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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The purpose of this work was to determine whether selected anticholinesterase compounds are likely to have effects on normal astroglial function in the mammalian central nervous system (CNS). To do this, we studied the effect of three organophosphates, diisopropyl-fluorophosphonate (DFP), paraoxon, and parathion, and the carbamate physostigmine on the ion transport, volume control, electrophysiological and monoamine transmitter uptake properties of primary astrocyte cultures. Compounds were studied at 1 μ M-1 mM concentrations and both acute and chronic effects were observed. Physostigmine and parathion inhibited uptake of 3 H-labelled serotonin at 10 μ M, but had no effects at concentrations of 1 μ M or less. The effects of parathion and paraoxon were irreversible within at least 1 hour after removal of the inhibitor. Parathion was an effective inhibitor of K^+ uptake (measured with $^{86}Rb^+$) catalyzed by the (Na+K) pump, but neither physostigmine nor DFP had any effect at concentrations up to 1 mM. Acute treatment with 1 mM, but not 50 μ M, physostigmine stimulated $^{36}Cl^-$ uptake. This appeared to involve stimulation of a furosemide and 4-acetamido-4-isothiocyano-stilbene					
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2,2'-disulfonic acid (SITS)-sensitive co- and exchange anion transport system respectively. Chronic exposure (1-3 weeks) to 50 and 100 μM physostigmine resulted in about 50% inhibition of $^{36}\text{Cl}^-$ uptake after 2 or 3 weeks exposure. After 2 weeks, 50 μM to 1 mM DFP or physostigmine had no effect on K^+ (measured as $^{86}\text{Rb}^+$) uptake. Consistent with their effects on K^+ uptake by astrocytes, parathion caused marked swelling of the cells, DFP caused much less swelling, and physostigmine had no effect on volume. The astrocytes in primary culture seem to possess muscarinic acetylcholine (ACh) receptors which result in inhibition of hormone-stimulated cyclic AMP increases and also cause up to 10 mV depolarization of the resting membrane potential.

In conclusion, it seems that some ~~micrometers~~ acetylcholinesterase (AChE) inhibitors have marked effects on monoamine transmitter uptake, ion transport, and volume regulation by astrocytes independent of their AChE inhibitor effects. However, the effects are only seen at concentrations of 10 μM or greater and thus are unlikely to be related to nonlethal toxic effects of these agents. In addition, increased muscarinic receptor stimulation caused by AChE inhibition-induced increases in ACh levels is also likely to occur on astrocytes, and this would presumably occur at concentrations of AChE inhibitors normally encountered. (HW)
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STUDIES ON THE EFFECTS OF ANTICHOLINESTERASE COMPOUNDS
ON FUNCTIONS OF NEUROGLIA

FINAL REPORT

HAROLD K. KIMELBERG

SEPTEMBER 1, 1987

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SUMMARY

The object of this work was to see whether acetylcholinesterase (AChE) inhibitors have effects on astroglial cells in the central nervous system (CNS) which may explain some of their neurotoxic effects. These effects would be in addition to the known effects of these agents due to AChE inhibition. These effects on a variety of astrocytic properties were studied in primary astrocyte cultures, considered to be good models for astrocytic function in situ.

The results we obtained were as follows.

We have shown that physostigmine, parathion, and paraoxon can inhibit the uptake of the neurotransmitters serotonin and norepinephrine, with IC_{50} values from 10 to 100 μM . However, this effect seems unrelated to inhibition of AChE, since diisopropylfluorophosphonate (DFP) at concentrations up to $10^{-3} M$ had no effects.

We found that parathion inhibited ouabain-sensitive or (Na+K) pump-dependent $^{86}Rb^{+}$ uptake into glial cells. However, physostigmine or DFP at concentrations up to $10^{-3} M$ had no effect on $^{86}Rb^{+}$ uptake.

Parathion at $10^{-3} M$ led to a 4.1-fold increase in cell volume associated with an approximate 2-fold increase in $^{22}Na^{+}$ uptake. This is probably related to the inhibitory effects of parathion on the (Na+K) pump just described. In contrast, physostigmine at $10^{-3} M$ had no effect on cell volume, while DFP at $10^{-3} M$ caused a more modest 2-fold increase in cell volume.

The electrophysiological studies show that $10^{-5} M$ acetylcholine (ACh) depolarized the cells by up to 10 mV. This may be due to activation of the same muscarinic receptor that inhibits the norepinephrine (NE)-stimulated cAMP response due to stimulation of cyclic nucleotide phosphodiesterase.

In terms of effects on ion transport, acute treatment with physostigmine at $10^{-3} M$ but not $10^{-4} M$ stimulates $^{36}Cl^{-}$ uptake, possibly by stimulating co- or exchange transport processes based on the effects of specific transport inhibitors. However, there was no effect of physostigmine on K^{+} (measured as $^{86}Rb^{+}$) transport. In contrast, chronic treatment with physostigmine showed 50% inhibition of $^{36}Cl^{-}$ uptake after 3 weeks exposure to $5 \times 10^{-5} M$ and $10^{-4} M$ physostigmine. Chronic treatment with physostigmine or DFP had no significant effects on $^{86}Rb^{+}$ uptake.

In conclusion, a major effect of the AChE inhibitors studied for this contract appears to be inhibition of monoamine uptake by physostigmine. Effects on ion transport are either absent or not seen except at very high concentrations (about $10^{-3} M$) of the anticholinesterase compounds. Thus, other than possible effects on ACh-mediated functions of astrocytes, the effects of these compounds do not appear likely to occur at toxic but nonlethal concentrations.

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) 86-23, Revised 1985).

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STATEMENT OF AND APPROACH TO THE PROBLEM

The toxic effects of anticholinesterase inhibitors in the central nervous system (CNS) may be due, in part, to direct effects on neuroglial cells. It has long been known that these cells are very numerous and are found at strategic locations in the CNS (1-3). Current research reveals that these cells, and specifically the astroglial subtype, have properties which would allow them to play critical roles in the modulation and perhaps control of CNS functions (3,4). Thus, they are a potential target for neurotoxic agents, including the organophosphate compounds, which have marked CNS effects. One cannot, however, exclude effects on the other major macroglial cell type in the brain, the oligodendroglia, or even on the more rarely encountered microglia. However, it was reasoned for the purposes of this contract that if the present studies showed marked effects of acetylcholinesterase (AChE) inhibitors on astroglia at the concentrations likely to occur in the body under nonlethal toxic conditions, the concept that these cells would be a potential site of action for such effects would be supported. If no effects were seen or effects occurred at inappropriately high concentrations, then it would enable us to draw the useful conclusion that these cells are unlikely to be a site of action. Thus, we studied a range of astroglial properties in which our laboratory has an acknowledged expertise. The effects of these agents could be unrelated to the known anti-acetylcholinesterase (anti-AChE) properties of these compounds, or since some astroglial cells also appear to have AChE and butyrylcholinesterase activities as well as acetylcholine (ACh) receptors (3,4), these agents may also affect such cells through their actions on such esterases or ACh receptors.

Knowledge of the effects of neurotoxins, such as the organophosphates, on glial cells is important for globally understanding the mechanisms of CNS toxicity. If clear effects in primary astroglial cultures are obtained, such cultures can provide a convenient means of screening such agents and of testing possible protective or therapeutic regimens. Apart from the data presented in this report, no such information has, so far, been available.

BACKGROUND

Up to about 15 years ago, concepts of glial function in the CNS were limited to the properties revealed by the pioneering studies of Kuffler and his colleagues on glial cells in leech ganglion and amphibian optic nerve (5-7), in which these cells were found to be always electrically silent and showed operationally a selective permeability to K^+ , leading to the idea that glial cells were responsible for selective uptake of K^+ released during neuronal activity. The myelinating role of oligodendrocytes had also been established prior to these studies. Views of the functions of astroglial cells or astrocytes were purely speculative.

A change has occurred in this limited view of astroglial function, although regulation of $[K^+]_o$ is still feasible and the subject of current investigation (8). This broadening of the view of astrocyte functions coincided in large part with studies on astrocyte cultures, especially primary cultures prepared from neonatal rodent brain which could be prepared to consist predominantly of astrocytes or oligodendrocytes, depending on conditions. These cultures can provide an important means whereby properties that may be exhibited by normal astrocytes in situ can be initially identified (3). Work with these cultures has already indicated that cultured astrocytes from the mammalian CNS, in

addition to showing a predominant permeability to K^+ and forming a syncytium by means of gap junctions, can take up and metabolize neurotransmitters and have receptors for transmitters, many but by no means all of which are linked to adenylate cyclase (3,4,9). Astrocytes in primary culture also have a number of electrically silent ion exchange and co-transport processes (3,10). They can also function for neuronal guidance during growth in vitro and these studies in vitro (11,12) further support prior work in situ (13). These cultures thus currently provide the best models for studying astroglial function, since the cultures show the same limited number of properties established so far for astrocytes in situ. These properties include the presence of the astrocyte-specific intermediate filament protein, glial fibrillary acidic protein (GFAP), glutamine synthetase (GS), uptake systems for glutamate and other transmitters, rectangular intramembrane particle assemblies seen by freeze-fracture studies, large negative membrane potentials determined by the K^+ diffusion gradient due to a high selective K^+ permeability, a large density of beta receptors, and the ability to guide and modulate neuronal migration.

The existence of Cl^-/HCO_3^- anion exchange and Na^+/H^+ exchange transport processes suggests that astrocytes may have an important role in controlling acid-base balance in the CNS (3,10). The CNS is protected from large changes in systemic pH except for respiratory acidosis when CO_2 increases, because CO_2 can rapidly penetrate the blood-brain barrier (14). The astrocyte may have a role in controlling the pH of the CNS since recent work on the changes in extracellular pH (pH_e) during ischemia (15), the effects of an anion exchange inhibitor on the levels of CNS HCO_3^- in chronic metabolic alkalosis (16), and the effect of lactic acid-induced acidosis on pH_e in ischemia (17) suggests that a glial response is involved. The uptake of Na^+ and Cl^- by coupled Na^+ in/ H^+ out and Cl^- in/ HCO_3^- out co-transport (10,18) is a possible mechanism for the swelling of astrocytes in response to a number of pathological conditions. This swelling occurs in a number of pathological states, such as ischemia, where it seems to be the basis of the observed cytotoxic edema. This edema involves mainly a shift of fluid from an extra- to an intracellular compartment, with a consequent decrease in the extracellular space (19).

An equally important area where astroglia may specifically affect CNS function by controlling seizure activity is in the uptake of excitatory and inhibitory transmitters. Control of the extracellular levels of such transmitters can have a vital role in controlling excitability. Astroglia take up glutamate and gamma-aminobutyric acid (GABA) by high affinity transport systems, both in situ and in primary cultures (3,9,20). Uptake of choline has also been shown in astrocyte cultures (9). We have recently found that primary cultures take up catecholamines by a Na^+ -dependent system that is inhibited by tricyclic antidepressants (21), and catecholamines are also taken up by a low affinity system (22). We have recently confirmed this uptake in astrocytes by autoradiography (23). We have also found high affinity uptake of serotonin (5HT) (24,25). The kinetics, Na^+ sensitivity, and inhibitor sensitivity of this 5HT uptake system are identical to those described for 5HT uptake in brain slices and subcellular nerve terminals. We have confirmed astroglial localization by a combination of autoradiography and staining of the same cells for GFAP (25). In relation to effects of GABA and diazepam on astrocytes, benzodiazepine binding sites of either high (26) or low affinity (27) type have been observed.

In addition, based on work in primary astrocyte cultures, it seems no longer possible to view mammalian astroglia as having a membrane potential that

is affected only by changes in $[K^+]_o$, since major conductance pathways other than for K^+ can be observed under certain conditions. We have found that norepinephrine (NE) depolarizes astrocytes in primary culture by an α -receptor-mediated process (28), and glutamate depolarizes these cells by a Na^+ -dependent mechanism (29). This effect of glutamate may represent either electrogenic uptake or a receptor-mediated event. Such effects of neurotransmitters on astrocytes are likely to be important in relation to seizure activity.

Methods and Materials

Cell culture

Cultures were prepared from the cerebral cortices of 1 to 2-day-old Sprague-Dawley rats after careful removal of the meninges. The cell suspension prior to plating was prepared by sequential dissociation of the tissue by means of the bacterial protease Dispase (30). The growth medium used was Eagles Basal Medium (BME) + 10% fetal calf serum with added supplements (4 ml of 100 x concentrated BME vitamin solution and 2 ml of 100 x concentrated BME amino acid solution per 100 ml growth medium), and antibiotics (1 ml penicillin at 10,000 units/ml plus streptomycin at 10,000 units/ml per 100 ml growth medium). The cells were grown in 12 well trays (Costar) or 60 mm diameter dishes. All tissue culture materials were from Gibco, Grand Island, N.Y. The cells were used when 3-5 weeks old since a stable, maximal level of expression of the activities studied in this project was found after this period of time in culture (refs. 3, 10, 21, 22 and unpublished observations). We did not observe any significant effects of different times in culture within this range of 3 to 5 weeks and did not, because of time limitations, study younger or older cultures. The cultures were at least 95% astrocytic as judged by staining for GFAP using a 1:200 dilution of a monoclonal antibody (Amersham) to human GFAP as the primary antibody and rhodamine conjugated rabbit immunoglobulin G (IgG) against mouse immunoglobulin (Accurate Chemicals) as the second antibody.

Iontransport and cell volume

These studies were performed under steady state conditions. The growth media was removed and the cells washed 3 times with HCO_3^- or in some cases HEPES-buffered balanced salt solution (Na^+ , 145 mM; K^+ , 4.5 mM; Mg^{2+} , 0.4 mM; Ca^{2+} , 1.3 mM; Cl^- , 123 mM; glucose, 10 mM; SO_4^{2-} , 1.2 mM). The solutions were buffered with either HCO_3^- 25 mM bubbled with 5% CO_2 /95% air to pH 7.4, or HEPES 25 mM adjusted to pH 7.4 with NaOH. The cells were then incubated in this solution at 37°C for 30 or 60 minutes and, for the HCO_3^- -buffered solution, in a 5% CO_2 /95% air atmosphere. Inhibitors were added during this period when preincubation was desired. This solution was then removed and 0.5-1.0 ml of the same medium, warmed to 37°C and containing appropriate radiotracers with or without inhibitors, was added to each well. After varying times, the medium was rapidly aspirated off and each well washed six to seven times with 1 ml ice-cold 0.29 M sucrose containing 10 mM Tris-Cl (pH 7.4) and 0.4 mM $Ca(NO_3)_2$, by rapid addition with an automatic pipette attached to a reservoir, followed by immediate aspiration with a tube attached to a vacuum pump (total washing time was approximately 10-15 seconds). Cell volume was measured by the uptake of the non-metabolized [^{14}C] labelled analogue of glucose, 3-O-methyl-D-glucose, using the same methodology as originally described by Kletzien et al. (31) and applied by us to primary astrocyte cultures (18). The cell monolayer from each well was then solubilized in 0.5 ml 1 N NaOH at room temperature for about 15-30 minutes.

This solution was then removed and the wells washed with 0.5ml distilled water which was added to the NaOH digest. Aliquots were taken for scintillation counting and protein determination by the Lowry- Folin method (32). $^{42}\text{K}^+$ was determined by Cerenkov radiation with 10 ml water using a Packard 3330 liquid scintillation counter. $^{36}\text{Cl}^-$ and ^{14}C were determined by beta emission using Liquiscint (National Diagnostics, Somerville, NJ).

Catecholamine and serotonin uptake

These studies were performed in an identical manner to the methods used for measuring uptake of ions (radionuclides) as described above. ^3H -labelled serotonin and norepinephrine were added to the final concentrations desired and uptake was determined after the appropriate times by rapid washing as described above.

Cyclic AMP (cAMP) determination

cAMP was determined using a commercially available ^{125}I radioimmunoassay kit (Schwarz/Mann, Orangeburg, NY) as previously described (33). Growth medium was poured off, and the cells were washed twice with Hank's balanced salt solution (Hanks BSS; Gibco, Grand Island, NY). 5% trichloroacetic acid (TCA) at 70°C was then added to the cells, the cells were removed by scraping, sonicated for 1.5 min and then centrifuged at 3000 rev./min for 10 min.. The supernatant from this centrifugation was extracted with water-saturated ether three times, and aliquots were measured for cAMP content by the radioimmunoassay. The precipitates were dissolved in 1 ml 1 N NaOH and assayed for TCA insoluble protein (33).

Materials

SITS (4-acetamido-4-isothiocyano-stilbene 2,2'-disulfonic acid) was from Pierce Chemical Company, Rockford, IL. HEPES was from United States Biochemical Corporation, Cleveland, OH. $^{42}\text{K}^+$ Cl was from New England Nuclear, Rockford, IL. Na^{36}Cl , methyl ^{14}C 3-O-methyl-D-glucose (specific activity, 40 mCi/mol), and ^{14}C L-glucose (specific activity, 210 mCi/mol) were from ICN. ^3H Sucrose (2-10 Ci/mmol) was from New England Nuclear. 5-Hydroxy $^{3\text{H}}$ tryptamine creatinine sulfate was from Amersham (specific activity, 9-23 Ci/mmol). L-[7- ^3H (N)]NE was obtained from New England Nuclear and had a specific activity of 2.7 Ci/mmol. Furosemide was a generous gift of Hoechst-Roussel Pharmaceuticals, Somerville, NJ, and bumetanide was from Hoffmann-La Roche Inc., Nutley, NJ. Pargyline hydrochloride was from Sigma Chemical Company, St. Louis, MO. All other reagents were of at least reagent quality and were obtained principally from Sigma.

RESULTS

Effects of AChE inhibitors on ^3H serotonin uptake

In our studies to date, we have found that physostigmine, parathion, and paraoxon, but not diisopropylfluorophosphonate (DFP), are inhibitors of ^3H 5HT uptake in primary astrocyte cultures. These data are shown in Figures 1 through 5. Figure 1 shows a dose-response curve for inhibition of ^3H 5HT uptake by physostigmine. There is a suggestion of inhibition at 10^{-6}M physostigmine, but only for the case of no pretreatment. For both pretreatment and no pretreatment with physostigmine inhibition is essentially complete at 10^{-3}M . As we have

recently described (24), primary astrocyte cultures show high affinity uptake of 5HT which is markedly dependent on external Na^+ and is inhibited by a variety of antidepressants. This system so far is identical to the high affinity uptake described in brain preparations and attributed to re-uptake into nerve endings (20,34,35). The low affinity uptake seen in the absence of Na^+ in the medium, which is not via the high affinity system, is essentially unaffected by physostigmine (Figure 1). DFP, even at concentrations as high as 10^{-3}M , had no effect on $[^3\text{H}]5\text{HT}$ uptake (Figure 2). However, other organophosphates with nitrophenyl substituents, namely, parathion and paraoxon, inhibit Na^+ -dependent $[^3\text{H}]5\text{HT}$ uptake in the 10^{-5} to 10^{-4}M range, with a small stimulation at lower concentrations (see Figures 3 and 4). This inhibition was not reversed when uptake was measured after 60 minutes in inhibitor-free medium (Figure 5). In figures 3 and 4 a slight stimulation of $[^3\text{H}]5\text{HT}$ uptake in the absence and presence of Na^+ was seen at 1 mM parathion or paraoxon. This may be some non-specific effect involving a general increase in permeability to $[^3\text{H}]5\text{HT}$, but it is clear that the Na^+ -dependent component of uptake is markedly inhibited at these concentrations of parathion or paraoxon.

Effect of acute exposure to physostigmine or DFP on $[^3\text{H}]$ norepinephrine uptake

No effect of physostigmine from 10^{-6} to 10^{-4}M was found on the Na^+ -dependent uptake (for 20 minutes) of $[^3\text{H}]$ NE (Figure 6). Inhibition of Na^+ -dependent uptake was observed at 10^{-3}M physostigmine, but there was no effect on Na^+ -independent uptake (Figure 6). This is similar to the effects seen for uptake of $[^3\text{H}]5\text{HT}$ (Figure 1), but in the case of $[^3\text{H}]5\text{HT}$, significant inhibition occurred at $5 \times 10^{-5}\text{M}$. For DFP, no effect at concentrations of 10^{-6}M to $5 \times 10^{-5}\text{M}$ was found (Figure 7). From 10^{-4}M to 10^{-3}M DFP, a gradual stimulation of both Na^+ -independent and Na^+ -dependent $[^3\text{H}]$ NE uptake was seen, suggesting that a nonspecific increase in permeability was occurring. As will be described later, we have found an increase in the $[^{14}\text{C}]3\text{-O-methyl glucose space}$ (measuring cell volume) after 10^{-3}M DFP. However, there was no effect of 10^{-3}M DFP on $[^3\text{H}]5\text{HT}$ uptake (Figure 2). Presumably the small increase in volume was insufficient to increase $[^3\text{H}]5\text{HT}$ uptake, and the stimulation of $[^3\text{H}]$ NE uptake must therefore be attributable to another cause.

Acetylcholine-induced effects

We have observed that 10^{-5}M ACh inhibits the large beta receptor-stimulated rise in steady-state cAMP levels seen in primary astrocyte cultures. This inhibitory effect of ACh appears to be mediated via a muscarinic receptor, since 10^{-6} to 10^{-5}M atropine, but not d-tubocurarine, blocks the effect (Table 1). This inhibition by ACh seems to be principally due to an increase in phosphodiesterase activity, which is responsible for hydrolysis of cAMP intracellularly, since an inhibitory effect of ACh was barely seen (bottom two rows in Table 1) in the presence of a phosphodiesterase inhibitor, 0.5 mM 3-isobutyl-1-methyl xanthine (IBMX).

Addition of 10^{-5}M ACh also resulted in depolarization (up to 10 mV) of the cells, and desensitization to this effect was seen upon a second addition of ACh (Figure 8). So far we have seen no effect of 10^{-5}M parathion on the resting membrane potential of these cells, and parathion did not potentiate the effect of ACh (Figure 8). Indeed, there may have been a slight inhibition. The lack of an effect of parathion on the ACh-induced depolarization could be due to several reasons: 1) there is no AChE activity associated with the muscarinic

receptor in these cells; 2) because of the large extracellular-intracellular volume ratio (several thousand to one), the hydrolysis of ACh never becomes rate-limiting; 3) 10^{-5} M parathion does not affect astroglial AChE (this seems unlikely); and 4) like other ACh inhibitors (36), parathion inhibits the ACh receptor channel which is open only in the presence of ACh.

Effects on ion transport processes

We have found that parathion, but not DFP or physostigmine, inhibits uptake of $^{86}\text{Rb}^+$ (an analogue for K^+); this effect seems to be due to inhibition of the ouabain-sensitive (Na+K) pump component. A dose-response curve is shown in Figure 9 for the effects of varying concentrations of parathion on 5-minute uptake of $^{86}\text{Rb}^+$, in the absence and presence of 1 mM ouabain to inhibit the (Na+K) pump. Both parathion and ouabain were added at $t = 0$. This dramatic effect does not seem to have been reported before, although there have been a number of studies on the effects of insecticides on ion transport in different systems (35). In contrast, physostigmine and DFP, even at 10^{-3} M, had no effect on $^{86}\text{Rb}^+$ uptake. Figure 10 shows a time course for up to 120 minutes for the uptake of $^{86}\text{Rb}^+$ in the presence of 5×10^{-5} and 10^{-3} M physostigmine. There is no consistent effect. Figure 11 shows the same type of experiment but in the presence of 5×10^{-5} and 10^{-3} M DFP. Again, there is no inhibition. The anticholinesterase agents were added at the same time as $^{86}\text{Rb}^+$ at $t = 0$.

Effects of acute and chronic exposure to physostigmine on $^{36}\text{Cl}^-$ uptake

The effects of acute exposure (physostigmine added at zero time) to physostigmine on the time course of $^{36}\text{Cl}^-$ uptake are shown in Figure 12. It can be seen that at 5×10^{-5} M physostigmine, there was no effect, while at 10^{-3} M physostigmine, there appears to have been some stimulation of $^{36}\text{Cl}^-$ uptake.

In Figure 13 we show again that 10^{-3} M physostigmine stimulated $^{36}\text{Cl}^-$ uptake measured over 20 minutes. The same effect was seen after 1-hour preincubation with physostigmine. However, no effect was seen when 10^{-3} M furosemide (a Na + K + 2Cl co-transport inhibitor and $\text{Cl}^-/\text{HCO}_3^-$ anion exchange inhibitor) or 10^{-3} M SITS (a $\text{Cl}^-/\text{HCO}_3^-$ anion exchange inhibitor) was also present. This suggests that physostigmine was stimulating the $\text{Cl}^-/\text{HCO}_3^-$ exchange transport system.

In contrast, chronic exposure to physostigmine showed inhibition of $^{36}\text{Cl}^-$ uptake (10 minutes) after 2 and 3 weeks but not after 1-week exposure to 10^{-6} to 10^{-4} M physostigmine (table 2).

Effects of 1 and 2 weeks chronic exposure to physostigmine and DFP on $^{86}\text{Rb}^+$ uptake

Time courses of $^{86}\text{Rb}^+$ uptake in cells exposed for 1 and 2 weeks to 10^{-6} M or 5×10^{-5} M physostigmine are shown in Figure 14. The data in Figure 14A indicate some slight inhibition of $^{86}\text{Rb}^+$ uptake, although the steady-state levels at 90 minutes were not affected. This effect did not persist, as it was not seen after 2 weeks exposure to physostigmine (see Figure 14B), when treated and control curves are essentially superimposable.

Exposure to DFP at 10^{-6} M, 5×10^{-5} M, and 10^{-3} M for 1 or 2 weeks had no effect on $^{86}\text{Rb}^+$ uptake (see Figure 15).

Effects on cell volume

We found marked swelling of the cells exposed to 10^{-3} M parathion, as measured by [^{14}C]3-O-methyl-D-glucose uptake (36), shown in Figure 16. There was also marked stimulation of $^{22}\text{Na}^+$ uptake at 10^{-3} M but not at 10^{-4} M parathion (Figure 17). This is consistent with increased uptake of Na^+ , probably with Cl^- . Since ouabain was present from zero time to block the (Na+K) pump, it indicates an increase in the permeability to Na^+ (and possibly Cl^-). This effect, coupled with inhibition of the (Na+K) pump (see Figure 9), explains the marked swelling seen with 10^{-3} M parathion present (Figure 16), since there is an increased influx of Na^+ (with Cl^-) which cannot be pumped out in the presence of ouabain. Physostigmine at 10^{-3} M had no effect on cell volume (Figure 18). However, as also shown in Figure 18, 10^{-3} M DFP produced a small, approximately 2-fold increase, but this is considerably smaller than the 4.1-fold increase shown in 10^{-3} M parathion. These effects at 1 mM concentrations are clearly toxic; it is likely that such concentrations will not be reached in the CNS before other fatal CNS effects are seen. However, the effects at 10^{-5} to 10^{-4} M may well be part of the toxic syndrome seen at lower concentrations.

CONCLUSIONS AND RECOMMENDATIONS

Although studies of the contribution of glia to CNS function are still in their infancy, it seems likely that glial cells have major roles in controlling excitability and seizure activity in the CNS by controlling the ionic and neurotransmitter environment of the neuron. There is also the possibility of direct effects of transmitters on glial receptors themselves such as those seen for NE, glutamate, and ACh, leading to chemical and electrophysiological changes. Thus, the effects of organophosphates and other AChE inhibitors on ion transport and transmitter uptake into glia, as well as possible alteration of transmitter receptor action in astroglia, become significant in terms of the effects of such compounds on the CNS.

In relation to the above questions, the conclusions we have come to, based on the foregoing work, are as follows:

1. We have shown that physostigmine, parathion, and paraoxon can inhibit the uptake of the neurotransmitter serotonin, and physostigmine inhibited the uptake of NE. Half-maximal inhibition values ranged from 10 to 100 μM . However, these effects seem unrelated to inhibition of AChE, since DFP at concentrations up to 10^{-3} M had no effects on the uptake of either of these two transmitters.
2. We found that parathion inhibited ouabain-sensitive or (Na+K) pump-dependent $^{86}\text{Rb}^+$ uptake into glial cells. However, physostigmine or DFP at concentrations up to 10^{-3} M had no effect on $^{86}\text{Rb}^+$ uptake.
3. Parathion at 10^{-3} M led to a 4.1-fold increase in cell volume associated with an approximate 2-fold increase in $^{22}\text{Na}^+$ uptake. This is probably related to the inhibitory effects of parathion on the (Na+K) pump just described. In contrast, physostigmine at 10^{-3} M had no effect on cell volume, while DFP at 10^{-3} M caused a more modest 2-fold increase in cell volume.
4. The electrophysiological studies show that 10^{-5} M ACh depolarized the cells by up to 10 mV (Figure 8). This may be due to activation of the same muscarinic receptor that inhibits the NE-stimulated cAMP response (Table 1) due to stimulation of cyclic nucleotide phosphodiesterase.

5. In terms of effects on ion transport, acute treatment with physostigmine at 10^{-3}M but not 10^{-4}M stimulates $^{36}\text{Cl}^-$ uptake, possibly by stimulating anion exchange transport processes based on the effects of specific transport inhibitors. However, there was no effect of physostigmine on K^+ (measured as $^{86}\text{Rb}^+$) transport. In contrast, chronic treatment with physostigmine showed 50% inhibition of $^{36}\text{Cl}^-$ uptake after 3 weeks exposure to $5 \times 10^{-5}\text{M}$ and 10^{-4}M physostigmine. Chronic treatment with physostigmine or DFP had no significant effects on $^{86}\text{Rb}^+$ uptake.

In conclusion, a major effect of the AChE inhibitors studied for this contract appears to be inhibition of monoamine uptake by physostigmine. Effects on ion transport are either absent or not seen except at very high concentrations (about 10^{-3}M) of the anticholinesterase compounds. However, these do not appear to be simply reflections of a toxic effect on cell membranes leading to a gain of Na^+ and Cl^- with resultant cell swelling, since 1) The acute effects of physostigmine on $^{36}\text{Cl}^-$ transport are inhibited by specific transport inhibitors; and 2) the effects of chronic exposure to physostigmine include inhibition of $^{36}\text{Cl}^-$ transport, but chronic exposure to DFP or physostigmine had no effect on K^+ (measured with $^{86}\text{Rb}^+$) uptake or steady-state content. Effects on $^{86}\text{Rb}^+$ transport should be a very sensitive measure of toxicity, since uptake of K^+ partly depends on the integrity of the membrane-bound ($\text{Na}+\text{K}$) ATPase, which depends on membrane integrity and which also requires ATP to function, and the steady-state content of K^+ depends on maintaining a balance between the pump-mediated K^+ influx and passive efflux and influx.

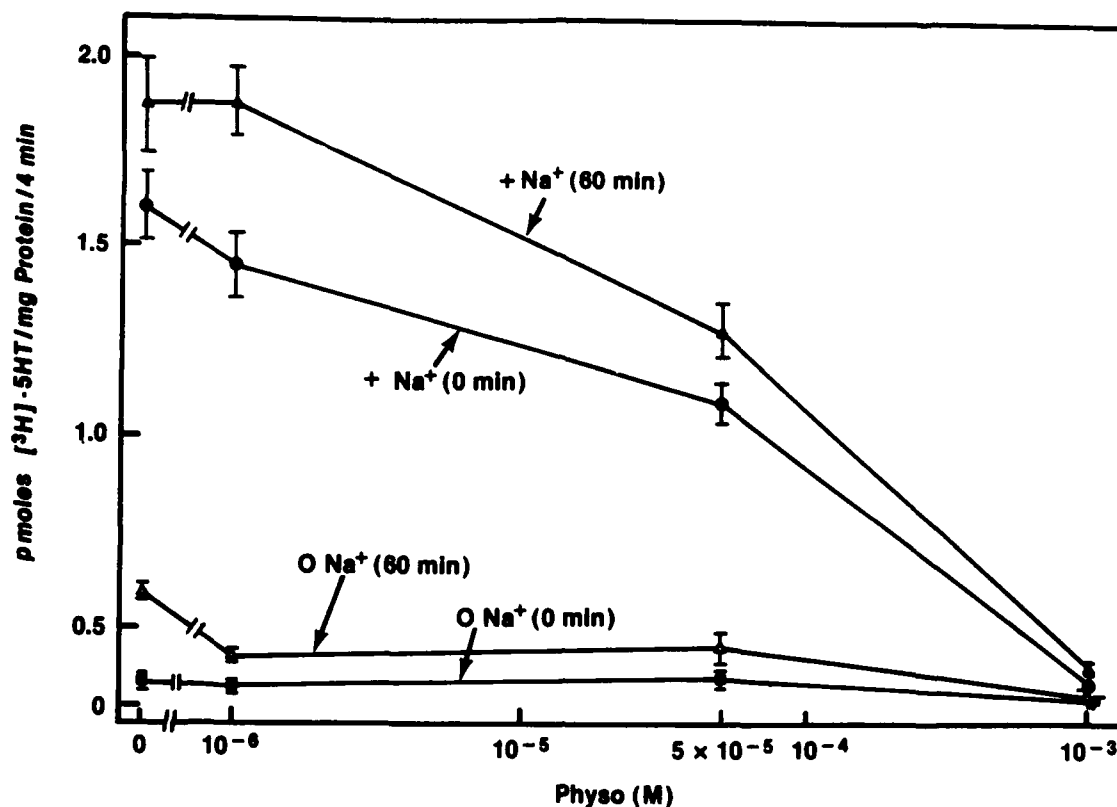


Figure 1. Dose-response curve for physostigmine inhibition of [³H]5HT uptake. Cells were grown for 25 days in 12 well trays (Costar). Growth medium was removed and the cells were pretreated for 60 minutes with the different concentrations of physostigmine indicated or physostigmine was added at zero time with [³H]5HT as indicated. Final concentration of [³H]5HT was 10⁻⁷ M (1 uCi in 1 ml total reaction volume). The reaction medium also contained 10⁻⁴ M pargyline to prevent oxidative deamination of 5HT by the cells due to monoamine oxidase (MAO). Uptake was measured as described in the Methods and Materials section. In Na⁺-free medium, Na⁺ was replaced by choline and NaHCO₃ by triethylammonium HCO₃⁻. Four-minute uptake of [³H]5HT was measured in the presence and absence of Na⁺ in the medium, as indicated on the graph. A zero time uptake (when [³H]5HT was added and immediately removed) was always subtracted. N = 3 wells ± S.D.

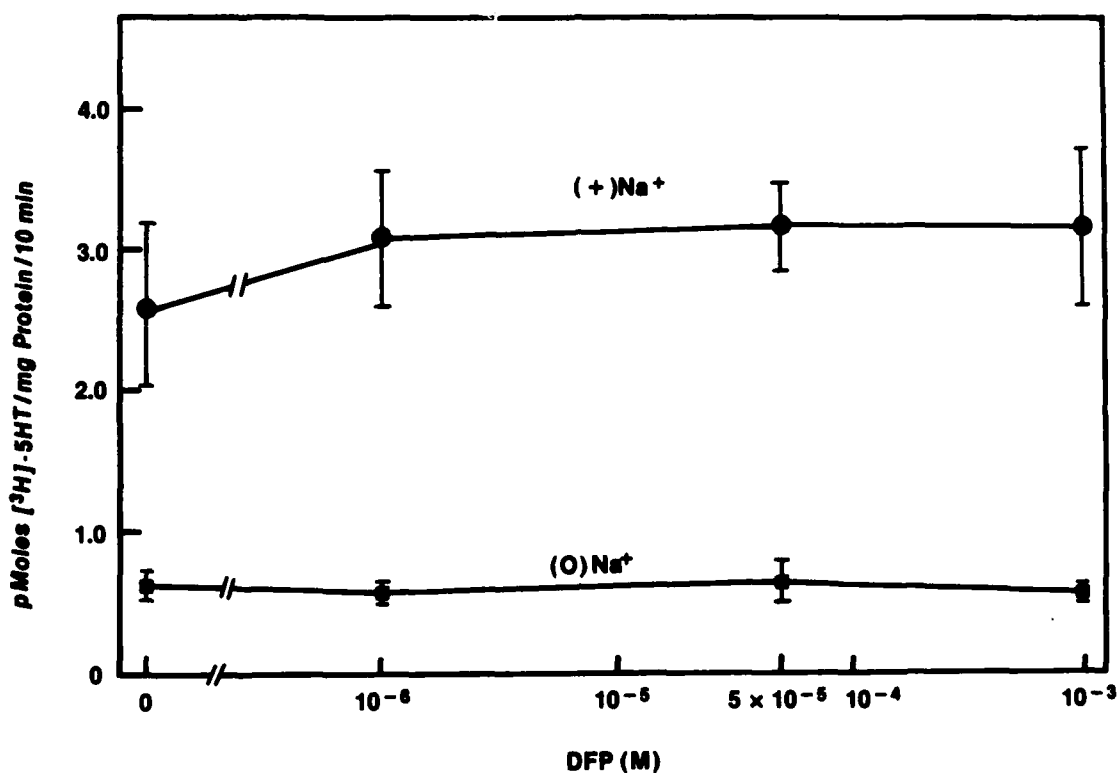


Figure 2. Dose-response curve for inhibition of [³H]5HT uptake by DFP. Ten-minute uptake of [³H]5HT in the presence and absence of varying concentrations of DFP and also in the presence and absence of Na⁺ in the medium was measured as described in Figure 1. [³H]5HT and DFP were added at zero time; cells (29 days old) were preincubated in +Na⁺-containing HCO₃⁻-buffered medium for 20 minutes prior to this. N = 3 wells ± S.D.

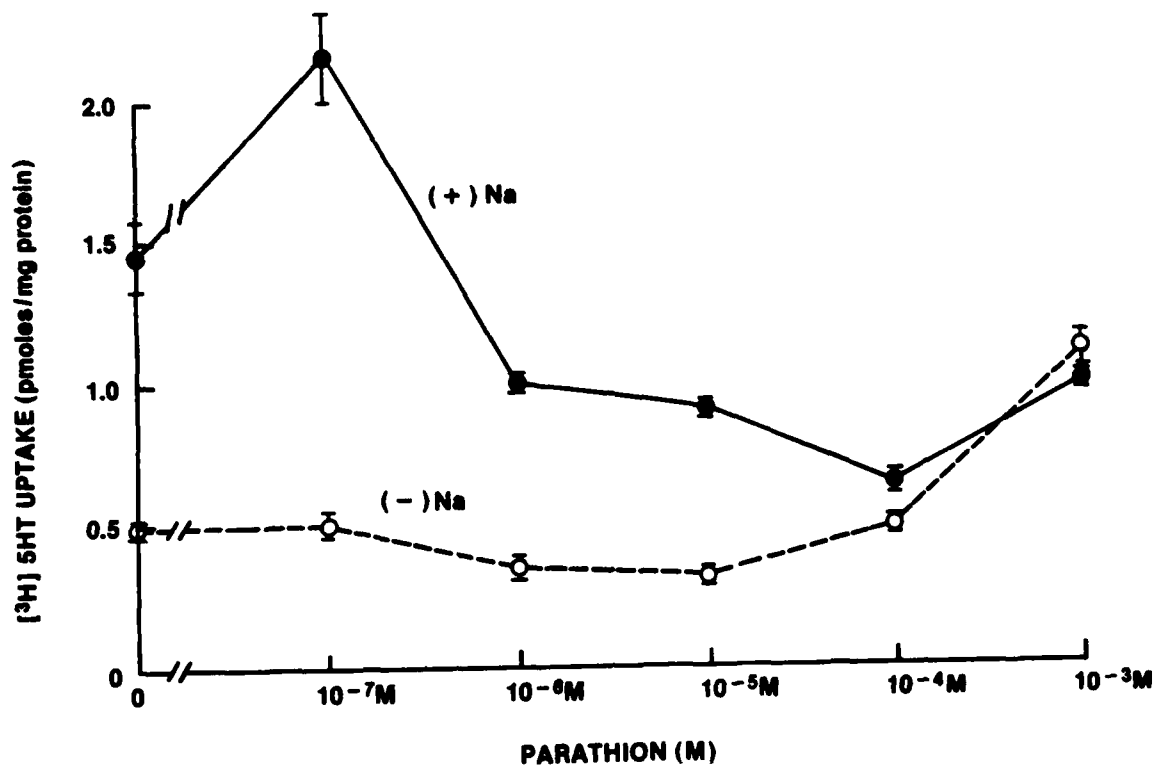


Figure 3. Dose-response curve for inhibition of [³H]5HT uptake by parathion. Uptake of 10⁻⁷ M [³H]5HT was measured over 4 minutes in HCO₃⁻-buffered medium with or without Na⁺, as described for Figure 1. Concentrations of parathion shown were present in a 20-minute preincubation period. Parathion was dissolved in ethanol (final concentration, 1%), which by itself had no effect on [³H]5HT uptake. N = 4 wells ± S.D.

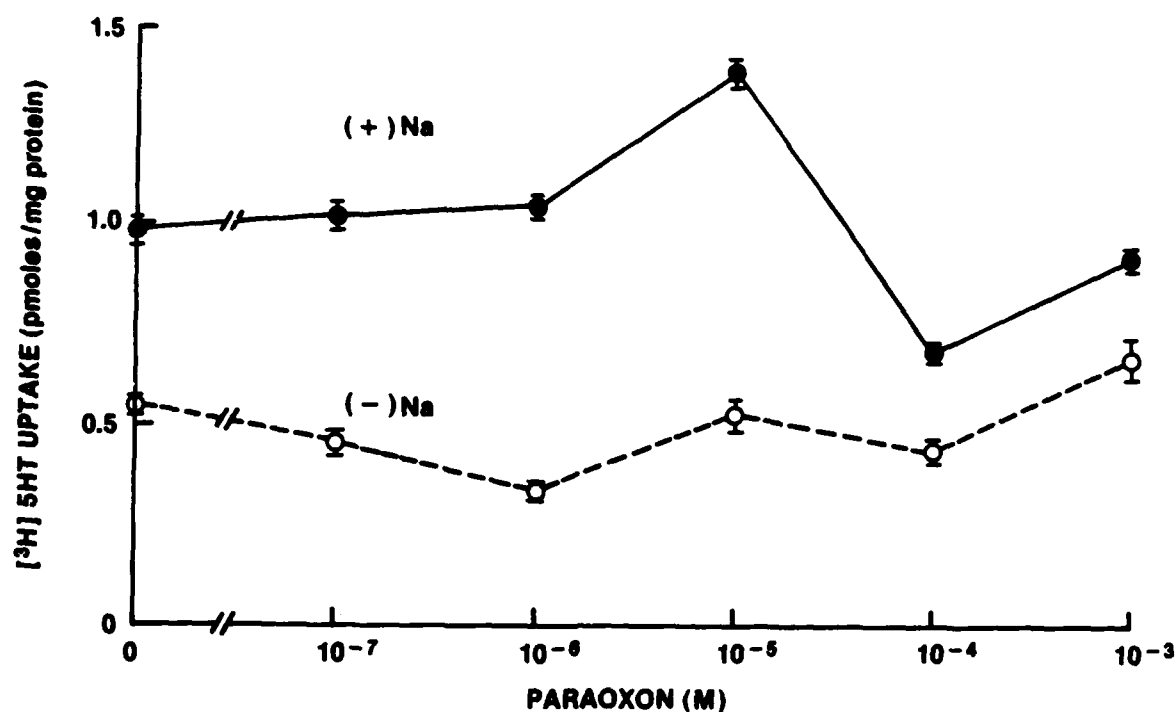


Figure 4. Dose-response curve for inhibition of [³H]5HT uptake by paraoxon. Uptake of 10⁻⁷ M [³H]5HT was measured over 4 minutes in HCO₃⁻-buffered medium with or without Na⁺, as described for Figure 1. Concentrations of paraoxon shown were present in a 20-minute preincubation period. Paraoxon was dissolved in ethanol (final concentration, 1%), which by itself had no effect on [³H]5HT uptake. N = 4 wells ± S.D.

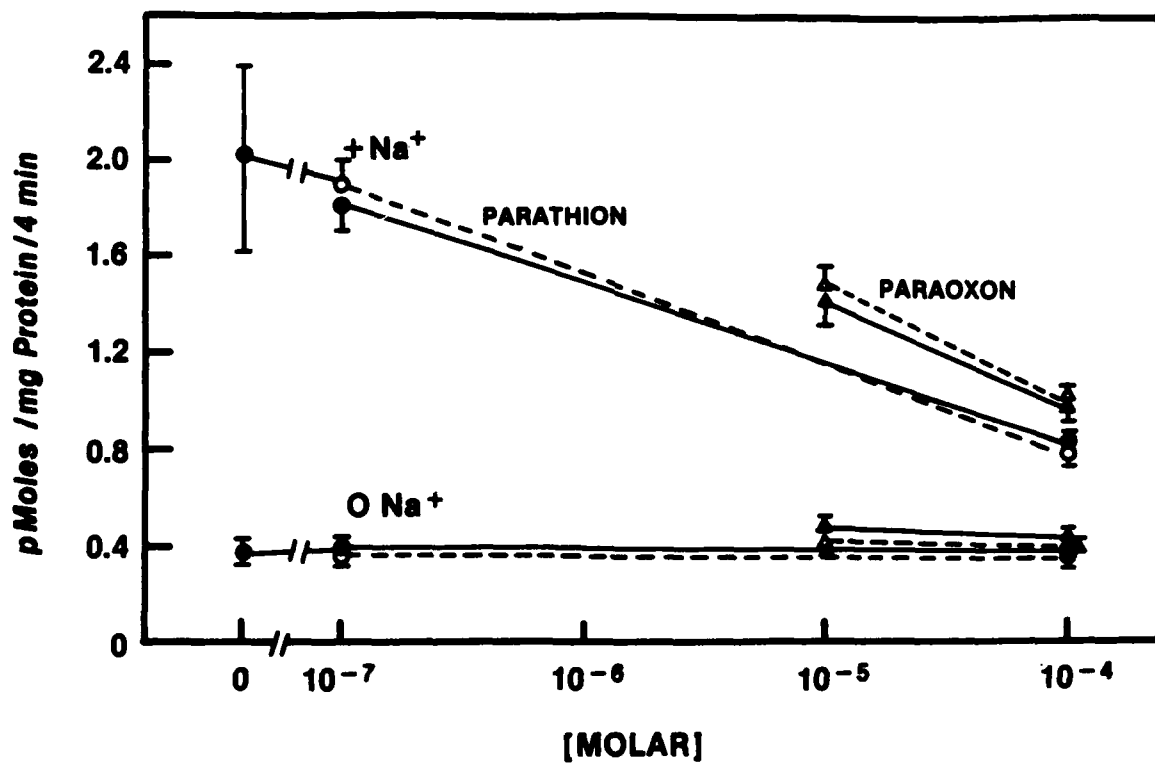


Figure 5. Dose-response curve for parathion and paraoxon inhibition of [³H]5HT uptake. [³H]5HT was added immediately after a 60-minute preincubation with inhibitors (●—●, parathion; ▲—▲, paraoxon) or after a further 60-minute incubation in inhibitor-free medium (○---○, parathion; △---△, paraoxon). Lower curves are for uptake measured in Na⁺-free medium. Other conditions were as in Figure 1. $n = 6 \pm$ S.D.

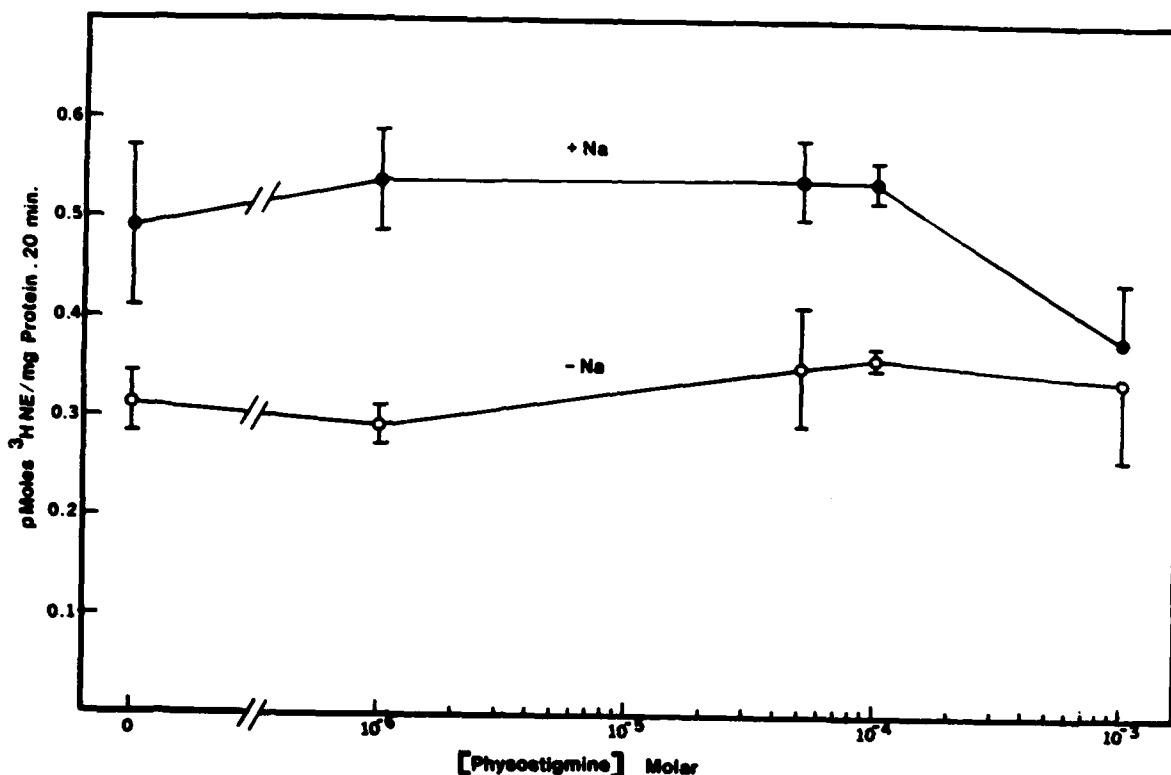


Figure 6. Dose-response curve for inhibition by physostigmine of [³H] norepinephrine uptake by primary astrocyte cultures. Uptake of [³H]NE was measured in the presence and absence of Na⁺ (Na⁺ replaced by choline) for 20 minutes as described for [³H]5HT in Figure 1. Final concentration of [³H]NE was 10⁻⁷M (1 uCi in 1 ml total reaction volume). The reaction medium also contained 10⁻⁴M pargyline plus 10⁻⁴M tropolone to prevent oxidative deamination of NE due to MAO and O-methylation by COMT respectively. In Na⁺-free medium, Na⁺ was replaced by choline and triethylammonium HCO₃. A zero time was always subtracted when [³H]NE was added and immediately removed. n = 3 wells + S.D.

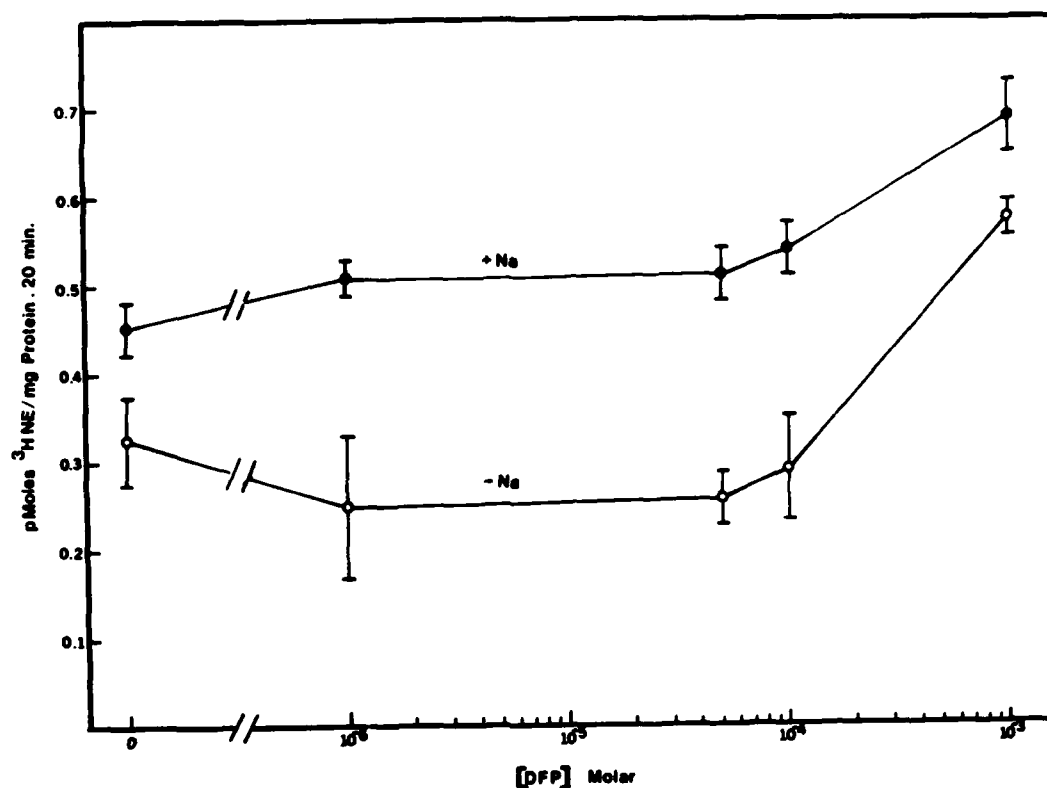


Figure 7. Dose-response curve for effects of DFP on [³H]norepinephrine uptake by primary astrocyte cultures. Uptake of [³H]NE was measured in the presence and absence of Na⁺ (Na⁺ replaced by choline) for 20 minutes as described for [³H]5HT in Figure 1. Final concentration of [³H]NE was 10⁻⁷M (1 uCi in 1 ml total reaction volume). The reaction medium also contained 10⁻⁴M pargyline plus 10⁻⁴M tropolone to prevent oxidative deamination of NE due to MAO and catechol O-methylation by COMT respectively. In Na⁺-free medium, Na⁺ was replaced by choline and triethylammonium HCO₃⁻. A zero time was always subtracted when [³H]NE was added and immediately removed. N = 3 wells ± S.D.

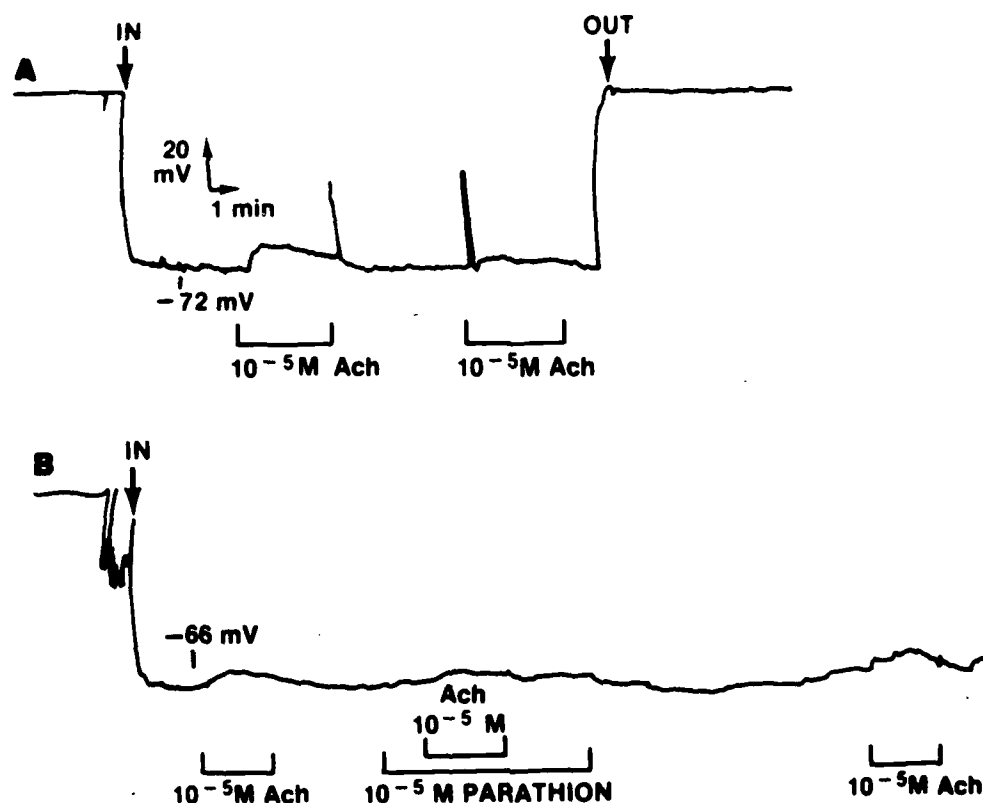


Figure 8. Effects of 10^{-5} M ACh on membrane potentials of cultured astrocytes. Cells were 8 weeks old. The reaction medium was the same as that for Figure 1 except that 25 mM HEPES buffer was used; temperature, 33-35°C.

A: IN refers to impalement of cell by the electrode, which measured a sharp change in potential from 0 to -72 mV. Medium containing 10^{-5} M ACh was perfused for the time shown (4 minutes). A depolarization of 10 mV was seen, which began to repolarize with ACh still present. The second ACh perfusion gave a response of only about 4 mV, suggesting some desensitization. OUT: electrode withdrawn.

B: A second cell shows a potential of -66 mV. 10^{-5} M ACh was perfused as before, and gave a depolarization of about 7 mV. In the presence of 10^{-5} M parathion, the response appears to be only 4 mV, while a third perfusion with 10^{-5} M ACh by itself gave an apparent 7-8 mV depolarization again. This suggests that this cell either showed no desensitization or was already desensitized.

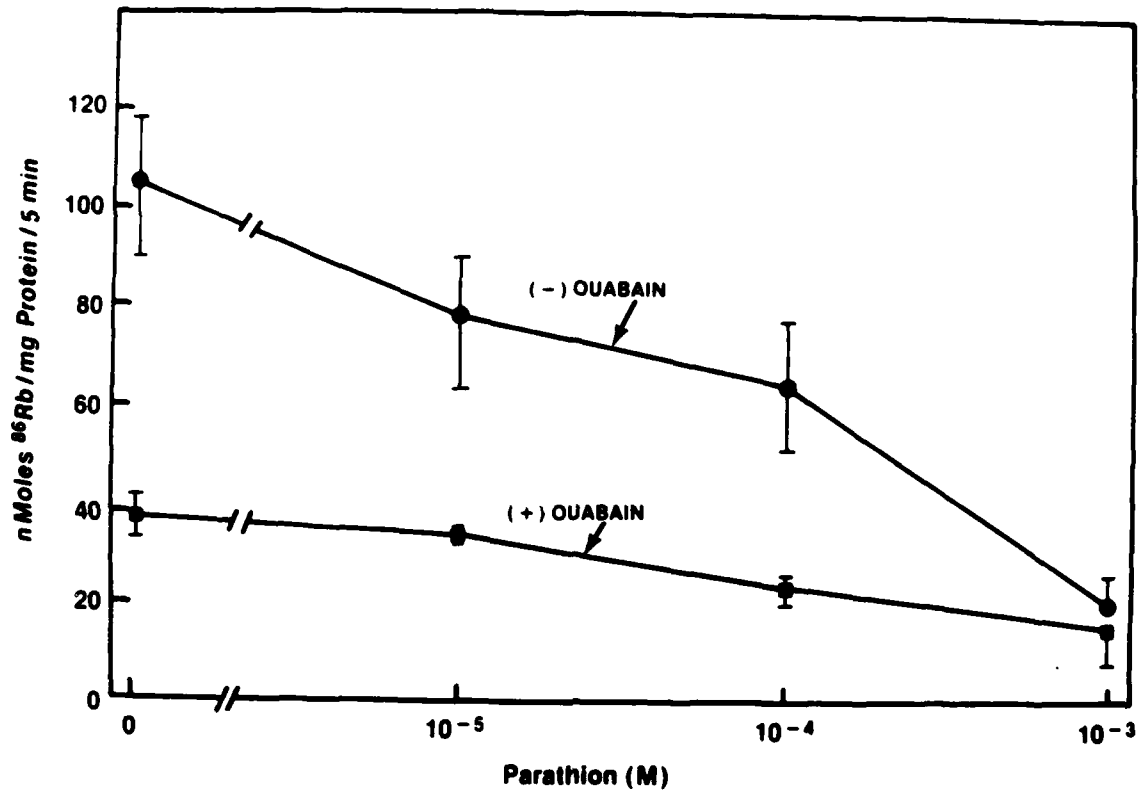


Figure 9. Dose-response curve for effect of parathion on $^{86}\text{Rb}^+$ uptake. Cells were first preincubated for 30 minutes in HCO_3^- -buffered medium (see Figure 1), and then changed to medium containing $^{86}\text{Rb}^+$, ± 1 mM ouabain and the concentrations of parathion shown; uptake was measured for 5 minutes so that only unidirectional influx was measured. Parathion was dissolved in ethanol. Final concentration of ethanol was 1%, which by itself had no effect on $^{86}\text{Rb}^+$ uptake. $N = 4$ wells $\pm$ S.D.

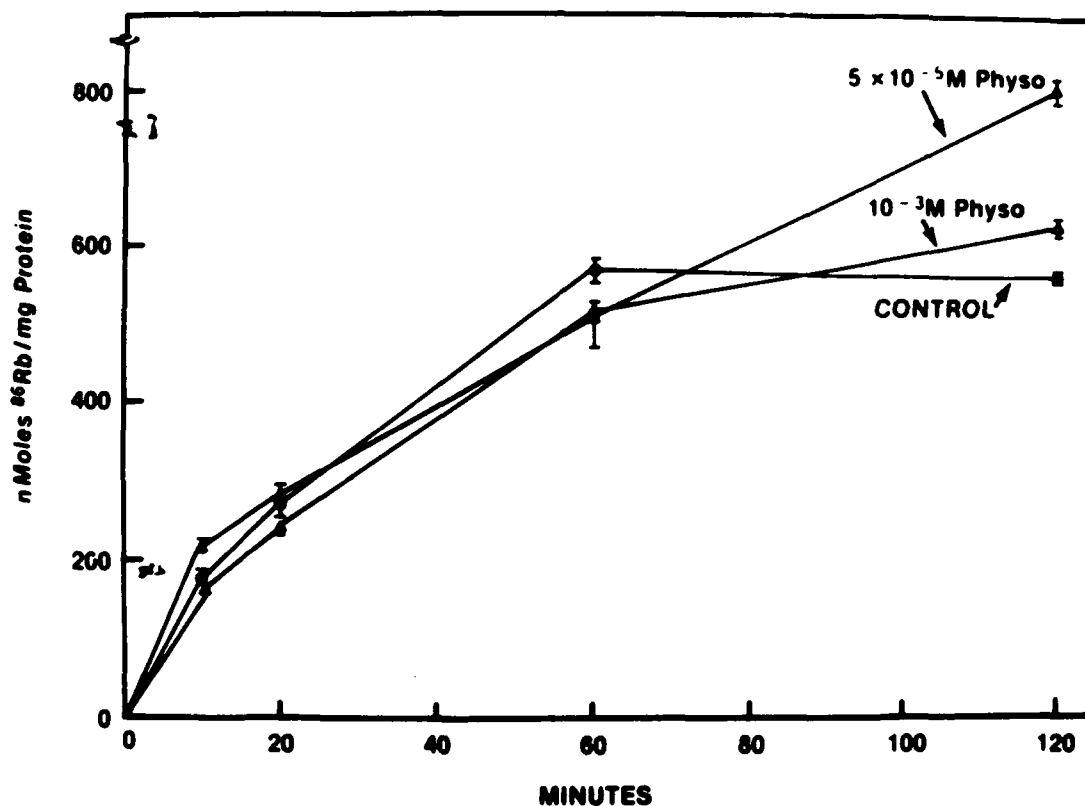


Figure 10. Time course of $^{86}\text{Rb}^+$ uptake in the presence of 5×10^{-5} and 10^{-3}M physostigmine. Cells were first preincubated for 30 minutes in HCO_3^- -buffered medium (see Figure 1), and then changed to medium containing $^{86}\text{Rb}^+$, + 1 mM ouabain and the concentrations of parathion shown; uptake was measured for 5 minutes so that only unidirectional influx was measured. Cells, cultured for 26 days, were preincubated for 30 minutes in reaction medium and physostigmine was added at the same time as $^{86}\text{Rb}^+$. $N = 4$ wells $\pm$ S.D.

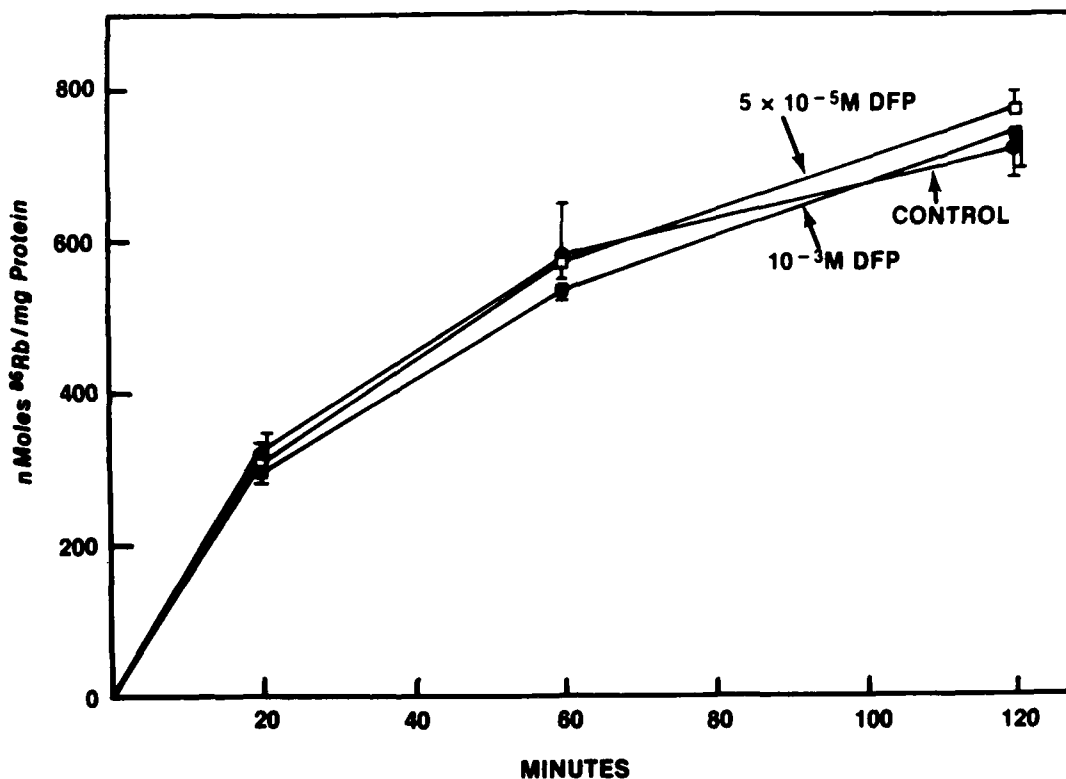


Figure 11. Time course of $^{86}\text{Rb}^+$ uptake in the presence of 5×10^{-5} and 10^{-3}M DFP. Cells were grown for 32 days. Other conditions were as in Figure 10 except that cells were preincubated for 30 minutes in reaction medium + DFP as indicated. $N = 4$ wells $\pm$ S.D.

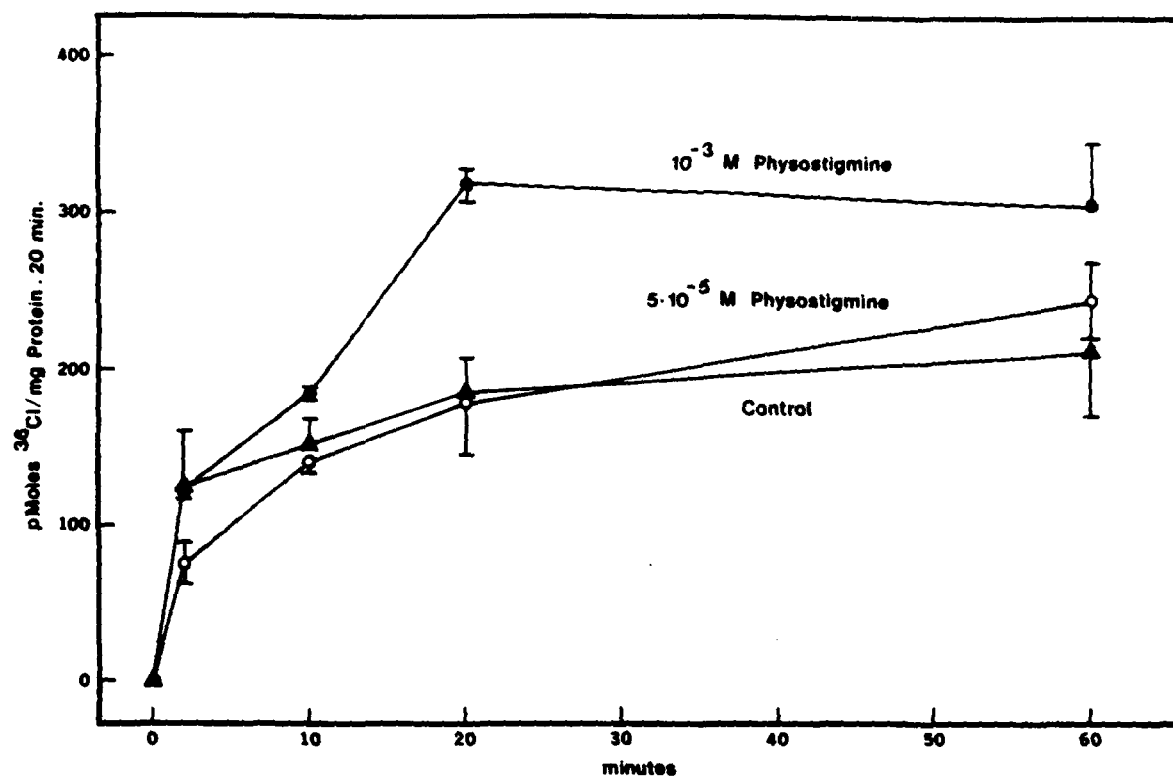


Figure 12. Effects of varying concentrations of physostigmine on time course of Cl^- uptake by primary astrocyte cultures. Cultures were preincubated in HEPES-buffered medium with 25 mM HEPES replacing HCO_3^- (see Methods and Materials). After 20 minutes, $^{36}\text{Cl}^-$ + $5 \times 10^{-5}\text{M}$ ($\blacktriangle$) or 10^{-3}M ($\bullet$) physostigmine was added at zero time and $^{36}\text{Cl}^-$ content measured at the times shown. $N = 3$ wells $\pm$ S.D.

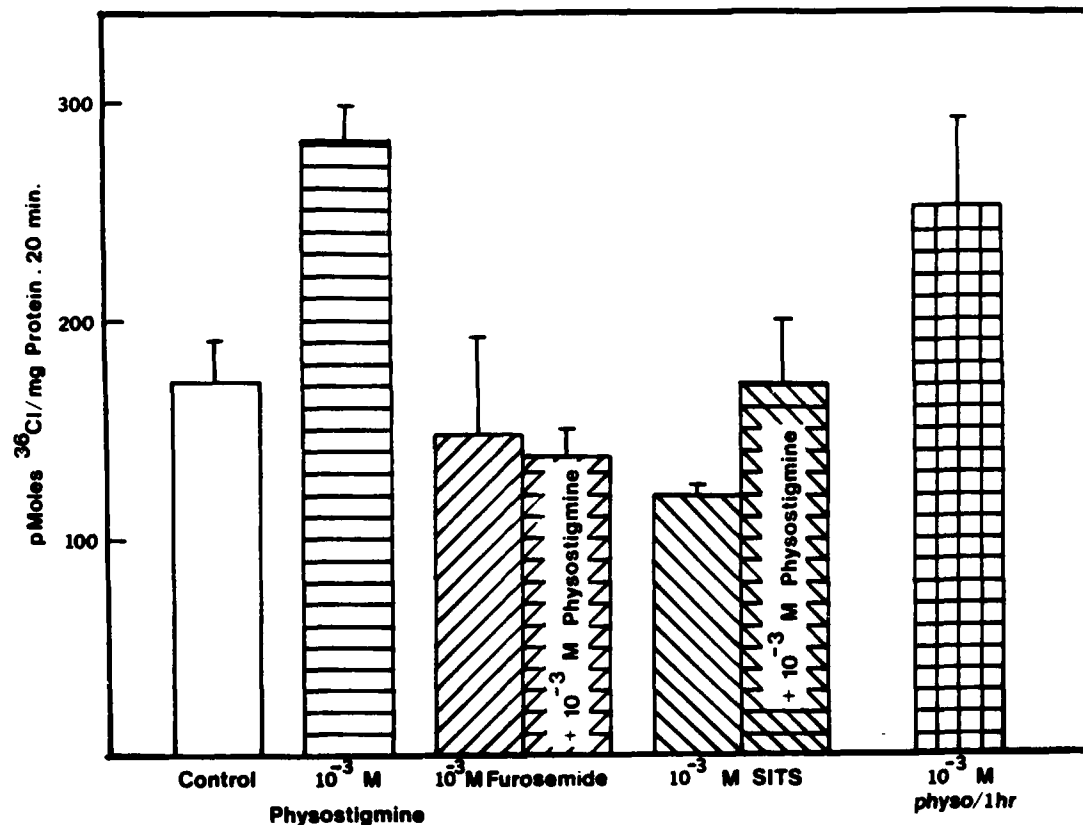


Figure 13. Effects of physostigmine on Cl^- uptake in the presence and absence of 10^{-3} M furosemide or SITS. Uptake of $^{36}\text{Cl}^-$ was measured for 20 minutes as described in Figure 12. During the 20-minute preincubation period, 10^{-3} M furosemide or SITS was present as indicated. In one case, physostigmine was present during a 1-hour preincubation period as indicated. $N = 3$ wells $\pm$ S.D.

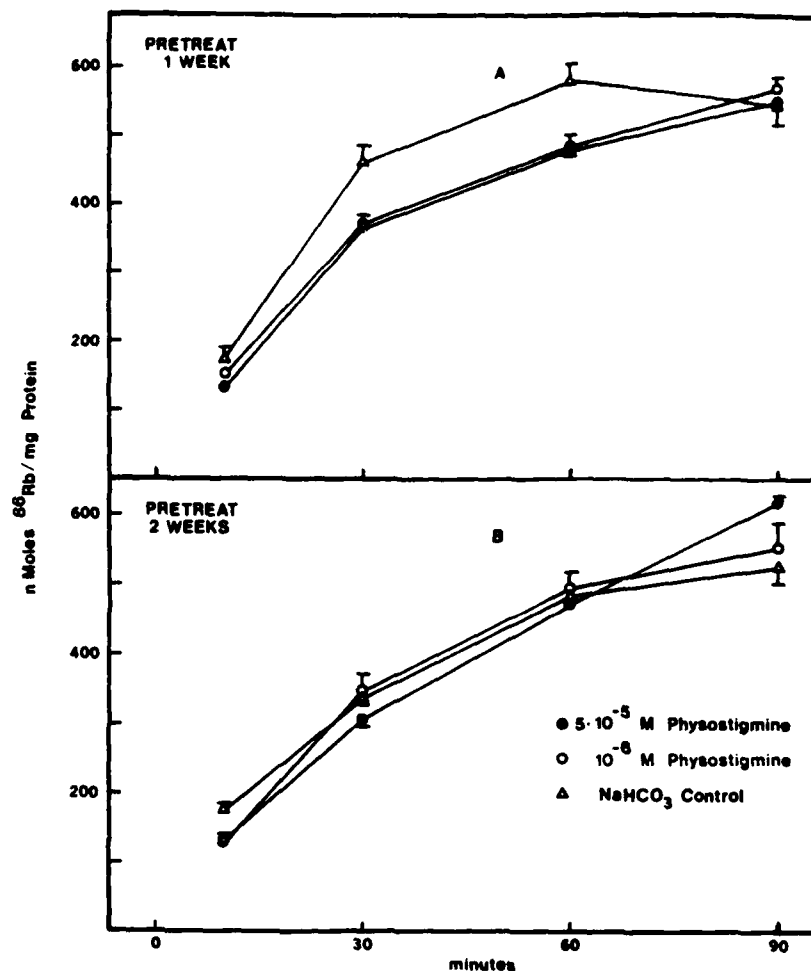


Figure 14. Effects of chronic exposure to physostigmine on uptake of K^+ (measured as $^{86}Rb^+$). Cells were grown in complete medium with physostigmine present at the concentrations shown. Cells were 2 weeks old before the procedure was started. Cells were assayed for uptake of $^{86}Rb^+$ as described in Figure 1 for $[^3H]NE$. Physostigmine was also present at the same concentrations during the uptake period as during the preexposure period. A) 1-week exposure to physostigmine, B) 2-week exposure to physostigmine. $N = 3$ wells $\pm$ S.D.

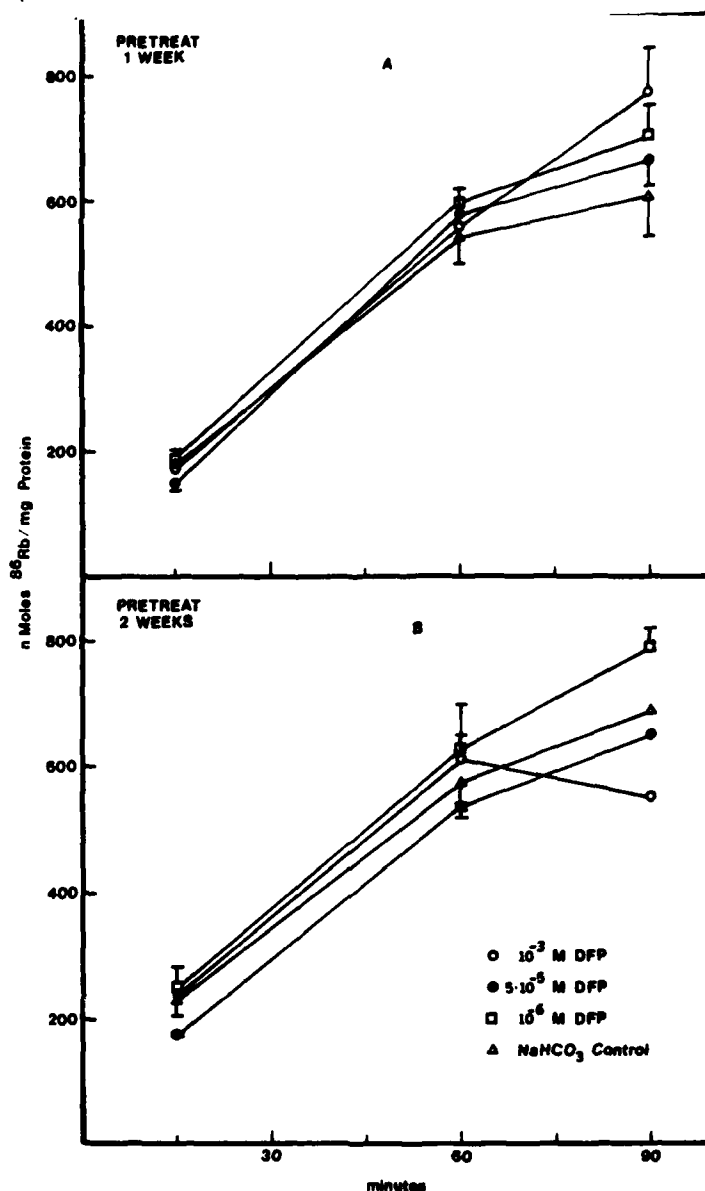


Figure 15. Effects of chronic exposure to DFP on uptake of K^+ (measured as $^{86}\text{Rb}^+$). The cells were pretreated with different concentrations of DFP, as indicated on the graph. A) 1-week exposure, B) 2-week exposure. Uptake of $^{86}\text{Rb}^+$ was then measured for the different times shown (see Figure 9). DFP was also present during the preincubation and uptake periods at the same concentrations as used for pretreatment. $N = 3$ wells $\pm$ S.D.

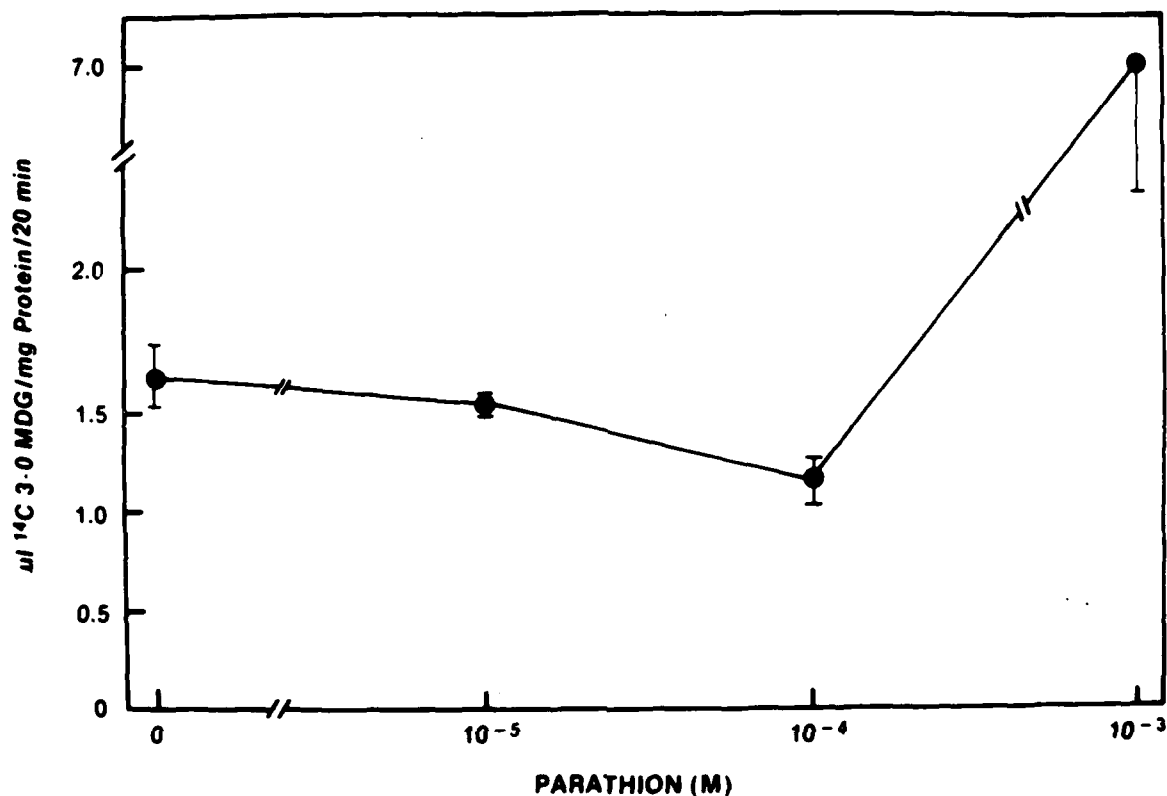


Figure 16. Dose-response effect of parathion on cell volume, as measured by ¹⁴C-labeled 3-O-methyl-D-glucose. 3-O-Methyl-D-glucose (3-O-MDG) space is expressed in terms of ul/mg protein (based on equilibration of 3-O-MDG inside and outside the cell - see text). Cells grown for 34 days were preincubated for 30 minutes in HCO₃⁻-buffered medium. Uptake of [¹⁴C]3-O-MDG (1 uCi/well/0.5 ml) was measured for 20 minutes. Parathion was added with [¹⁴C]3-O-MDG at zero time.

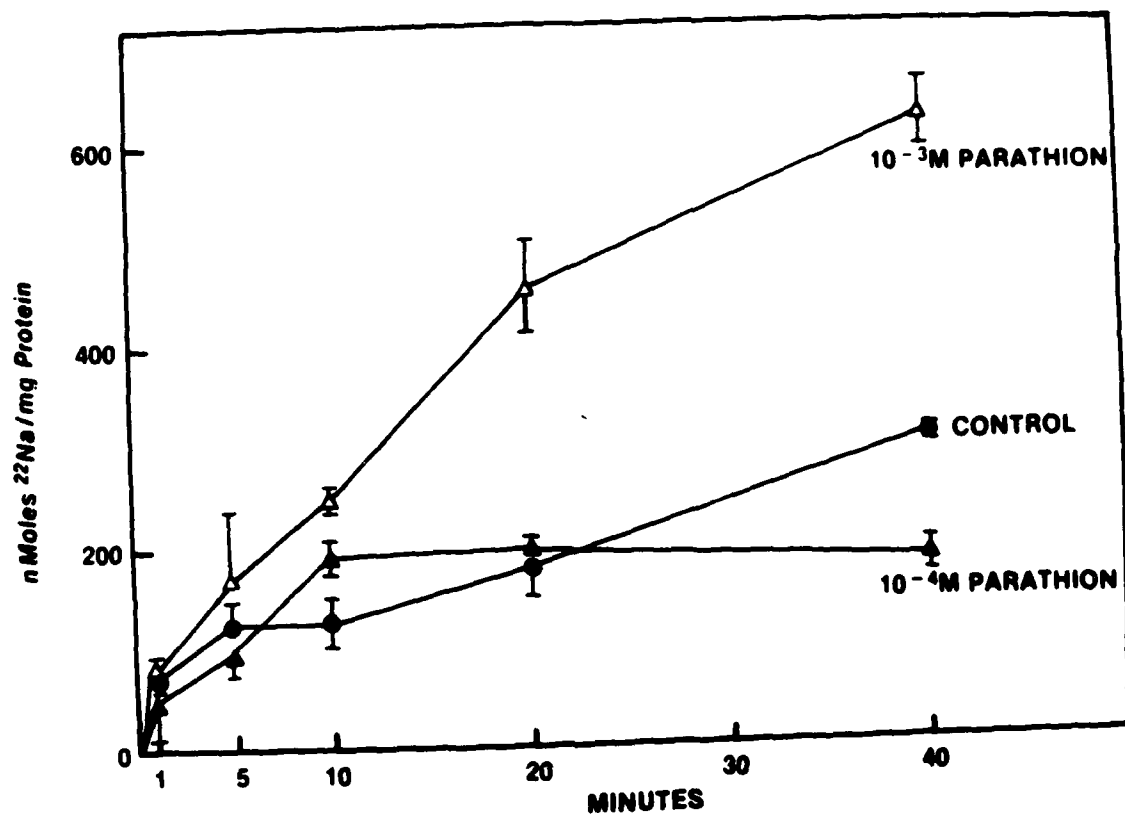


Figure 17. Time course of effect of parathion on uptake of $^{22}\text{Na}^+$. Cells (35 days old) were preincubated in HCO_3^- -buffered medium for 30 minutes. At zero time, medium was replaced with identical medium but containing 2 μCi $^{22}\text{Na}^+$ and 1 mM ouabain with the indicated concentrations of parathion. Final concentration of ethanol was 1% (v/v), which had no effect by itself. Uptake was then measured for the times shown. $N = 3$ wells $\pm$ S.D.

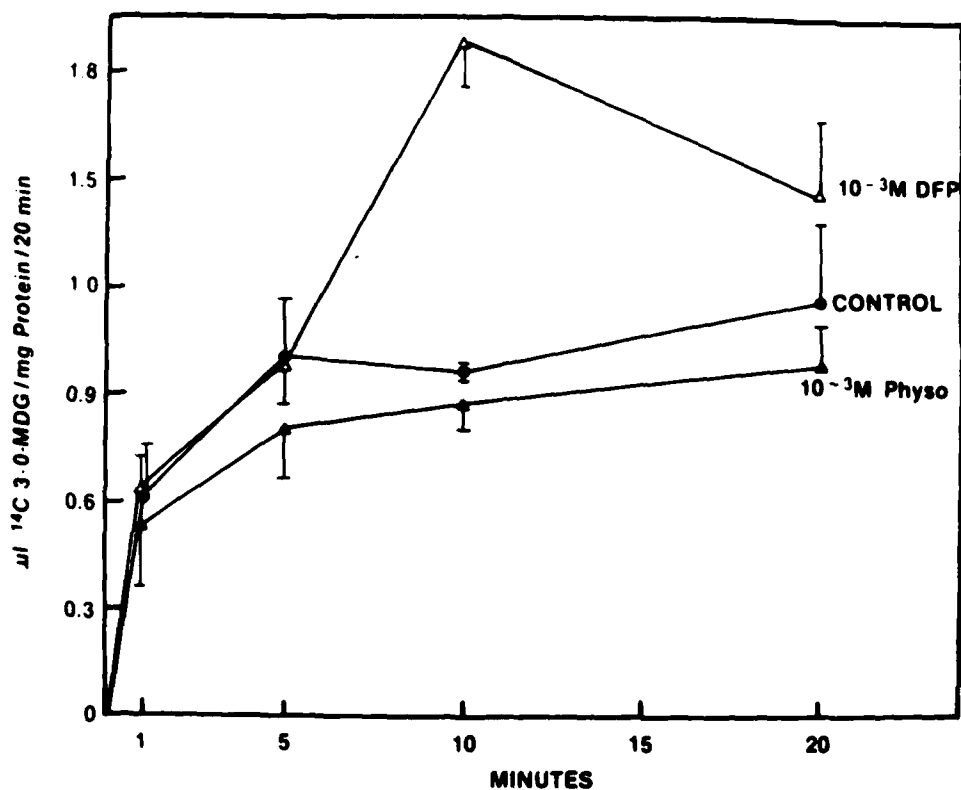


Figure 18. Time course of effect of 10^{-3} M physostigmine or DFP on cell volume as measured by [14 C]3-O-methyl-D-glucose (3-O-MDG). Cells cultured for 28 days were preincubated for 20 minutes in HCO_3^- -buffered medium with physostigmine or DFP. 1 μCi 3-O-MDG/well/0.5 ml. $N = 3$ wells $\pm$ S.D.

TABLE 1

ACH INHIBITS NE-INDUCED INCREASE IN cAMP: EFFECTS OF ACh RECEPTOR
AND IBMX, A PHOSPHODIESTERASE INHIBITOR

Condition	Additions	cAMP Content ^a (pmol/mg protein/10 min)
Culture 1		
Control		725
Control		760
10 ⁻⁵ M NE		8100
10 ⁻⁵ M NE	10 ⁻⁵ M ACh	4915
10 ⁻⁵ M NE	10 ⁻⁵ M ACh and 10 ⁻⁵ M atropine	7285
10 ⁻⁵ M NE	10 ⁻⁵ M ACh and 10 ⁻⁶ M atropine	6000
10 ⁻⁵ M NE	10 ⁻⁵ M ACh and 10 ⁻⁵ M d-tubocurarine	4925
10 ⁻⁵ M NE	10 ⁻⁵ M ACh and 10 ⁻⁶ M d-tubocurarine	4490
Culture 2		
10 ⁻⁵ M NE		2000
10 ⁻⁵ M NE	10 ⁻⁵ M ACh	1100
10 ⁻⁵ M NE	0.5 mM IBMX ^b	3700
10 ⁻⁵ M NE	10 ⁻⁵ M ACh + 0.5 mM IBMX ^b	3370

^a cAMP content of cells was measured after exposure to 10⁻⁵M NE with or without the additions shown. Results from two cultures. Magnitude of cAMP response varies between cultures.

^b IBMX: 3-isobutyl-1-methyl xanthine.

TABLE 2 EFFECTS OF 1,2 OR 3 WEEK EXPOSURE TO VARYING CONCENTRATIONS OF
PHYSOSTIGMINE ON UPTAKE OF $^{36}\text{Cl}^-$ IN PRIMARY ASTROCYTE CULTURES

Time of Exposure (weeks)	Control Uptake (nmol $^{36}\text{Cl}^-$ / 10 min/mg protein)	Concentrations of Physostigmine		
		10^{-6}M	$5 \times 10^{-5}\text{M}$ (% control uptake)	10^{-4}M
1	152.59 $\pm$ 6.13	140.05 $\pm$ 15.08	165.34 $\pm$ 28.42	133.46 $\pm$ 12.83
2	140.93 $\pm$ 11.80	122.81 $\pm$ 12.10	121.14 $\pm$ 11.01	94.98 $\pm$ 6.53
3	181.48 $\pm$ 3.32	138.88 $\pm$ 11.03	101.13 $\pm$ 5.01	97.81 $\pm$ 9.8

Cultures were grown in complete medium with concentrations of physostigmine shown. Cell medium was changed twice per week. Uptake of $^{36}\text{Cl}^-$ measured as in Figure 3 for 10 minutes. All uptake is as nmol $^{36}\text{Cl}^-$ /10 min/mg protein. N = 3 wells $\pm$ S.E.M.

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