine inhibition on eserine concentration

Similar experiments were conducted, with similar results, using echothiophate, an ionic organophosphate. This drug, applied to sections, produced inhibition of staining. Experiments were performed using the same protocols as the experiments with eserine; echothiophate was added to the ATCh incubation medium. The experiment was repeated three times with similar results; one of these experiments, using two concentrations of echothiophate and a control group, with one slide per time/concentration point, is shown. Results with echothiophate are shown in Fig. 7.
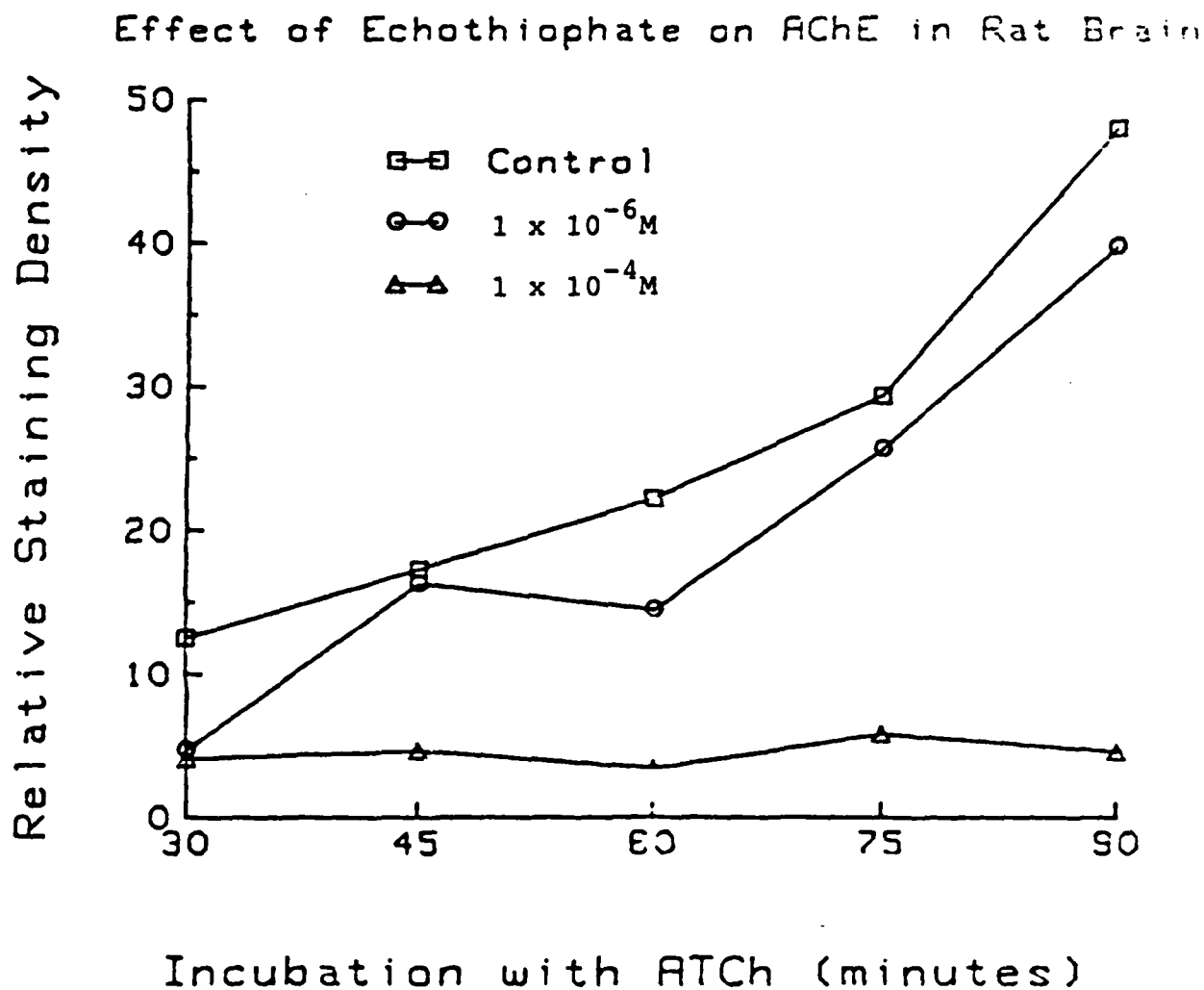
Fig. 7. Inhibition of histochemical staining by echothiophate

Experiments were conducted with tissues other than brain. Skeletal muscle, heart, liver, and spleen were tested. The skeletal muscle was obtained from the thigh region of the rat. Blood was drained from the tissues, but no special attempt was made to perfuse the tissues to remove erythrocytes; since the tissues are sectioned and stained without fixation, erythrocytes seem to be washed from the slides fairly effectively during the staining procedure. Only low levels of staining, not readily inhibited by AChE inhibitors, were seen in liver and spleen. Skeletal muscle gave results like those in brain; we expect to continue to measure muscle and brain staining routinely. However, the AChE levels in muscle are less than those in brain; for this reason, longer ATCh incubation times were used for muscle than the 45 minutes used with brain. Results with muscle tissue are shown in Fig. 8. Fig. 8a shows an experiment using reaction times from 1 to 3 hours, while Fig. 8b shows the effect of eserine with the same reaction times (simultaneous eserine and ATCh). Fig. 9 shows inhibition of AChE in heart tissue by echothiophate.
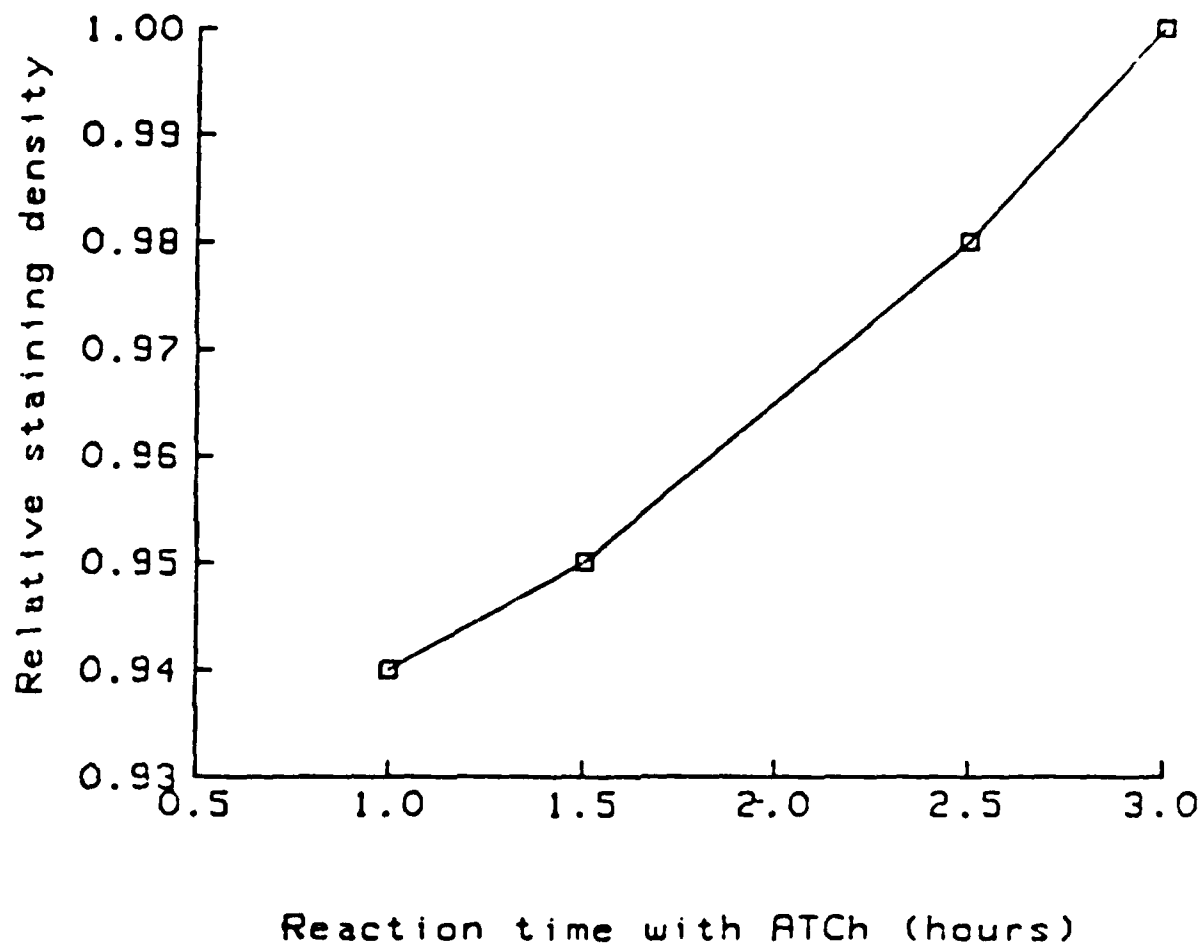
MUSCLE

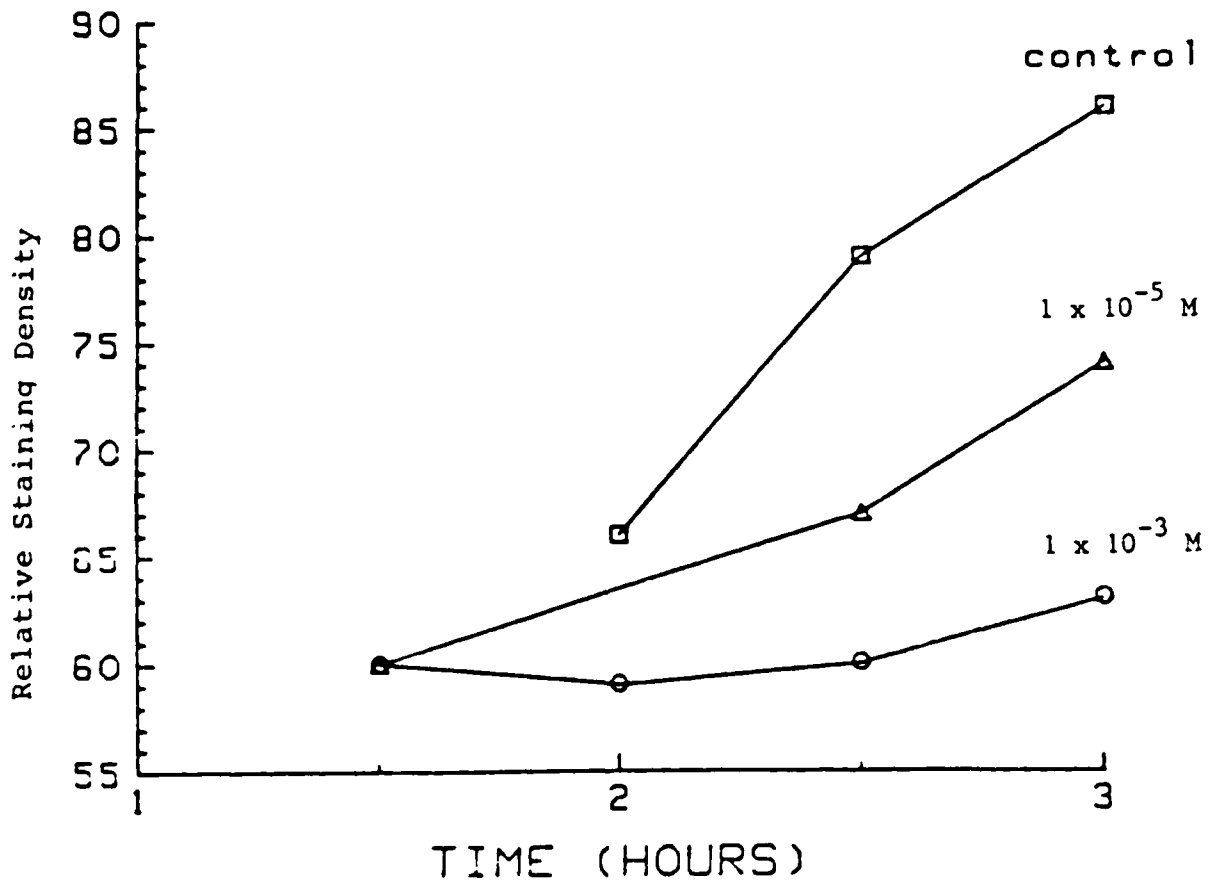Fig. 8a. Time course of staining of AChE in skeletal muscle tissue slices.

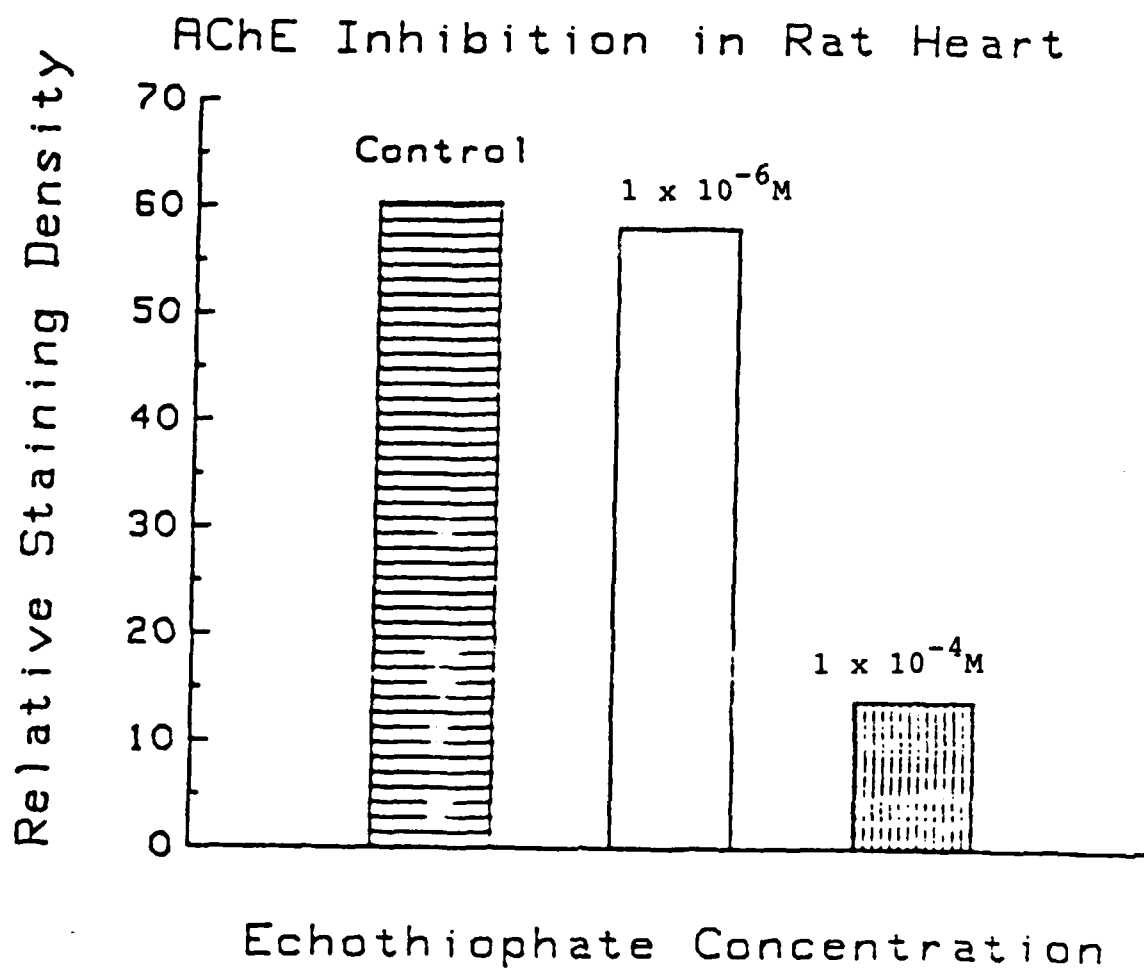Fig. 8b. Inhibition of staining of skeletal muscle by eserine

Fig. 9. Inhibition of staining of AChE in heart tissue by echothiophate

In Vivo Experiments

We have performed three preliminary in vivo experiments with rats injected with diisopropylfluorophosphonate (DFP). The DFP-treated rats, given doses which did not produce lethality after injection, showed substantial inhibition of cholinesterase in brain slices. An experiment of this type is shown in Fig. 10. Female rats weighing about 175 grams were injected with DFP by the intraperitoneal route. The DFP solution was prepared by adding DFP (Sigma Chemical Co.) directly to 0.9% saline, to a concentration of 1 mg/ml., which is within the limits of solubility of DFP in water. The dose given to the rats was 1 mg/kg. This dose produced some visible toxic reactions (salivation, shaking), but no fatalities. At 15 minutes or 1 hour after DFP dosing, a rat was killed and the brain sectioned and stained. In one experiment the level appeared to recover significantly after a week; these results are not yet clear and require much more study before they can be reported.
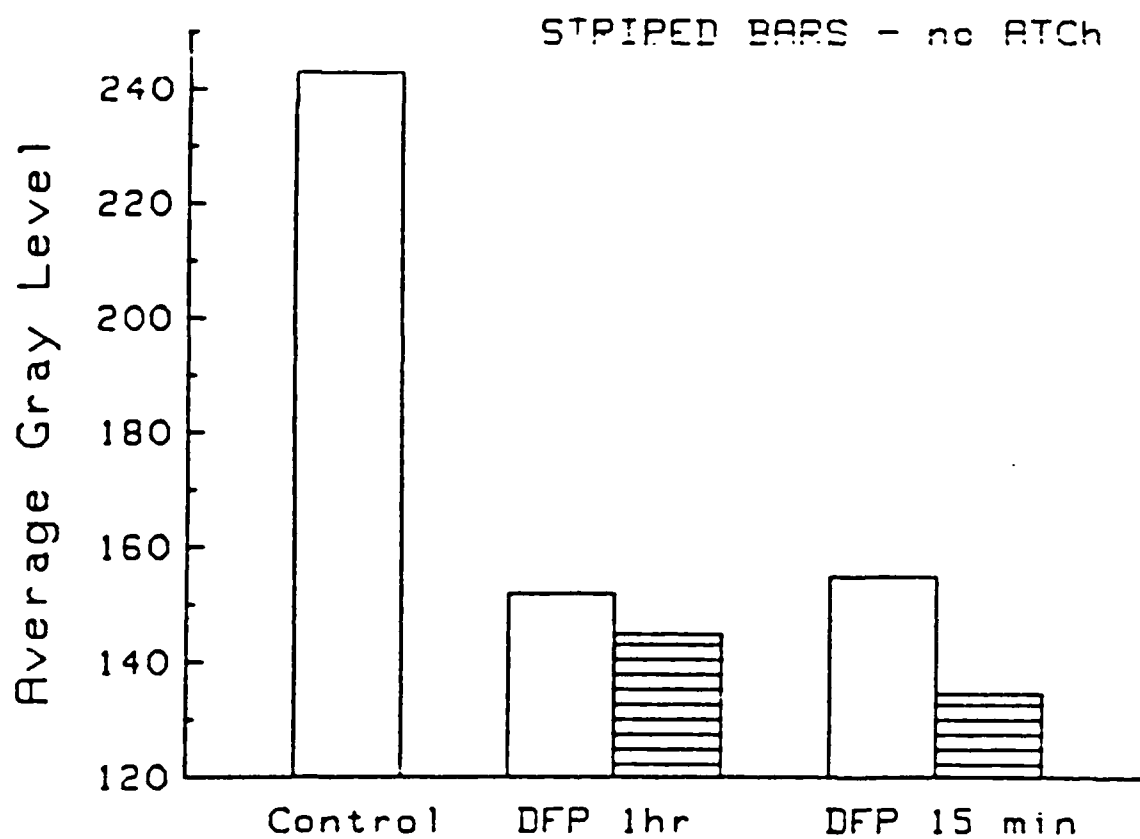
Fig. 10. Inhibition of brain AChE by *in vivo* injection of DFP

Discussion and Future Plans

Our activities for the first year have led to the conclusion that it is feasible to measure AChE in frozen tissue sections by computer image analysis. Methods for preparation of tissue sections and for staining the sections have been worked out, and a preliminary method of analysis of the stained sections has been established. Several experiments have been completed whose purpose is to demonstrate that inhibitors of the enzyme do inhibit the staining, and that sections that appear inhibited do, in fact, give analytical results in agreement with their appearance. Results are reported that show that the computer-based staining measurements can be correlated with chemical determinations of enzyme levels. A few in vivo experiments with DFP have shown that changes are seen in rats treated with sublethal doses of an organophosphate. This protocol is the basis for future work.

During the second year of the project, we expect to begin in vivo treatments of rats with soman. These rats will be used in experiments examining the disappearance and recovery of AChE after sublethal dosage of soman. The methods developed during year 1 will be subject to continued improvement, but we feel that most of the methods development is complete. Some improvement seems possible in the algorithms that are used to quantitate the staining. We hope that the proposed arrangements for soman treatment at Baylor will give reliable results. Our initial experiments will be directed at determining the reproducibility of inhibitory effects, and at determining the time course of recovery of staining after a soman dose; dosages of 90ug/kg will be used at the outset.

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