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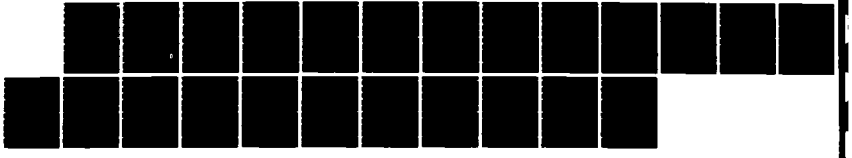

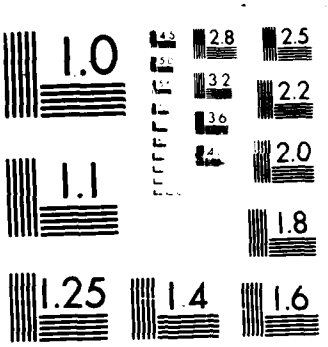

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NEUROCHEMICAL MECHANISM OF ORGANOPHOSPHORUS COMPOUNDS: EFFECT ON NEUROMEMBRANE

ANNUAL REPORT

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SUMMARY

Our work over the past year has focussed on two major areas: (1) an attempt to identify an endogenous enzyme in brain capable of inactivating diisopropylfluorophosphate (DFP) and other organophosphorus compounds; and (2) effects of DFP on in vivo brain protein synthesis. In addition, we have begun a new project in which the effects of DFP administered in vivo on brain content of several endogenous opioid peptides is to be examined.

The possibility of a DFP-inactivating enzyme in brain tissue was examined by incubating mouse brain homogenates and subcellular fractions with DFP under various conditions. DFP hydrolysis was followed indirectly, by testing the effects of supernatants to inhibit acetylcholinesterase (AChE). Our results suggest that the 100,000g supernatant of brain homogenate (S_3) may contain a DFP-hydrolyzing enzyme, but further work is needed to establish this conclusion.

Our results in the brain protein synthesis study, which are still very preliminary, suggest that DFP does alter the synthesis of specific polypeptide species in brain. ~~Our~~ study of the effect of DFP on endogenous opioid peptides has just begun; we are still perfecting the methodology.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) 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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BACKGROUND AND STATEMENT OF THE PROBLEM

Organophosphate compounds (OP's) include the deadly nerve gasses soman, sarin and tabun. It is generally believed that these substances act by inhibiting acetylcholinesterase (AChE), for the symptoms of OP poisoning are similar to those of known AChE inhibitors, and the LD_{50}s of OP's parallel their effectiveness in inhibiting the enzyme (1). In addition, antidotes based on reversing or counteracting AChE inhibition are the most successful ones developed to date (2-4).

Nevertheless, much recent evidence indicates that other mechanisms may contribute to OP toxicity. OP's have been shown to affect many other tissue components, including other enzymes (5); OP's react directly with the cholinergic receptor (6), as well as with a number of other cell membrane components of unknown function (7). A "neurotoxic esterase" to which radioactive OP's bind is believed to be involved in neurotoxic effects, including neuronal degeneration, produced by these substances (8).

These considerations make it important to study other possible mechanisms of action of OP toxicity. It is likely, particularly in non-lethal cases, that OP's act by a combination of several processes, with the relative contributions of each depending on the individual. One of our major research interests has been the structure and function of biological membranes, particularly synaptosomal plasma membrane (SPM), which surrounds nerve terminals in the brain. Accordingly, we have been examining the possible interactions of OP's at this site of the nervous system. Specifically, we are studying the effects of OP's on brain membrane protein synthesis in vivo.

In addition, during the course of this work we pursued two other, related approaches to this problem. We attempted to identify and characterize an enzyme in brain capable of hydrolyzing OP's. This grew out of our studies of OP-binding components, since such an enzyme would be a special form of the latter. We also have begun to study the effects of OP's on the brain content of endogenous opioid peptides -- beta-endorphin, the enkephalins, and dynorphin -- which are another major interest of our laboratory. This project is a natural extension of our studies on the effects of these agents on membrane components. Furthermore, endogenous opioids might be thought to have a role in OP action, because they are known to be involved in the body's response to stress (9), and because they have links to brain cholinergic systems (10).

EXPERIMENTAL DESIGN AND METHODS

Diisopropylfluorophosphate (DFP) preparation. Great difficulty was encountered in the acquisition and assay of pharmacologically/toxicologically active DFP. DFP preparations from different companies were found to vary markedly in their activities, as measured by LD_{50} in mice, and there was sometimes even substantial variation in two preparations from the same company. Furthermore, DFP stored in aliquots exhibited a steady decline in toxicity over time. This loss of activity significantly impeded our research, not only because a batch had to be assayed prior to every experiment, but because of the steepness of the dose/response relationship. A dose of 1/3 the LD_{50} might produce no

mortality, so that a very accurate assessment of dose was necessary to produce non-lethal effects.

Similar problems have been reported by others (11). In fact, the activity of a single DFP preparation, stored at -20°C, may fluctuate markedly over a period of months; that is, when tested at various intervals, it does not simply exhibit a decline in toxicity, but may actually increase at certain times. These fluctuations may result from differences in the susceptibility of different batches of mice used to bioassay the DFP, or possibly even reflect the operation of natural long-term cycles in DFP susceptibility. With respect to the latter, it is known that DFP sensitivity varies over a 24 hour cycle, reflecting cyclical changes in activity of brain AChE and other cholinergic components (12). The existence of longer-term cycles would have considerable relevance to prophylactic/therapeutic approaches, particularly since the changes in sensitivity are quite significant, as great as 2- or 3-fold.

Finally, we have observed that although the activity of DFP as measured in vitro -- that is, its ability to inhibit AChE -- also varies, it does not parallel the in vivo variations. For example, some DFP preparations which showed markedly different in vivo activities had similar in vitro potencies. This offers further support of the idea, discussed in the Background section, that the LD_{50} of DFP and other OP's is indeed the result of more than just AChE inhibition.

Preparation of brain fractions. Mouse (Simonsen ICR) brain homogenates were prepared by placing one brain in a Potter-Elvejhem homogenizer with 2.5 ml. of 0.05 M Tris, pH 7.4, and homogenizing with 20 strokes by hand. In experiments in which homogenates were to be incubated at other pH's, they were prepared in distilled water and the appropriate buffer added to the incubation medium.

Nuclear (P_1), mitochondrial (P_2), microsomal (P_3) and soluble (S_3) fractions were prepared by differential centrifugation of brains homogenized in 0.32 M sucrose - 2 mM Tris, pH 7.4 (13). The particulate fractions from five brains were suspended in 6-8 ml. of the 0.05 M Tris buffer.

Protein assay. This was performed as described by Lowry et al. (14).

SDS polyacrylamide gel electrophoresis. This was carried out as previously described (13).

Radioactivity assay. Samples were added to 7 ml Scintiverse (Fisher) and counted for 5 min in a liquid scintillation counter. Efficiency was 38-45%.

In vivo administration of tritiated leucine. To study the effects of OP's on brain protein synthesis, we used intracerebroventricular (icv) injections of ^{3}H-leucine. Ten uCi containing 50 uCi were injected, followed by three different dose regimens, each employing 3 animals injected intraperitoneally (ip) with 4 mg/kg of DFP: 1) ^{3}H-leucine, followed 3 hours later by DFP, followed an hour later by sacrifice; 2) DFP followed one hour later by sacrifice; and 3) as in 2), except that 3 hours

after the leucine, another dose of DFP was given, followed one hour later by sacrifice.

After sacrifice, brains were removed, homogenized in 0.32 M sucrose, and membranes prepared as described above. Samples of membranes were then subjected to SDS gel electrophoresis and the gels dried and exposed to x-ray film.

DFP analysis. In our initial work, we used a DFP assay based on that of others (11). However, we found that DFP inhibition of AChE is more complex than many published reports would imply. In such studies (11), AChE activity is assayed immediately after DFP addition; however, if DFP is allowed to preincubate with the membrane, the toxin becomes progressively more potent, for preincubation times up to at least 30 min. (Fig. 1). Even after a 5 min. preincubation, the IC_{50} of DFP is about 1.1 ug/ml, which is about one-tenth what others have reported (11). After 15 min, it is down to about 0.3 ug/ml.

Because of this time-dependence, we modified our assay so that DFP was always preincubated with tissue for 10 min before assaying for AChE activity. While inhibition is not maximal at this time, the values obtained are very reproducible, and a standard curve with an IC_{50} of about 0.4 ug/ml could be constructed. By adjusting samples of unknown DFP concentration so that their AChE inhibition fell in the 70-30% range, we could assay DFP with good precision from experiment to experiment.

AChE assay. AChE was assayed essentially by the procedure of Ellman et al (15). Assay mixtures contained 1.0 ml 1.0 M Na phosphate, pH 8.0; 0.1 ml 0.03 M acetylthiocholine iodide (ASChI); 0.1 ml 0.01 M dithiobisdinitrobenzoic acid (DTNB); and 50 ul of P_3 (about 100 lg). ASChI and DTNB were first added to a cuvette, followed by P_3 in the phosphate buffer. The DFP preincubation was carried out in the phosphate buffer. After addition of all components, the change in absorbance at 412 nm was measured for two minutes.

DFPase assay. To test for a DFP-inactivating enzyme, our basic procedure was to incubate brain homogenates or other brain fractions with DFP at 37°C for various lengths of time. Following incubation, samples were cooled to 0°C, centrifuged to remove particulate material, then the supernatants analyzed for DFP. Among the variables we studied were a) length of incubation time; b) amount of tissue; c) DFP concentration; and d) pH. In control experiments, DFP was incubated in buffer alone, to measure the rate of spontaneous hydrolysis. Further details are given in Results.

DFP binding. In addition to spontaneous hydrolysis, a second non-enzymatic factor that might contribute to inactivation of DFP was simply binding of it to the tissue during incubation. This binding would remove DFP from the supernatant and thus lower its concentration. In order to estimate the contribution of this factor, ^{3}H-DFP was added directly to some of the samples prior to incubation. Following incubation and centrifugation, aliquots of both pellets and supernatants were assayed for radioactivity. In the case of DFP binding to S_3, the incubation mixture was passed down a Sephadex G-25 column, separating bound from free radioactivity.

Because of the aging process, by which bound DFP is thought to lose an alkyl group (16), radioactivity measurements probably underestimate the amount of DFP bound. Our radioactive DFP contains tritium in its alkyl groups; thus, during aging, it is expected that 50% of all bound radioactivity will be lost. Accordingly, to make a maximum estimate of the amount of DFP bound, we doubled the percentage of radioactivity found in the pellet. This calculation assumes that at any given time, all bound DFP is aged. While this assumption may be incorrect, it allows us to place an upper limit on the amount of DFP removed from the medium by aging.

Materials. Sources of most of the materials used in these studies have been given in a previous publication from this laboratory (13). DFP was from Sigma Chemical Corp., St. Louis, Mo., unless otherwise stated. All other OP's were supplied by the U.S. Sephadex was from Pharmacia, Uppsala, Sweden. Tritiated leucine and ^{3}H-DFP were from New England Nuclear, Boston, Mass. X-ray film used in developing autoradiograms was from Eastman Kodak, Rochester, N.Y.

Animals. Male ICR mice (25 g) were used in all studies for those determining endogenous opioids, where male Charles River rats (250 g) were used. All animals were housed in the University of California, San Francisco Animal Care Facility and fed ad libitum.

RESULTS

Brain Protein Synthesis Studies

Because full development of the X-ray films requires six months or more with the relatively low levels of radioactivity we are working with, we do not have the full results of these experiments yet. However, observations made after four months indicate that individual protein bands were labelled, and that the extent of labelling of some bands was altered by DFP administration.

Inactivation of DFP by Brain Tissue.

When DFP was incubated at 37°C with mouse brain homogenates, a definite inactivation occurred (Fig. 2 a-c). In one experiment, using relatively high concentrations of both DFP and homogenate, this inactivation appeared to be time-dependent (Fig. 2a). However, we were unable to reproduce this finding. Typically, a large degree of inactivation occurred within the first 15 minutes of incubation, followed by less or none at all (Fig. 2b,c). We initially thought that this might be due to the fact that most of the DFP was inactivated after 15 min, but adjusting DFP and homogenate concentrations so that the initial rate of inactivation would be slower did not eliminate this plateau. A similar effect was observed at other pH's and with P_1, P_2, P_3 and S_3 fractions (data for P_2 and P_3 are shown in Fig. 2d). Data for one hour incubation do suggest tissue- and temperature-dependence, however (Fig. 2c).

Two kinds of control experiments were run to insure that this loss of DFP was due to enzymatic inactivation. First, to determine the rate of spontaneous DFP hydrolysis, DFP was assayed after incubation at 37°C in buffer without tissue. As shown in Fig. 3a, a significant amount of

inactivation was detectable within 30 min at pH 7.4, and after 2 hours about 25% of the DFP had been inactivated. However, this amount of inactivation could not account for all of the inactivation observed with high concentrations of homogenates.

Spontaneous inactivation appeared to be slightly retarded at pH 5, while it was markedly enhanced at pH 9 (Fig. 3a). At 0°C, it was insignificant after 2 hours. However, assays of DFP stored at 4°C showed a marked decline in potency within 24 hours (Fig. 3b).

The other non-enzymatic factor that might be expected to contribute to DFP inactivation is binding of the toxin to tissue during incubation. As shown in Fig. 4, this binding occurred rapidly to all particulate fractions, reaching a maximum within 30 min, and declining thereafter. This decline was probably due to aging, which, as previously noted, should result in a loss of bound radioactivity.

The rapid kinetics of particulate binding is consistent with its accounting for much of the DFP inactivation observed, which also was almost complete in 15-30 min. The amount of binding can also account for much of the inactivation. As explained in the Approach section, the phenomenon of aging makes it necessary to assume that twice as much DFP is bound as the radioactivity bound would indicate. When the latter value is used and added to the amount of DFP inactivated spontaneously, it appears that all DFP inactivation in the presence of P_2 amd P_3 is adequately accounted for, but not all of that in the presence of homogenate is (Table I).

Table 1

Theoretical Rates of DFP Inactivation Due to Spontaneous Hydrolysis and to Binding. Comparison with Observed Rates.

Fraction	DFP Bound (A)	DFP Spontaneously Hydrolyzed (B)	Theoretical Inactiv. (A)+(B)=(C)	Actual Inactiv. (D)
Homogenate (4.25 mg)	0.28+0.05	.020+0.004	0.048	0.078+0.010
Homogenate (0.50 mg)	0.007+0.003	.020+0.004	0.027	0.035+0.005
P_2 (1.50 mg)	0.026+0.002	.020+0.004	0.046	0.042+.004
P_3 (1.00 mg)	0.012+0.004	.020+0.004	0.032	0.026+.003

All values are for 1 hour incubation at 37°C, pH 7.4, and are expressed as the mean ~ standard deviation of 2 determinations (mg protein). Bound DFP is calculated as twice the % of radioactivity in the pellets.

These results imply that there may be some DFP-inactivating material in S_3, the soluble portion of the brain homogenate. To test this idea, we first determined the binding of ^{3}H-DFP to S_3, by incubating them at 37°C for one hour, then passing the mixture down a Sephadex G-50 column. As shown in Fig. 5, all detectable radioactivity eluted in a single peak coincident with the position of free ^{3}H-DFP (totally retained on the column). No radioactivity was observed in the void volume, where any protein larger than 10,000 molecular weight should have appeared. From similar experiments with Sephadex G-10, which voids still smaller species, we conclude that DFP is not binding in appreciable quantities (5% of the total) to any other molecule in S_3.

Having established that DFP binding to S_3 components would not significantly interfere with inactivation, we next tested for the latter. As with particulate fractions, DFP was incubated withh S_3 for various times, then assayed for AChE-inhibiting ability. Since S_3 and DFP could not be rapidly separated, the mixtures were assayed. As a control, S_3 was also assayed by itself, since it contains a significant amount of AChE.

Results of this experiment are shown in Table 2. Though some inactivation above that of spontaneous levels was observed, this inactivation was not time-dependent.

Table 2

Inactivation of DFP by S_3

Time of Incubation	DFP Spontaneously Hydrolyzed	Total Inactivation	Inactivation Due to S_3
30 min	0.014+0.0	0.030+0.003	0.016+0.005
1 hr	0.025+0.006	0.043+0.007	0.018+0.009
2 hrs	0.027+0.005	0.042+0.008	0.015+0.010

All values are in mg. Further details are given in text.

If S_3 did contain a DFP-inactivating enzyme, it might require some ion for its full activity. Accordingly, we tested the effect of adding various ions, all at a final concentration of 2 mM, to S_3 during incubation with DFP. These ions included Ca^{++}, Mg^{++}, Mn^{++}, Cu^{++}, Co^{++} and ethylenediaminetetracetic acid (EDTA). Of these, two seemed to have a significant effect on DFP inactivation. Cu^{++} greatly increased subsequent DFP inhibition, while Mn^{++} reduced it (data not shown).

The effect of Cu^{++} was subsequently shown to result from a direct poisoning effect on AChE itself, rather than an effect on DFP. Mn^{++}, however, seemed to enhance DFP inactivation; as control experiments showed, it had no effect on AChE itself, nor on spontaneous DFP hydrolysis. Subsequent experiments, however, showed that neither heating nor treatment with the proteolytic enzyme trypsin had any effect on S_3's DFP-inactivating ability.

These results suggest that the latter is not an enzyme. Furthermore, dialysis, which should have no effect on an enzyme, reduced the DFP-inactivating ability of S_3.

Preliminary Studies of Endogenous Opioid Peptides

Development of procedures for the determination of beta-endorphin, leucine-enkephalin and dynorphin contents of various regions of rat brain following OP administration have been in progress. The first attempts were based on similar procedures developed in this laboratory and found successful for measurements of brain Substance P levels. In these experiments, rat brains were removed immediately after sacrifice, and dissected into cortex, striatum, diencephalon, medulla and cerebellum.

The extraction medium consisted of a freshly prepared solution of PMSF (phenylmethyl sulfanylfluoride, 30 mg.) and iodoacetamide (30 mg.), which were dissolved in 1 ml. of anhydrous ethylalcohol. 99 ml. of 2 M acetic acid were then added. A weighed amount of nervous tissue was homogenized in the extraction medium (500 u./50 mg. wet tissue) in an ice bath with 10 strokes, and the homogenizer was rinsed with another 40% of this volume of medium. Each sample was then cenrifuged in an Eppendorf centrifuge for 15 min. at 12,000 g in the cold room. The supernatants were decanted and then lyophilized. The lyophilized powders were redissolved in 1 ml. buffer D per 50 mg. original wet tissue. Five liters of buffer D contained 13.8 g. $NaH_2PO_4 \cdot H_2O$, 43.75 g. NaCl, 0.5 g. BSA, 5.0 g. gelatin (type III, calf), 5.0 g. Triton X-100, and 0.5 g. Thimerosal. Among these ingredients, $NaH_2PO_4 \cdot H_2O$, NaCl, and BSA were first dissolved in 4 l. distilled water, then gelatin predissolved in 200 ml. warm distilled water and Triton in 100 ml. water were added, and then Thimerosal. The pH of the solution was adjusted to 7.5 with 5N NaOH, and water added to a total volume of 5 l. The composition and method of preparation of buffer C were the same as of D, except that Triton was not included.

The radioimmunoassays used antisera supplied by Peninsula Laboratories. The antisera were diluted as suggested by the manufacturer, and used at 100 ul. per assay, the amount of ^{125}I-peptide used was about 15,000 cpm/tube, and the total incubation volume was made up to 500 ul. by the addition of 100 ul. of redissolved extract or peptide standard solution, and 350 ul. of buffer D. After incubating at 4° C for 16 hr., 500 ul. of ice-cold 2% charcoal in buffer C were added. After incubating 10 additional minutes at room temperature, the charcoal mixture was centrifuged at 4000 rpm for 4 min. in an Eppendorf centrifuge. Finally, 800 ul. of the supernatant were taken from each tube, and 10 ml. of scintillation fluid added for counting. Standard curves using solutions of cold peptide in buffer D indicated that the respective assays were sensitive to beta-endorphin, leucine-enkephalin or dynorphin in the range of 10^{-9} to 10^{-11} M.

The initial experiments using frozen rat brain regions for the tissue extracts yielded no measurable peptide levels. The lyophilized extracts were not completely soluble in buffer D, even with much greater dilutions, with homogenization and with sonication.

Considering that the procedure might result in degradation of peptides and consequently in no detectable levels in any brain region, extracts were prepared to which known quantities of ^{125}I-labelled peptides were added. Recovery of radioactivity was less than 20%, indicating a serious flaw in the procedure. In another experiments designed to limit degradation, the lyophilization step was avoided by extracting in ice-cold 0.1 M acetic acid-PMSF-iodoacetamide, centrifuging, then assaying the soluble supernatant fractions immediately. Again, no detectable levels were observed in the brain extracts, though standard curves were appropriate.

According to the published values of rat brain opioid peptide levels, the antibodies which we have been using should beof sufficiently high affinities to conduct these assays, even if 80% loss of activity, as suggested above, occurred. Thus there may be some constitutent of the brain extract which interferes with the radioimmunoassay. We are currently working on this problem.

CONCLUSIONS

Our results in the studies of DFP effects on brain protein synthesis and endogenous opioid content of brain are still preliminary. Our most promising work so far is therefore our studies of a possible endogenous DFPase.In this study, we sought to demonstrate the existence of a brain enzyme capable of inactivating DFP and other OP's. Such enzymes have already been described in other tissues including squid nerve (17), but their existence in brain would be of especially great significance, in that they might make new therapeutic approaches possible.

The binding of DFP to brain tissue components (Figs. 4) makes demonstration of true enzymatic inactivation difficult. This is even more so because we could only make a maximum estimate of binding, by assuming that the aging process -- in which one of the DFP molecule's two methyl groups is lost -- is rapidly completed. This assumption is almost certainly incorrect, however, for the amount of radioactivity bound declines after 30 min (Fig. 4); this suggests that aging, the most likely source of this lost radioactivity, continues after this time. A more accurate estimate of DFP bound might be obtained by doubling the percentage of radioactivity bound at a plateau, which presumably must occur within several hours after incubation. On the other hand, we can't be sure that during this time, some bound DFP does not undergo spontaneous inactivation, though according to the literature, this process is not supposed to occur at a significant rate (16).

In order to avoid the problem of binding, we attempted to saturate DFP binding sites on the membrrane by pre-incubating tissue with unlabelled toxin for one hour before carrying out the regular incubation; subsequently, the membranes were thoroughly washed to remove unbound DFP. Studies with radioactive DFP, however, showed that the pre-incubation had no effect at all on subsequent DFP binding. Apparently, most of this binding is non-specific, probably resulting from dissolution of DFP into the lipid bilayer of the membrane.

Another problem arises from our method of measuring DFP inactivation. Since at present we can do this only by assaying DFP itself, we must demonstrate the existence of an enzyme by measuring the rate of disappearance of substrate, rather than by rate of increase of product. This means that the reaction must proceed well towards completion, preferably at least 30 to 50%, in order to reveal a significant decrease; and even then there is a large error factor, since both starting and ending DFP conentrations must be assayed. Linear kinetics of any enzyme, however, are observed only during the initial reaction, when most of the substrate is still present. In theory, this problem can be avoided by use of a fluoride electrode, which can measure the appearance of fluoride resulting from DFP hydrolysis. This is feasible only if the enzyme does hydrolyze DFP, however; if it acted in some other way, such as by removing an alkyl group, then its activity could not be assayed by fluoride release.

Despite these problems, we feel that our findings are sufficiently encouraging to warrant furtherr work. Our data, using both unfractionated homogenate and S_3, suggest that soluble DFP-inactivating material is present in brain. While further work will be needed to confirm this, as well as to characterize this putative enzyme, this study has defined and overcome the initial problems.

FIGURE LEGENDS

Fig. 1. Effect of pre-incubation on inhibition of AChE. Brain membranes were incubated with various concentrations of DFP at 25°C for various lengths of time in phosphate buffer, then assayed for AChE activity, as described in the Approach section. (a) AChE activity as a function of time of DFP pre-incubation: (1) 0.05 lg DFP/1.25 ml; (2) 1.0 lg; (3) 2.0 lg; (4) 4.0 lg; (b) the same data plotted as a function of DFP concentration: (1) 5 min. pre-incubation; (2) 10 min; (3) 15 min. Control AChE activity was 0.200 - 0.400 A-412/min, depending on the membrane preparation used. Each point is the mean of 3-5 determinations. Standard deviations were 5-7% of the means.

Fig. 2. Inactivation of DFP during incubation with mouse brain fractions at 37°C. The general procedure is described in the Approach section. (a) 1.0 mg DFP incubated with 17 mg homogenate in 1 ml at pH 7.4; (b) same, except that 0.1 mg DFP used; (c) same as (b), but pH varied: o, 7.4; o, 5.0; x, 6.7; +, 8.0; 9.0; o, 7.4 at 0°C. These experiments were done with only one hour incubation with DFP. (d) 0.1 mg DFP incubated with 4 mg brain mitochondrial (-o--o-) or 2.5 mg microsomal (-c--o-) fraction per ml. In Figures 7-9, each point represents the mean of 2-3 determinations. Standard deviations are 10-15% of means.

Fig. 3. Spontaneous inactivation of DFP in solution. (a) 0.1 mg/ml DFP incubated in buffer at 37°C for various lengths of time, then its concentration assayed as described in the Approach section. o, pH 7.4; o, pH 5.0; , pH 9.0; o, pH 7.4 at 0 C. (b) DFP at a concentration of 10 mg/ml in water was stored at 4°C and its activity assayed at several times.

Fig. 4. Binding of ^{3}H-DFP to brain fractions at 37°C and pH 7.4. DFP (0.1 mg/ml) was incubated with mouse brain homogenate (a) or mitochondrial or microsomal fractions (b) for various lengths of time, then bound radioactivity determined as described in Approaches. In (b), o---o, mitochondrial, o---o, microsomal fraction.

Fig. 5. Analysis of DFP interaction with brain soluble (S_3) fraction. ^{3}H-DFP (0.02 1Ci) was incubated with 1 mg S_3 at 37°C and pH 7.4 for 30 min, then the mixture was passed down a Sephadex G-50 column. 1 ml fractions were collected and assayed for radioactivity.

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FIGURE 1

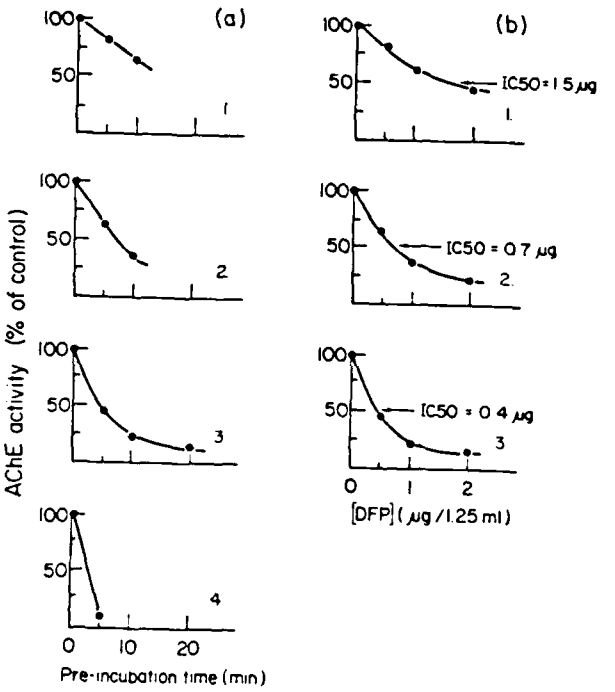

(a)
(b)
100
50
1
2
3
4
IC50=1.5 µg
IC50 = 0.7 µg
IC50 = 0.4 µg
AChE activity (% of control)
0
10
20
Pre-incubation time (min)
0
1
2
[DFP] (µg/1.25 ml)

FIGURE 2

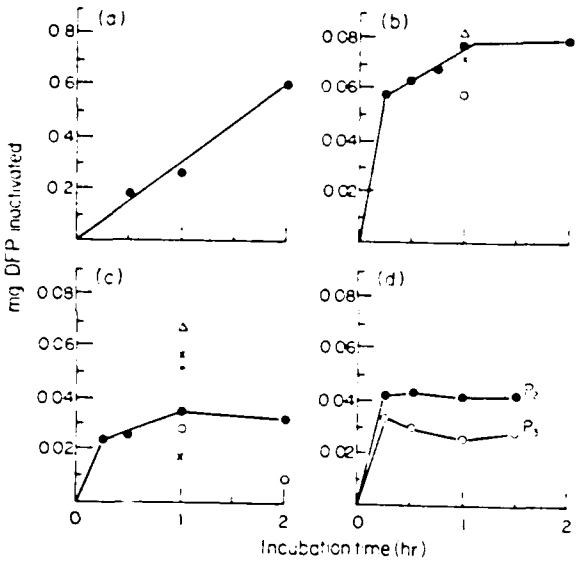

(a)
(b)
(c)
(d)
0.8
0.6
0.4
0.2
0.08
0.06
0.04
0.02
mg DFP inactivated
0
1
2
Incubation time (hr)
P2
P3

FIGURE 3

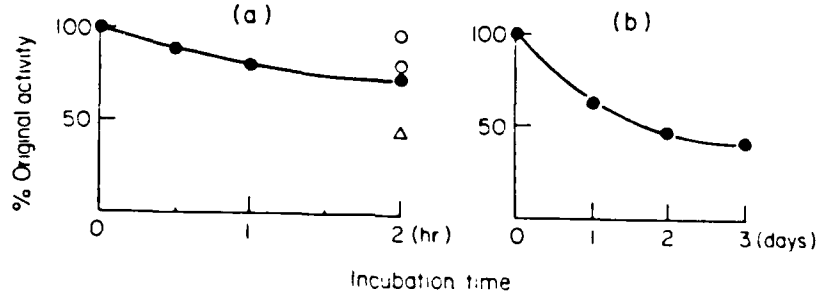
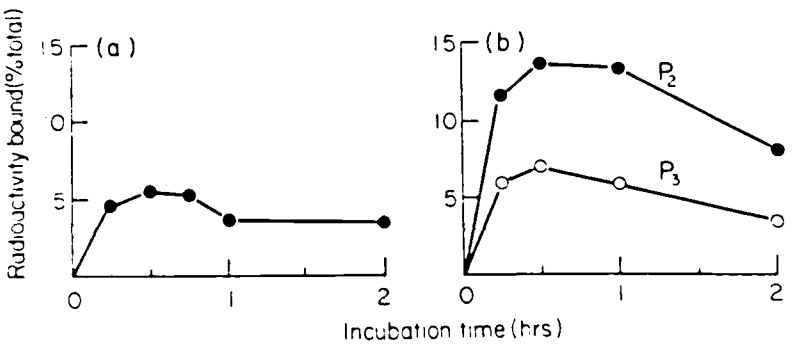


FIGURE 4


Radioactivity bound(% total)
15
(a)
0
5
0
1
2
15
(b)
10
5
P_2
P_3
0
1
2
Incubation time (hrs)

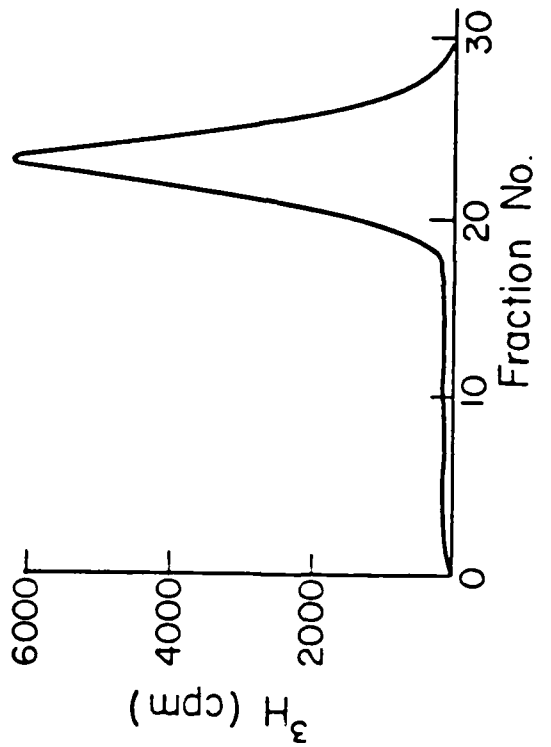


FIGURE 5

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