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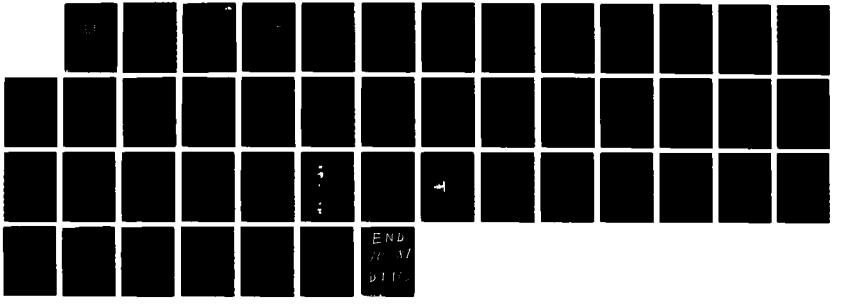

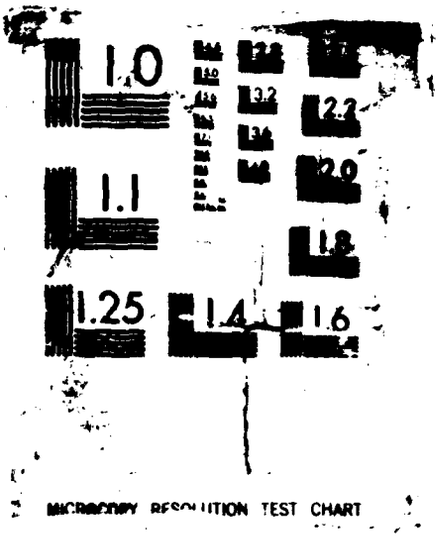
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CLONING OF ACETYLCHOLINESTERASE GENE IN A MICROBIAL VECTOR

Annual Report

1 May 1982-31 January 1983

WILLIAM J. HARRIS, Ph.D.

15 February 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD 17-82-C-2144

Inveresk Research International Limited
Inveresk Gate, Musselburgh, EH21 7UB Scotland

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Techniques to distinguish between the activities of human acetylcholinesterase and pseudocholinesterase have been developed. Human neuroblastoma cell lines have been shown to synthesise human acetylcholinesterase and the level of enzyme within these cells can be induced by treatment of cells with sodium butyrate or dibutyryl cAMP. These cell lines should be suitable as a source of mRNA for the cloning of the human gene into E. coli.

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SUMMARY

Nature of Project

As an aid to the design of effective antidotes to chemical agents, the U.S. Army would like to study in detail the structure of the human enzyme acetylcholinesterase (AChE). This requires the availability of reasonable quantities of the pure enzyme. One way in which this may be achieved is the introduction of the gene from human cells into bacteria by genetic engineering techniques. This is the aim of the current project.

The approach proposed in this study was as follows:

1. Obtain human neuroblastoma cells expressing high levels of AChE activity.

2. Manipulate growth conditions so that expression of enzyme levels is maximised.

3. Isolate mRNA from such cells and transcribe into cDNA.

4. Insert into a plasmid vector which will give low expression.

5. Transfer the gene into a high expression system.

Progress to Date

Objectives 1 and 2 described above have been achieved and work upon Objective 3 is under way.

Cell line CHP-126 has been found to produce 10-fold higher basal levels of AChE activity compared with cell line IMR-32.

Moreover, the AChE activity can be induced 3-4-fold by treatment of cells with either sodium butyrate or dibutyryl cAMP. Data relating to the induction of mRNA synthesis by these agents and the detection of *in vitro* translation of the mRNA into AChE-related protein should be forthcoming in the next 3 months.

FOREWORD

In conducting the research described in this report, the investigators adhered to the Cruelty to Animals Act, 1876 and to the guidelines issued by the Advisory Committee on Genetic Manipulation, formerly the Genetic Manipulation Advisory Group, as defined in the health and Safety (Genetic Manipulation) Regulations, 1978.

INTRODUCTION

Acetylcholine is an example of a transmitter substance which is released from nerve endings in response to electrical impulses and, by diffusing across gaps, causes excitation of post-synaptic cells and continuation of nerve impulses. Nerves which transmit impulses by release of acetylcholine (cholinergic) include motor fibres to striated muscle, parasympathetic fibres to smooth muscle, and fibres connecting the central nervous system to sympathetic ganglia. While acetylcholine acts as a transmitter substance in small concentration, it is a paralytic substance in high concentration. Careful control of the amount of acetylcholine is therefore essential. This is achieved by the action of the enzyme acetylcholinesterase (AChE) which hydrolyses acetylcholine to acetate and choline before another impulse can be transmitted. Agents which inhibit AChE, therefore, cause a variety of toxic effects, often fatal. Such agents include various chemical agents and organophosphorous insecticides.

The U.S. Army would like to produce effective antidotes to chemical agents suitable for both treatment of personnel after exposure to such agents and also possible treatment to protect, prior to such potential exposure. Such agents act by binding to the active site of human AChE. Consequently, from a thorough knowledge of the structure and 3-dimensional arrangement of active chemical groupings within the active site of the human AChE, it would be possible to custom-build chemicals to replace or prevent binding of chemical agents within the active site.

Properties of AChE

The most detailed information regarding the structure and properties of AChE has been obtained from studies with electric eel (Electrophorus electricus) and the electric ray (Torpedo marmorata), though general properties have been studied with impure preparations from rat, mouse and chicken skeletal muscle; bovine brain; and human erythrocytes and plasma (1). In most vertebrate tissues, AChE is extracted along with a related enzyme which hydrolyses either butyrylcholine or propionylcholine in addition to acetylcholine. This enzyme is generally referred to as cholinesterase (EC 3.1.1.8.). Human AchE can be distinguished from cholinesterase in blood samples; AChE is found only in erythrocytes and is inhibited by high concentrations of acetylcholine. Cholinesterase, on the other hand, will hydrolyse butyrylcholine, propionylcholine and benzoylcholine (2). A comparison of AChE from fish and non-human vertebrate tissue has been described by Massoulie (3), and while the enzymes are similar in overall structure and organisation, it is clear that significant species variations exist at the molecular level in terms of size and shape. Generally, the enzyme exists in three globular forms and three collagen-tailed forms, the globular forms being monomers, dimers and tetramers of uniform 70-80,000 dalton peptides. Similar data from human muscle or ganglia are not available but the human erythrocyte enzyme is generally membrane bound and is extracted as a glycoprotein

with CHO:protein ratio 0:16 (4). Purification is achieved using the property of the anionic site by affinity chromatography with trimethyl-p-phenyl ammonium chloride. Extraction of the enzyme in the presence of Triton X-100 results in a homogenous preparation of 6.5-7.0S consisting of a dimer of subunits of molecular weight 80,000. On removal of Triton X-100, the enzyme aggregates into at least 8 multiple molecular weight species equivalent to 6-14 subunits, though aggregation can be prevented by chaotropic ions (5, 6).

Unfortunately, it is not possible to prepare large quantities of pure human AChE from human material or from human cells in cell culture. In this contract, therefore, it is proposed to transfer the gene for human AChE from human cells into the bacterium E. coli, thereby permitting the routine preparation of large quantities of the pure human enzyme for structural studies.

Rationale for Project Design

The methodology of cloning directly from the genome of human neuroblastoma cells was rejected. It was considered that the human genome is too complex and too many colonies would need to be screened. Also, it is possible that the AChE gene within the genome is "split" and would not be transcribed and translated in bacteria into a protein recognisable as AChE. We proposed the following approach:

1. Obtain a neuroblastoma cell line which expresses a high level of AChE activity.

2. Manipulate growth conditions so that expression of AChE activity is maximised.

3. Isolate mRNA from such cells and subfractionate polyA$^+$ -RNA to enrich for species containing AChE or mRNA.

4. Transcribe this into cDNA.

5. Insert into plasmid pAT153, which will give low expression.

6. Transfer into a high expression vector host system by a method based upon insertion of the AChE gene into bacteriophage M13 mp-7 and induction of transcription through the lac operon by isopropyl thiogalactoside (IPTG).

PROGRESS TO DATE

Several discoveries have necessitated a degree of deviation from the main line of the research.

First, the discovery that foetal and new-born calf serum possess highly active and stable AChE activity necessitated careful examination of the origin of such activity detected in human neuroblastoma cell extracts. Evidence that AChE activity was a product of the cells themselves and obtained from three lines of investigation: It was found that AChE activity continued to accumulate even if neuroblastoma cells were maintained for up to 10 days in serum-free medium. Further, the levels of enzyme activity within cells could be increased by external agents such as sodium butyrate and dibutyryl cAMP. Finally, the human and bovine enzymes could be distinguished by migration rates in polyacrylamide gel electrophoresis (PAGE).

Second, it was essential to clearly establish that the acetylcholine-hydrolysing activity observed within neuroblastoma cell extracts was due to AChE rather than pseudocholinesterase. It was hoped that a rapid solution to this problem would be provided by direct PAGE analysis by the technique which has been used so successfully to distinguish these enzyme activities in amniotic fluid (7). However, clear band separations with cell extracts from neuroblastoma cells, red blood cells or commercial preparations were not obtained with this technique. Instead, a smear of activity extending approximately 1 cm into the gel was obtained. Eventually, as described in Section IV, an electrophoretic technique was developed.

Third, the publication of data describing the isolation of monoclonal antibodies to AChE of human erythrocytes (8) raised the possibility of immunoassay detection of human AChE. In-house preparation of rabbit antisera was delayed until evaluation of these monoclonals. One such monoclonal, termed AE-2, has been obtained from the American Type Culture Collection. However, preliminary analysis has failed to provide evidence that this monoclonal binds to human AChE from either red blood cells or IMR-32. Also, Brock (personal communication) was unable to demonstrate binding of this monoclonal to AChE enzyme in amniotic fluid. Further evaluation is under way but in-house preparation of rabbit antisera has also been initiated.

METHODOLOGY

Materials

Human neuroblastoma cell line IMR-32, PG3, Lot 27961, was obtained from Flow Laboratories, Irvine, Scotland, on 8 June 1982. Neuroblastoma cell line CHP-126 was received from Professor M. Glick, University of Pennsylvania, on 20 May 1982. RPMI-1640, foetal calf serum and nonessential amino acids were purchased from Gibco Europe Limited, Paisley, Scotland. Anti-erythrocyte membrane antibody and anti-human cholinesterase were obtained from Dakopathy, Denmark. Human serum cholinesterase and Proteinase K were products of Boehringer. Lysivane was obtained from Professor D. Brock, University of Edinburgh. All other enzymes and associated reagents were purchased from Sigma, Poole, England, and general laboratory chemicals were either from BDH or Sigma. Phenol for RNA extractions was AnalaR grade from BDH and was redistilled before use.

Growth and Maintenance of Neuroblastoma Cells

a) Cell Culture

Neuroblastoma cells were routinely grown as a monolayer at 37°C in an atmosphere of 5% CO_2 (100% humidity). The cells were cultured in plastic Falcon flasks or 1 litre glass Roux bottles containing RPMI-1640 medium supplemented with foetal calf serum (10% v/v), non-essential amino acids (1% v/v), glutamine (297 $\mu g.ml^{-1}$), pyruvate (200 $\mu g.ml^{-1}$), penicillin (6.3 $\mu g.ml^{-1}$) and streptomycin (10 $\mu g.ml^{-1}$). In some experiments "serum-free" medium was used which lacked foetal calf serum and contained insulin (20 $\mu g.ml^{-1}$), transferrin (20 $\mu g.ml^{-1}$), ethanolamine (20 μM), sodium selenite (2.5 nM) and mercaptoethanol (10^{-5} M). Medium was changed every 2-3 days until the cells formed a confluent monolayer, spent medium was decanted and a small volume of fresh medium added. Cells were deteched from the surface of the culture flask by gentle shaking with a few glass beads, diluted with fresh medium and seeded into new flasks or tissue culture multi-well plates at a split ratio of 1:2.

b) Freezing Cells

Cells were cultured to confluence and suspended in a volume of medium equal to that in which the cells were grown. They were collected by centrifugation at room temperature at 1,000 r.p.m. for 5 min and resuspended in fresh medium (with serum) at a concentration of at least 10^6 cells.ml^{-1} (1 ml of medium per 25 cm^2 flask). The cell suspension was added to a plastic ampoule for freezing and sterile dimethylsulphoxide added to a final concentration of 10% (v/v). Ampoules were cooled slowly by placing them within a polystyrene box at -70°C overnight. Ampoules were finally immersed in liquid nitrogen.

c) Thawing Cells

Ampoules were removed from liquid nitrogen and the contents thawed quickly by placing in a water bath at 37°C. Cells were then centrifuged for 5 min at 1,000 r.p.m., and medium containing dimethylsulphoxide removed with a sterile pasteur pipette; cells were resuspended in fresh medium in a flask of the same size as that in which the cells were originally grown.

Induction of AChE Activity in Neuroblastoma Cells

A range of chemicals known to cause induction of enzyme activities and/or differentiation of cell cultures was compared with IMR-32 and CHP-126. Cells were seeded at 1:2, split from confluent flasks into 24-well plates and grown for 4-7 days in standard medium with 10% foetal calf serum and supplemented, where appropriate, with varying concentrations of the potential inducers dimethylsulphoxide (DMSO), 5-bromodeoxyuridine (5-BrdU), papaverine, sodium butyrate, dibutyryl cAMP (dB-CMP), nerve growth factor, prostaglandin E1 (PgE1) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA).

Preparation of Cell Extracts

A number of variations of a standard method have been described for disrupting mammalian cells and releasing AChE activity (e.g., 9, 10). The method involves non-ionic detergent treatment (Triton X-100, Lubrol WX) together with mechanical disruption (sonication, homogenisation, osmotic shock) and removal of cell debris by centrifugation. The effects of sonication, homogenisation and osmotic shock on the release of AChE activity from IMR-32 cells were compared. Confluent monolayers from three 150 cm^2 Falcon flasks were suspended in 2.25 ml phosphate buffer (0.1 M, pH 7.4) containing 1% (v/v) Triton X-100, and the suspension was divided into three aliquots and (a) left at 4°C for 1 h; (b) placed in a Kerry sonic water bath for 1 min, then held at 4°C for 1 h; and (c) homogenised with 6 strokes of a ground-glass pestle and held at 4°C for 1 h.

All three samples were centrifuged at 100,000 g for 1 h in a Beckman Model L5-50 ultracentrifuge at 5°C. Liquid assays demonstrated little difference in AChE activity among the preparations. Thus, for routine preparation, gentle methods of cell disruption such as (a) were used.

Enzyme Assays

a) Acetylcholinesterase (AChE) Assay

A spectrophotometric assay to detect the liberation of thiocholine using Ellman's reagent with increased absorbance at 412 nm (11).

AchE activity was distinguished from pseudocholinesterase (ChE) activity with specific inhibitors, AChE being inhibited by 8 μM 1,5-bis-4-allyldimethylammonium-phenylpentone-1,3-dibromide

(Figure 1) (BW 284C51), in agreement with data of Austin and Berry (12). Lysivane (ethopropazine hydrochloride) at 11.4 μm specifically inhibited ChE activity (Figure 1) (13).

b) ChE Assay

Cholinesterase activity was monitored by the decrease in absorbance at 240 nm with benzoyl choline as substrate (14). The assay was performed in quartz glass semi-micro cuvettes with a reaction mix containing 120 mM sodium phosphate, pH 7.2/0.16% (v/v) Triton X-100/0.25 mM benzoyl choline. AChE was unreactive in this assay (personal observation).

c) Assay of AChE in Microtitre Plates

The general assay described in a) above was modified to permit rapid assay of samples in 96-well microtitre plates. The final concentration of all reagents in the reaction mix was the same except that the substrate concentration was reduced to 0.6 mM and total assay volume was 0.25 ml. All reagents except substrate were placed in the wells and reaction was started by the addition of substrate and mixing. If bubbles formed during this stage, they were removed by gentle blowing on the plate or in severe cases by placing the plate under vacuum for 2-3 min. The absorbance of each well was measured as soon as possible at 405 nm in a Titertek multiscan and again after 10 min incubation of plates at room temperature. The multiscan recorded the absorbance within the 96 wells in 10-20 sec. Normally lysivane was included at 28 μM to inhibit ChE activity.

d) Protein Estimation

Protein assays were carried out according to Lowry et al. (15) using bovine serum albumin as standard. When non-ionic detergent was present in cell extracts, an equivalent amount was added to standards and reagent blank. Prior to measuring absorbance, samples were centrifuged at 500 g for 10 min to remove the precipitate.

Polyacrylamide Gel Electrophoresis

The method used for non-denaturing gels was the simplified procedure described by Clark (16). A gel system described by Gratzl et al. (17) was also used. Denaturing gel electrophoresis was varried out as detailed by Laemmli (18).

Activity Staining of Gels

After electrophoresis, AChE was detected in the gels using specific activity stains. Three different methods were used:

a) The method routinely used was that of Koelle (19) as described by Chubb and Smith (20). Gels were soaked for 1 h at room temperature in a pre-incubation mixture of 1.69 M sodium sulphate/ 70 mM sodium maleate, pH 6.5. Sometimes gentle warming (<37°C) was required to keep the sodium sulphate in solution. The incubation mixture (100 ml/gel) consisted of 1.69 M Na_2SO_4/70 mM sodium maleate, pH 6.5/4 mM $CuSO_4$/20 mM glycine/33 mM $MgCl_2$/0.4 mM Tri-Cl, pH 6.5/2 mM acetylthiocholine iodide, and was freshly prepared each time. The gels were incubated at 37°C between 3 and 16 h before washing twice in distilled water and soaking in saturated, aqueous dithiooximide for 30 min.

   To specifically stain AChE 14.2 µM lysivane was included in the incubation mix. When only cholinesterase activity was required, 10 µM BW284C41 was used.

b) A modification of the method of Gratzl et al. (17) was also tried. After electrophoresis the gel was washed twice for 30 min each in 100 mM sodium phosphate, pH 7.4/0.1% Triton X-100 and then placed in fresh buffer containing, in addition, 0.5 mM DTNB and 0.5 mM acetylthiocholine iodide. The gel was incubated at 37°C until yellow bands appeared, corresponding to the AChE activity.

c) The method of Galbraith and Watts (21) was used. The gel was soaked in a solution of 6.4 mM N-methylindoxyl acetate/0.2 M sodium phosphate, pH 7.4, containing 50 $mg.ml^{-1}$ Fast Blue RR at 30°C for 1 h. Some difficulty was experienced in keeping the reagents in solution.

Protein Staining of Gels

Gels were soaked in 0.1% Coomassie Blue R250 in 25% (v/v) methanol/10% (v/v) acetic acid for 1 h and destained in several changes of 25% (v/v) methanol/5% (v/v) acetic acid until the background was clear.

Gels to be dried were soaked for 1-2 h in 2% DMSO and dried on a Bio-Rad Dual Temperature Slab gel drier, Model SE 1125B.

Crossed Immunoelectrophoresis

The method of Galbraith and Watts (21) was used. Glass slides (5 x 7 cm) were painted with 1% agarose in a buffer containing 9 mM sodium barbital, 1.4 mM barbital, 94 mM glycine, 465 mM Tris base and 3.5 mM sodium azide, pH 8.8. Triton X-100 was added to give a final concentration of 1% (v/v). Gels 1.5 mm thick were poured on the prepared glass slide. Samples (one per gel) were loaded into 2 mm diameter holes cut in the gels. Samples were electrophoresed for 90 min at 120 V and 18 mA; then the agarose gel was cut away, leaving only a 1.5 cm strip along which the protein had been separated. This was replaced with agarose gel of the same composition, containing 30 µl of anti-serum (Dako A/S). Gels were then electrophoresed at right angles for 16 h at 50 V and 7 mA. Gels were washed in 0.1 M NaCl at least 6 times, then stained for AChE activity or for general protein.

RESULTS

Development of Specific Assays for AChE

a) Liquid Assay

A comparison of the kinetics of hydrolysis of acetylthiocholine by AChE (as represented by bovine erythrocyte AChE) and ChE (as represented by human serum ChE) is described in Figure 2. Bovine erythrocyte AChE has a Km of 0.20 mM compared with 0.66 mM for human ChE. The inhibitions of AChE by BW284C51 and of ChE by lysivane are described in Figure 1. On the basis of these data, 8 μM BW284C51 and 11.4 μM lysivane were chosen as suitable inhibitory concentrations for routine studies.

That these two inhibitors do not cross-react and can be used successively in direct kinetic assays to distinguish the presence of a mixture of AChE and ChE activities is demonstrated by Figure 3. It can be seen that human serum ChE is unaffected by addition of 40 μM BW284C51, a concentration which completely abolished AChE activity (Figure 2). Conversely, 70 μM lysivane, which abolished ChE activity (Figure 2), has no effect upon AChE activity (Figure 3).

b) Polyacrylamide Gel Electrophoresis (PAGE)

The above inhibitors have been used successfully *in situ* to distinguish AChE and ChE activities in polyacrylamide gels of amniotic fluid (22). Confirmation of this finding is illustrated by Figure 4. While both inhibitors do give specific inhibition, a clear band separation of AChE and ChE activities is not obtained with commercial preparations of AChE and ChE. Rather, a smear of activities is obtained extending approximately 1 cm into the gel, much of the enzyme failing to penetrate the gel at all. This is consistent with reports in the literature of multimeric forms of AChE activity bound to various membranous components. Collagenase treatment improved resolution to some extent (results not presented). Modifications of the gel systems were examined and a modification based on the gel system of Clarke (16) gave improved resolution. Each sample was prepared with 1% (v/v) Triton X-100 prior to electrophoresis and 0.1% (v/v) Triton X-100 was included in all gel buffers. One example is described in Figure 5 which demonstrates good resolution of AChE activities. This figure also demonstrates different migration of bovine and human AChE.

Detection of AChE Activity in Neuroblastoma Cells

Preliminary studies were carried out in the absence of specific inhibitors and thereby measured both AChE and ChE activities. Table 1 describes an example of the levels of enzyme activity in IMR-32 and CHP-126 after growth in complete and serumless media. CHP-126 contains 3-4-fold higher levels of activity that IMR-32, confirming

the data of Glick et al. (23). These data also demonstrate that both these cell lines possess both AChE and ChE enzymic species. Growth for up to 7 days in serumless medium had no effect on AChE levels in CHP-126, though IMR-32 cells seem less well suited to maintenance in the serumless medium. It should be noted that the serumless medium was supplemented with appropriate growth factors as described in the Methods section.

PAGE of the extracts in gels containing 0.1% (w/v) polyoxyethylene ether W-1 helped confirm these results (data not presented). Activity staining of the cell extracts resulted in a smear at the top of the gel. The level of activity in CHP-126 was greater than that in IMR-32, although equal protein was not added in the 2 wells.

Treatment with 1 μM BW284C51 inhibited the bovine erythrocyte AChE almost completely and reduced the activity in human red blood cells. The farthest migrating activity in IMR-32 extracts was also removed and overall activity was reduced. CHP-126 cell extract activity was not substantially altered by treatment with BW284C51 because insufficient inhibitor was used. Lysivane did reduce much of the least mobile material and led to the appearance of a band of activity at approximately the same position as that in the bovine erythrocyte AChE and human red blood cell enzyme.

Samples digested with DNase or glucuronidase before electrophoresis did not alter behaviour upon gels but treatment with collagenase increased the mobility of material which tended to bind to the origin (results not presented).

Acetylcholinesterase Activity in Animal Sera

During development of the assay systems, it was found that foetal calf serum, used to sustain the growth of neuroblastoma cells, possessed AChE activity. A survey of various commercial sera samples revealed the presence of the following activities:

Foetal calf serum	100% AChE activity
New born calf serum	100% AChE activity
Adult horse serum	50% AChE:50% ChE activity
Adult human serum	10% AChE activity:90% ChE activity
Human red blood cell extracts	100% AChE activity 100% AChE activity

Data from which these results were established are provided in Figure 3 along with data indicating that CHP-126 cell extracts possess both AChE and ChE activity.

The level of enzyme activity detected in animal sera would be sufficient to account for AChE activity detected in neuroblastoma cell extracts if the enzyme was absorbed from the growth media into the cells or attached to the outside of membranes. It has already been

reported that AChE activity in A. aegypti cells correlates with the amount of foetal calf serum present in growth media (24).

An attempt was made to distinguish human and bovine AChE activity immunologically. Extracts of CHP-126 cells, human erythrocyte membrane preparation and calf sera were mixed with varying amounts of human erythrocyte membrane anti-sera, incubated for 10 min at 37°C and analysed on PAGE. The anti-sera retarded the migration of AChE activity with all extracts, though the effect was clearly more marked with the erythrocyte membrane preparation (results not presented). A similar result was obtained using crossed immunoelectrophoresis, though a precipitin band was obtained only with the erythrocyte membrane enzyme preparation. Presumably calf sera and neuroblastoma AChE preparations have enough common determinants to cross-react with the anti-sera, but insufficient to form a precipitin band. Thus, the origin of AChE activity in neuroblastoma cell extracts could not be determined immunologically.

Induction of Acetylcholinesterase Activity in Neuroblastoma Cells

Using mouse neuroblastoma cells it has been shown that culture conditions which induce neurite outgrowth usually also induce AChE activity (25), neurite outgrowth is induced by factors enhancing interaction between the cell surface and the culture dish (passage from suspension to monolayer culture, serumless medium, 5-BrdU). Elevation of intracellular cyclic AMP in neuroblastoma cells also induces many characteristic differentiated functions such as AChE activity.

Some agents known to cause induction of enzyme activities and/or differentiation in cell lines was examined for their ability to induce AChE activity in IMR-32 and CHP-126 cells. Although previous studies (23) indicated that CHP-126 had higher basal levels of enzyme than IMR-32, this does not suggest that induction should be less effective in the latter cell line. Furthermore, IMR-32 cells grow better than CHP-26 and hence study of induction was carried out with both cell lines. In the first experiment, DMSO and 5-BrdU, papaverine and soidum butyrate were compared, as well as the effect of growth or maintenance of cells in serum-free or serumless medium. As can be seen from Table 2, growth in serum-free or serumless medium did not effect enzyme levels.

Visual examination of the cells revealed that only two treatments caused significant alteration in cellular morphology. Growth in the presence of 25 or 50 $\mu g.ml^{-1}$ papaverine markedly restricted growth and caused large numbers of cells to round up and slough off the dish. Serum-free conditions led to extensive differentiation. Cells grew in tight clumps with many long neurites and formation of an extensive network.

Levels of total esterase activity (AChE and ChE) were measured using the 96 well plate assay technique described in Methods (Figure 7). DMSO at concentrations up to 2% (v/v) depressed the specific activity of the enzymes, while 5-BrdU up to 25 μM had very little effect on enzyme activities. Although papaverine treatment caused an apparent increase in specific activity, the loss of material from these cultures resulted in low levels of both enzyme activity and protein. These data must therefore be treated with caution. Exposure to sodium butyrate produced a steady increase in specific activity of AChE as the concentration of inducer increased. Similar data generated after treatment of cells with nerve growth factor or TPA are presented in Figure 7. Visual inspection of cells did not reveal any morphological alterations to the cells, nor was there any significant increase in levels of esterase activity.

Increasing the length of exposure to inducer to 10 days and assaying cell extracts in the presence of lysivane showed significant elevation of levels of AChE after treatment of cells with sodium butyrate and 5-BrdU (Figure 9). Cells treated with these two agents assumed a more cobbled appearance and were more densely packed than those in control cultures. At concentrations higher than 500 μM, sodium butyrate caused cell death (data not presented).

A summary of these and additional data is presented in Table 3, from which it can be seen that similar data can be obtained with CHP-126 cells.

The optimum concentration of sodium butyrate was chosen and time course of the effect upon AChE activity performed (Figure 9). The increase in AChE activity is maximal 4-6 days after induction.

DISCUSSION

On the basis of available data, a number of conclusions can be drawn:

1. Both IMR-22 and CHP-126 synthesise both AChE and ChE enzymes. Evidence that AChE activity detected in cell extracts is not due to contamination with enzyme absorbed from serum stems from two sources:

   a) AChE activity accumulates in cells maintained for up to 10 days in serum-free medium.

   b) AChE activity can be induced by external agents such as sodium butyrate and dB-cAMP.

   Immunological evidence is still being sought.

2. CHP-126 has up to 10-fold (non-induced) and 28-fold (induced) higher levels of AChE activity than non-induced IMR-32 (Table 3). CHP-126 would seem, therefore, to be the cell line of choice for gene cloning studies.

3. The measurement of enzyme levels by catalytic activity is very sensitive. As shown in Figure 5, we have consistently failed to detect any protein-staining material at positions in PAGE that stain for AChE activity.

   Although AChE activity can be readily detected, this does not mean that AChE mRNA will be easily found and isolated.

FUTURE WORK

It is considered that the overall strategy outlined in the original proposal is viable and will be pursued. It is suggested, however, that a number of minor modifications and additions be introduced, as detailed below.

Rationale for Proposed Modifications

a) Change of Vector Systems

In the proposal it was suggested that initial cloning use pAT153 and then a high expression vector based upon mp-7. However, new expression vectors are being developed at such a rate that we and our advisors, Professor Burke and Professor Sherratt, proposed that initial selection use puC8/9, developed by Messing (26). These vectors contain all the cloning sites from M13 and mp-8 and mp-9 with a portion of the lac 7 gene which complements in the JM103 host cell. Cloning into the vector inactivates the lac gene rendering the host cell lac$^-$. These vectors are derived from pBr322, and Professor Sherratt is developing equivalent vectors from his pAT153 which may be used subsequently. Furthermore, it is suggested that selection of a high expression system be delayed until required. New expression systems are being described at an increasing rate and appropriate consideration should be given to bacterial, yeast and eukaryotic systems. No difficulty is envisaged in selecting a suitable high expression system at the appropriate time.

b) Screening of Clones

In the proposal, primary screening of bacterial clones would be by expression of enzyme activity or immunological techniques or ^{3}H-DFP binding, as well as competitive hybridisation of clones to mRNA prepared from induced and non-induced neuroblastoma cells. Recent studies by Dr. Doctor of the Walter Reed Institute for Research, Washington, have revealed a part of the amino acid sequence of an active-site-containing peptide from AChE of Electrophorus. This sequence is Trp-Asp-Pro-Asp-Arg-Glu-Met. Since it is possible that the region around the active site of AChE has been preserved during evolution, it is possible that human enzyme will be similar to this sequence. We propose, therefore, in addition to primary screening as defined in the original proposal, to prepare synthetic oligonucleotides relevant to this sequence as probes of primary clones. For example, one suitable consensus oligonucleotide sequence in E. coli would be the 12-mer TGGGACCCGGAT.

c) Gene Amplification Study

The primary aim of this project is to develop a system allowing the preparation of large quantities of AChE. While it is likely

that this will be achieved by the above gene cloning technology, it would be expedient to consider alternative technologies. Our experience in handling the enzyme to date has emphasised its complex structure in vivo: association with collagen, isolation in membrane complexes, tendency to aggregate and hydrophobic nature. Even if the cloning project is successful, it would be valuable to be able to compare the cloned product with "natural" material.

We propose, therefore, in parallel with present studies, to attempt to increase significantly the amount of enzyme produced by neuroblastoma cells. This will be achieved by selecting variants which have greatly amplified the AChE gene. Gene amplification is a well-established phenomenon whereby mammalian cells respond to stress by making multiple copies of appropriate genes to relieve the stress. Thus, methotrexate-resistant cells were selected which had amplified the gene for dihydrofolate reductase many hundreds of times. Similarly, Wilson (personal communication) has succeeded in isolating variants of Chinese hamster ovary cells which have amplified the gene for glutamine synthetase some 1500-fold. In these cells, glutamine synthetase accounts for about 20% of the total cell protein. It may be possible by the technique described below to select variants of human neuroblastoma cells which have greatly amplified the number of gene copies of AChE so that this enzyme will represent a considerable fraction of total cellular protein.

METHODOLOGY

Screening of Clones by Hybridisation to Synthetic Oligonucleotides

Oligonucleotides (12-15 mer) will be synthesised by the phosphotriester method (27). Such synthesis is likely to be carried out manually, although Inveresk Research International Limited is considering the purchase of an automatic gene synthesiser. It is likely that 4-6 oligonucleotides containing appropriate code degeneracies will be synthesised. These will be labelled at the 5'-end with ^{32}P using polynucleotide kinase (28) and used as hybridisation probes by *in situ* hybridisation to bacterial colonies transferred to nitrocellulose filters (29). Positive colonies will be selected and compared with those selected by other screening techniques.

Gene Amplification

Selection of cell variants with increased levels of AChE will be based upon the essential requirement for choline in growth media of cell cultures. Choline is routinely added to cell culture growth media as an essential nutrient, and preliminary studies will establish that human neuroblastoma cells have such a requirement. The aim of the technique is to gradually subject cells to an increasingly depleted choline environment so that variants are gradually selected from the population. Choline-depleted growth media will be supplemented with acetylcholine, thereby requiring the presence of AChE or ChE for growth. AChE and ChE activities will be removed from animal sera, probably by dialysis against difluorophosphate, and lysivane will be added to inhibit ChE produced by cell lines. The growth media content of acetylcholine will be adjusted so that cell growth rate approximates 70% of that in fully supplemented medium and growth and subculture will be continued in this medium until a rapid growth rate is restored. Subsequently, the amount of acetylcholine supplement will again be reduced and the process repeated through several cycles. It is envisaged that this selection procedure will take about 6 months.

AChE is a highly active enzyme, and it may not be possible to add a sufficiently low concentration of acetylcholine to restrict cell growth. If this is the case, restrictive growth conditions will be established by supplementation of growth medium with acetylcholine and an appropriate competitive inhibitor of choline kinase, the enzyme essential for choline utilisation.

Success by this procedure cannot be guaranteed or predicted. However, in view of the relatively small additional commitment in staff time, it is worthy of pursuit.

This work will draw upon the advice and assistance of Dr. R.H. Wilson, Department of Genetics, University of Glasgow, who has successfully amplified the gene for glutamine synthetase.

TABLE 1

Levels of AChE and ChE in Neuroblastoma Cell Lines IMR-32 and CHP-126

Cell Line	Growth Conditions	Sp. Act. nmoles.min^{-1}.mg protein^{-1}		
		No Inhibitor	+ Lysivane	+ BW284C51
CHP-126	RPMI-1640 + 10% FCS	14	NM	NM
CHP-126	Serumless; 3 days	21	NM	9
CHP-126	Serumless; 7 days	16	9	9
IMR-32	RPMI-1640 + 10% FCS	5	NM	2
IMR-32	Serumless; 3 days	5	NM	NM
IMR-32	Serumless; 7 days	0*		

* = This preparation had low protein content. Possibly the activity was below the level of detection.
NM = Not measured
Sp. Act. = Specific activity

TABLE 2

Levels of AChE and ChE in Neuroblastoma Cell Lines Under Different Growth Conditions in 24-Well Plates

Medium	Av. Sp. Act. (arbitrary units.mg protein^{-1})	Number of Samples
Standard + 10% FCS	0.60	8
RPMI "serum-free"	0.58	4

All experiments with IMR-32 cell line in a 4 day period.

Av. Sp. Act. = Average specific activity

TABLE 3

The Effects of Chemicals on AChE Levels in Neuroblastoma Cells

Cell Line	Treatment	Av. Sp. Act. (No. of Samples)	% of Control
CHP-126	Control	11.48 (8)	100
CHP-126	+ 200 $\mu g.ml^{-1}$ dB-cAMP	26.25 (2)	229
CHP-126	+ 400 $\mu g.ml^{-1}$ dB-cAMP	28.46 (2)	248
CHP-126	+ 800 $\mu g.ml^{-1}$ dB-cAMP	25.28 (2)	223
CHP-126	+ 0.5 $\mu g.ml^{-1}$ PGE_1	12.00 (2)	104
CHP-126	+ 1 $\mu g.ml^{-1}$ PGE_1	7.75 (2)	68
CHP-126	+ 2 $\mu g.ml^{-1}$ PGE_1	8.85 (2)	77
CHP-126	+ 4 $\mu g.ml^{-1}$ PGE_1	7.94 (2)	69
CHP-126	+ 8 $\mu g.ml^{-1}$ PGE_1	10.52	92
IMR-32	Control	1.01 (5)	100
IMR-32	+ 200 $\mu g.ml^{-1}$ dB-cAMP	2.36 (1)	234
IMR-32	+ 400 $\mu g.ml^{-1}$ dB-cAMP	3.17 (2)	314
IMR-32	+ 800 $\mu g.ml^{-1}$ dB-cAMP	3.95 (2)	391
IMR-32	+ 0.5 $\mu g.ml^{-1}$ dB-cAMP	3.95 (2)	391
IMR-32	+ 0.5 $\mu g.ml^{-1}$ PGE_1	1.22 (1)	121
IMR-32	+ 1 $\mu g.ml^{-1}$ PGE_1	1.30 (1)	129
IMR-32	+ 2 $\mu g.ml^{-1}$ PGE_1	1.17 (1)	116
IMR-32	+ 4 $\mu g.ml^{-1}$ PGE_1	1.51 (2)	150
IMR-32	+ 8 $\mu g.ml^{-1}$ PGE_1	1.45 (2)	144
CHP-126	Control	16.60 (4)	100
CHP-126	+ 0.313 mM Na butyrate	38.19 (4)	230
CHP-126	+ 0.625 mM Na butyrate	22.69 (4)	137
CHP-126	+ 1.25 mM Na butyrate	11.12 (4)	67
CHP-126	+ 2.5 mM Na butyrate	7.54 (4)	45
CHP-126	+ 5.0 mM Na butyrate	5.30 (4)	32
IMR-32	Control	1.99	100
IMR-32	+ 0.313 mM Na butyrate	4.04	204
IMR-32	+0.625 mM Na butyrate	4.45	224
IMR-32	+ 1.25 mM Na butyrate	2.29	115
IMR-32	+ 2.5 mM Na butyrate	2.37	120
IMR-32	+ 5 mM Na butyrate	2.81	142

Av. Sp. Act. = Average specific activity

FIGURE 1

Effects of Specific Inhibitors of ChEs

(a) ChE. Human serum ChE (Boehringer) was assayed using the 96-well microtitre plate system described in the methodology section. Each assay contained 40 mU of enzyme and 0.6 mM acetylthiocholine as substrate. Various amounts of lysivane were included in the assays.

(b) AChE. Bovine erythrocyte AChE was assayed in cuvettes by the standard procedure using Ellman's reagent. Each assay contained 20 mU of enzyme and 0.83 mM acetylthiocholine. Different levels of BW284C51 were tested in the assays.

FIGURE 1 (continued)

Effects of Specific Inhibitors on ChEs

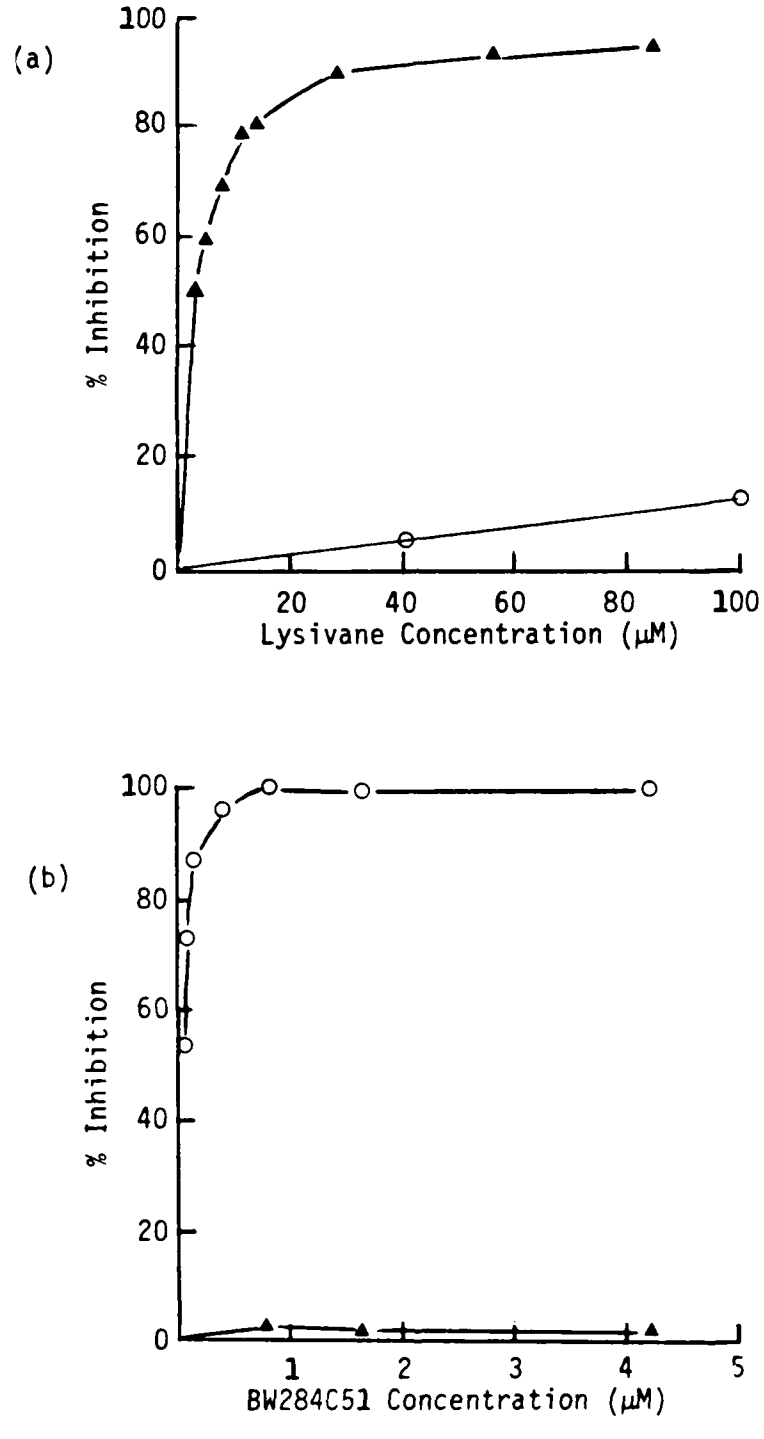


FIGURE 2

Lineweaver-Burke Plots of ChE (Human Serum) and AChE (Bovine Erythrocyte) Using Acetylthiocholine as Substrate

(a) ChE. The reactions were carried out as described in the methodology section using Ellman's reagent. Assays were at room temperature (22°C) and each contained 0.25 U of enzyme (human serum ChE; Sigma).

(b) AChE. The reactions were carried out as above except that each contained 20 mU of enzyme (bovine erythrocyte AChE; Sigma).

FIGURE 2 (continued)

Lineweaver-Burke Plots of ChE (Human Serum) and AChE (Bovine Erythrocyte) Using Acetylthiocholine as Substrate

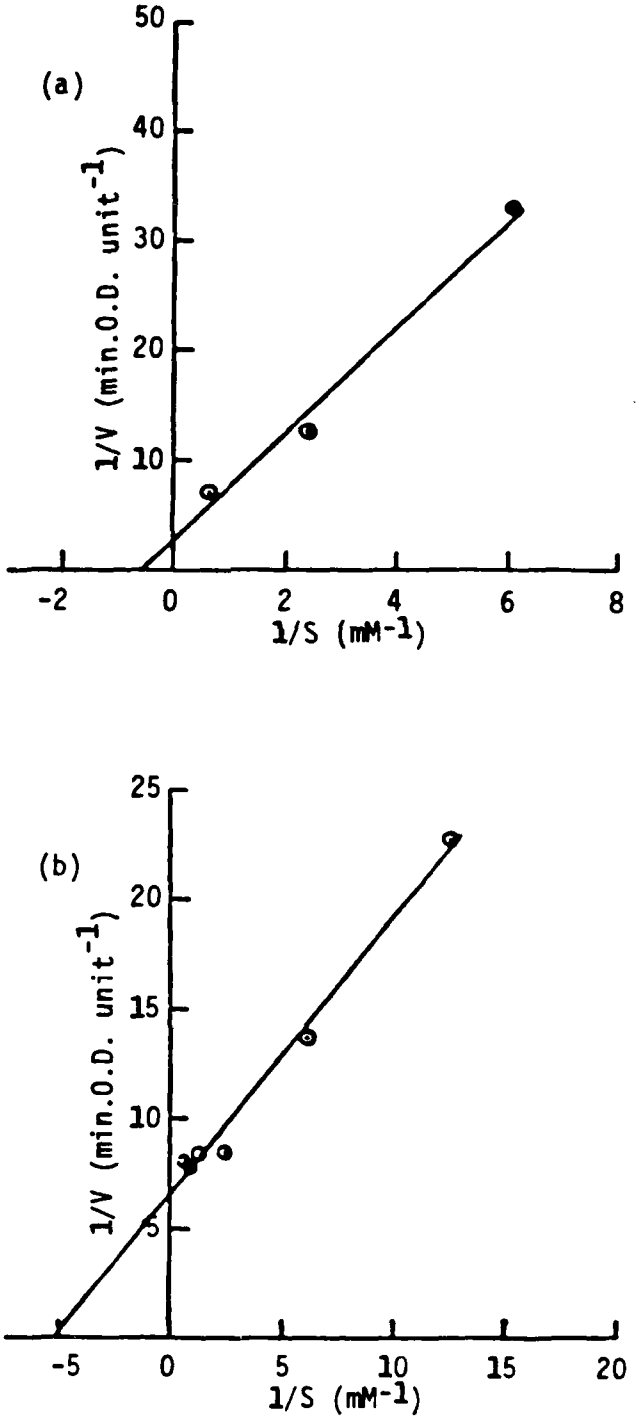


FIGURE 3

Liquid Assay of AChE in Cell Extracts and Commercial Serum Samples

1 ml of assay mix containing 0.1 M sodium phosphate buffer, pH 7.4, 0.2 mM DTNB, 0.05% v/v Triton X-100 and 0.05-0.2 ml of enzyme preparation was placed in a semi-microcuvette (path length 1 cm) and pre-incubated in a Unicam SP800 spectrophotometer at 24°C for 1-2 min. 0.1 ml of substrate (1 mM acetylthiocholine, final concentration) was then added and the rate of reaction recorded at 412 nm. After sufficient time to record reaction rate, either BW284C51 (4×10^{-7}) M, final concentration) or lysivane (7×10^{-7} M, final concentration) was added and the reaction rate recorded.

1: Foetal calf serum
2: Human serum ChE (Sigma)
3: Horse serum
4: Bovine red blood cell AChE (Sigma)
5: Human red blood cell extract
6: Foetal calf serum
7: Extract of CHP-126 grown 7 days in serum-free medium

A: BW284C51; B: Lysivane added.

FIGURE 3 (continued)

Liquid Assay of AChE in Cell Extracts and Commercial Serum Samples

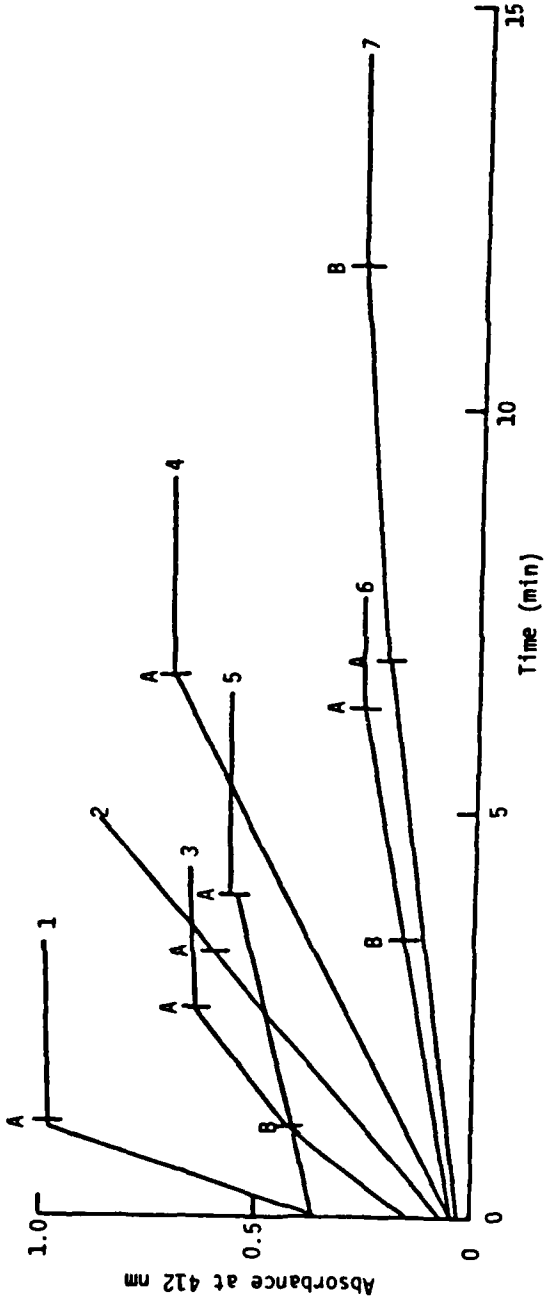


FIGURE 4

Effects of Specific Inhibitors on ChE Activity in Polyacrylamide Gels

Gel electrophoresis was carried out using a composite acrylamide/agarose gel and Tris/glycine/Triton X-100 buffer system. A 3% acrylamide/0.5% agarose gel was run on an LKB 2117 Miltiphor apparatus. The gel buffer was 50 mM Tris/0.38 M glycine/0.2% Triton X-100 and electrophoresis buffer was a 1:1 dilution of this. Electrophosesis was overnight at 5 mA plus 1 h at 30 mA. Cooling water was used throughout.

Activity staining was as described in the methodology section, using the inhibitor concentrations detailed therein.

Tracks 1, 5, 9	4 µl of foetal calf serum
Tracks 2, 6, 10	4 µl of human red blood cell extract
Tracks 3, 7, 11	40 mU of serum ChE
Tracks 4, 8, 12	4 µl of red blood cell extract + 40 mU human ChE
Tracks 1-4	no inhibitor
Tracks 5-8	plus BW284C51
Tracks 9-12	plus lysivane

FIGURE 4 (continued)

Effects of Specific Inhibitors on ChE Activity in Polyacrylamide Gels

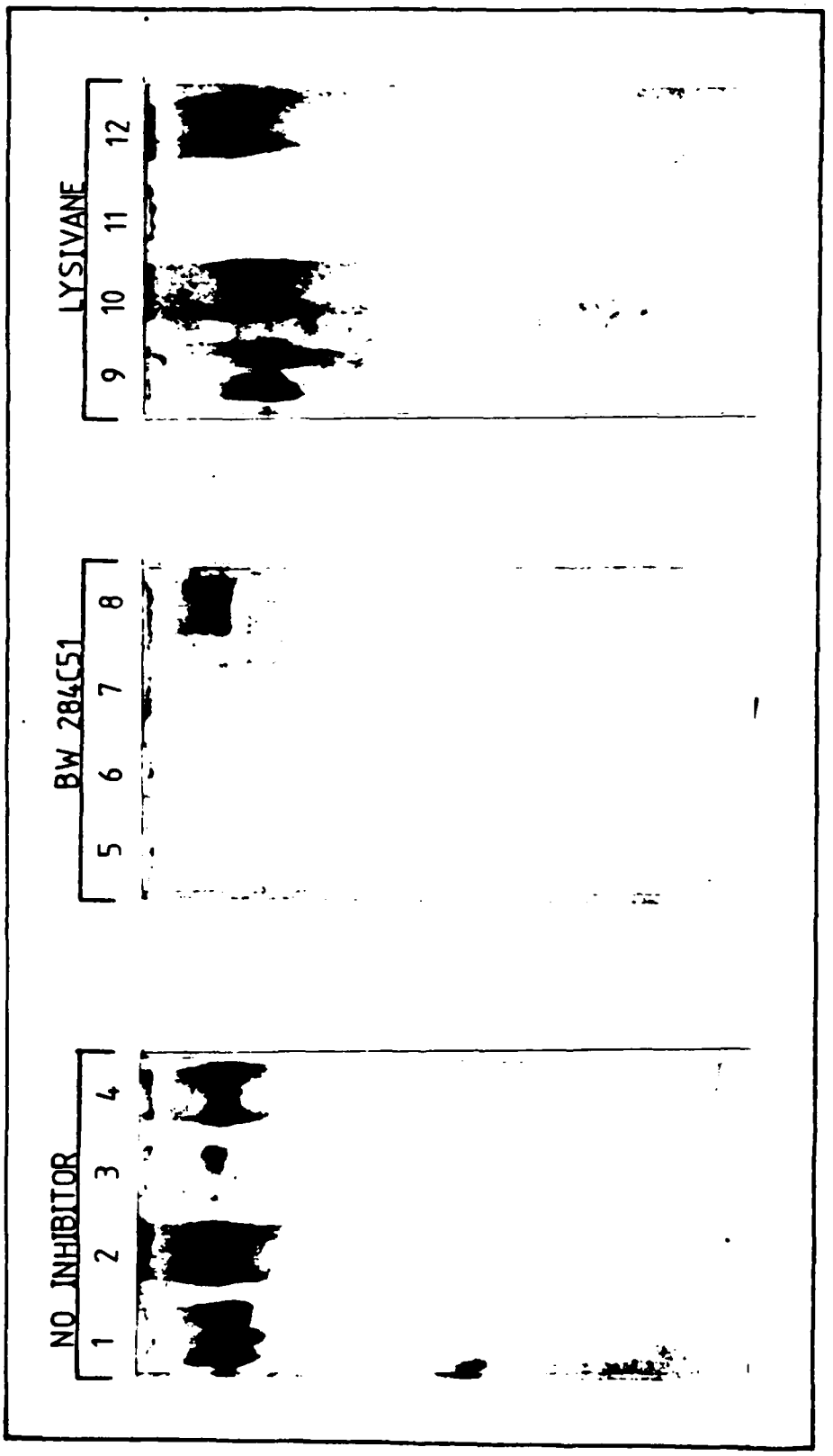


FIGURE 5

Foetal Calf Serum and Human Erythrocyte AChE in Polyacrylamide Gels

Gel electrophoresis and staining were carried out as described in the methodology section. The gel system was based on that of Clarke (16). Electrophoresis was carried out in a Bio-Rad Protein 32 cm slab cell with 1.5 mm spacers. Current was 3 mA for 18 h.

Tracks 1, 6	5 µl foetal calf serum
Tracks 2, 7	10 µl foetal calf serum
Tracks 3, 8	20 µl foetal calf serum
Tracks 4, 9	20 mU bovine erythrocyte AChE
Tracks 5, 10	5 µl human red blood cell extract

Tracks 1-5 were activity stained.
Tracks 6-10 were stained for protein.

FIGURE 5 (continued)

Foetal Calf Serum and Human Erythrocyte AChE in Polyacrylamide Gels

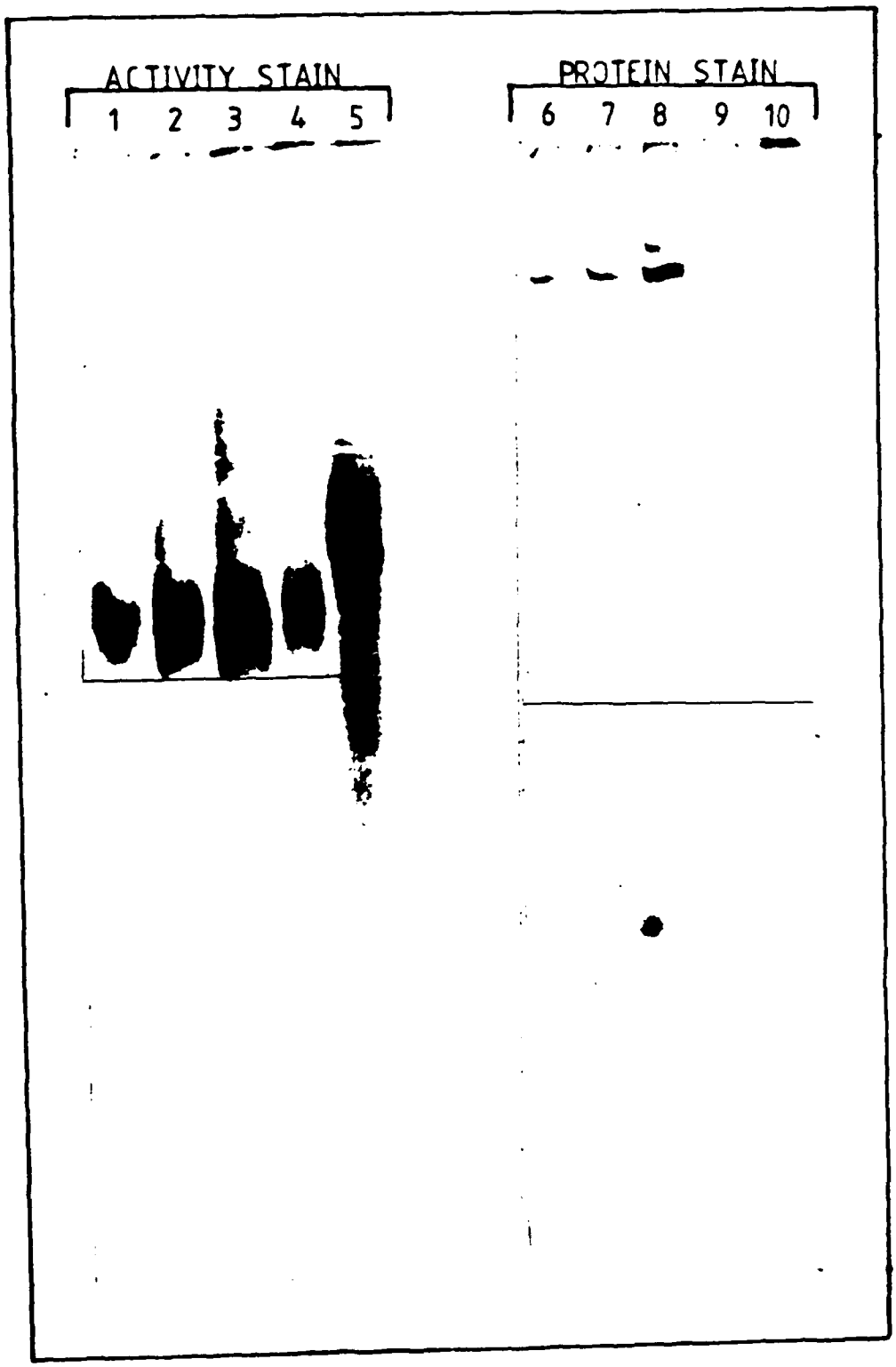


FIGURE 6

Effect of Inducers on ChEs in IMR-32 Neuroblastoma Cells
After 4 Days of Exposure

Each of the inducers was added to confluent IMR-32 cells in a 24-well Linbro plate at the concentrations indicated on the graphs. After 4 days the cells were harvested and assayed using the 96-well microtitre plate assay described in the methodology section. No inhibitor was included, so the results represent the sum of all ChEs in the extracts. The units of AChE are arbitrary. Protein was estimated by the Lowry procedure (15).

(a) Dimethylsulphoxide (DMSO)
(b) 5'-Bromodeoxyuridine (5-BrdU)
(c) Papaverine
(d) Sodium butyrate

FIGURE 6 (continued)

Effect of Inducers on ChEs in IMR-32 Neuroblastoma Cells
After 4 Days of Exposure

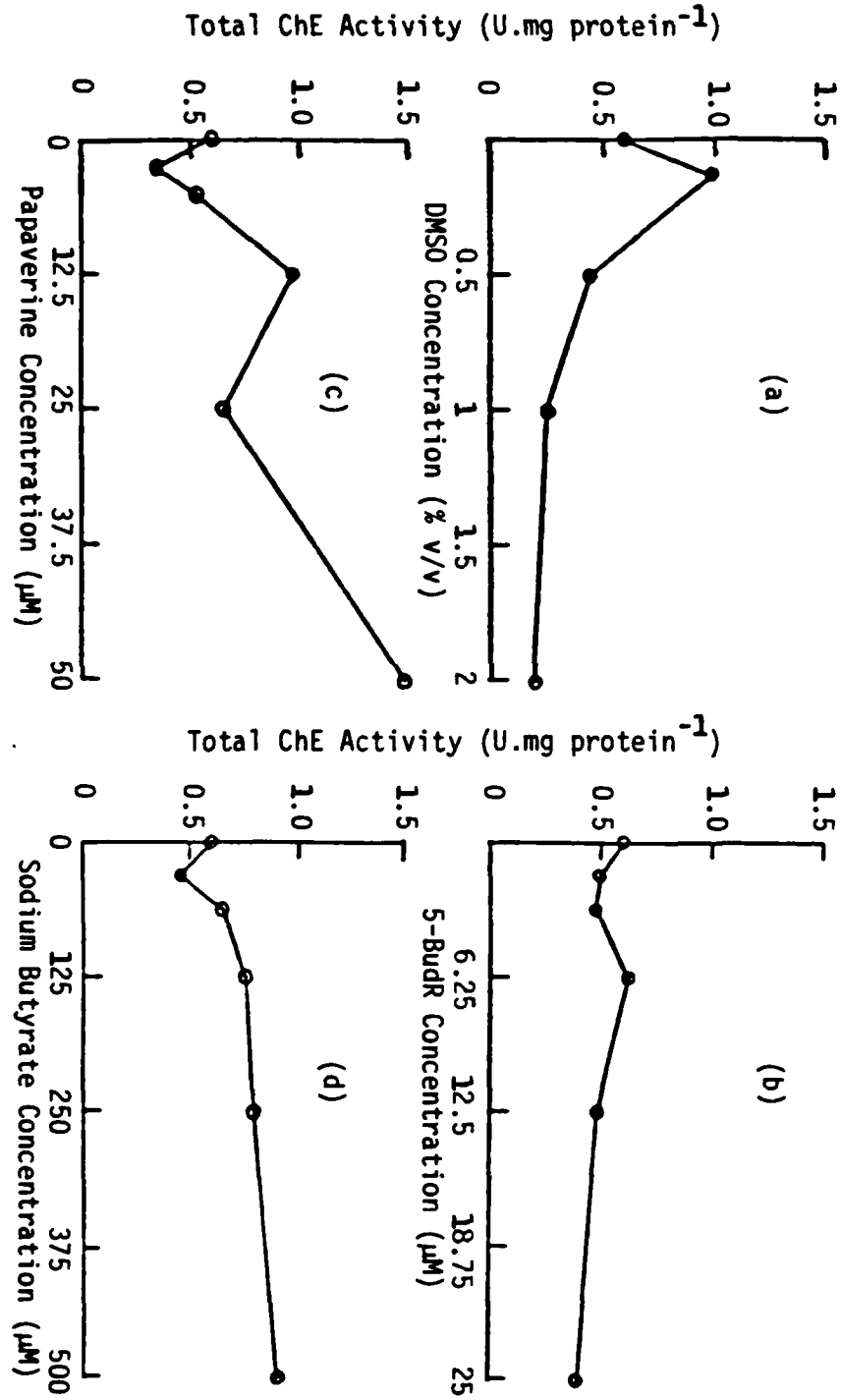


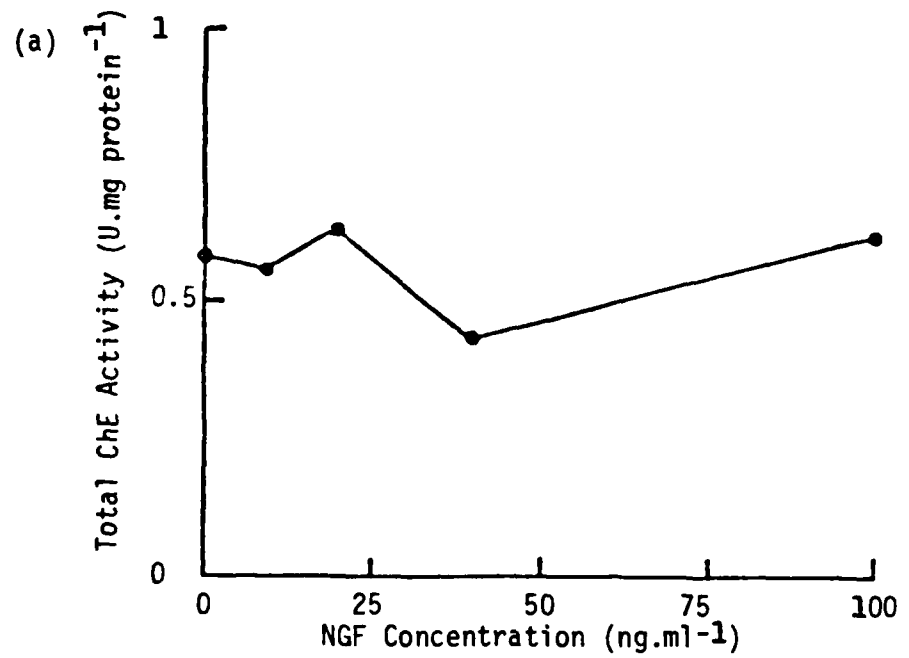
FIGURE 7

Effect of Nerve Growth Factor and 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) on Cholinesterases in IMR-32 Cells

(a) Nerve Growth Factor (NGF) (2.25 S form)
(b) 12-O-tetradecanoyl-phorbol-13-acetate (TPA)

FIGURE 7 (continued)

Effect of Nerve Growth Factor and 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) on Cholinesterases in IMR-32 Cells



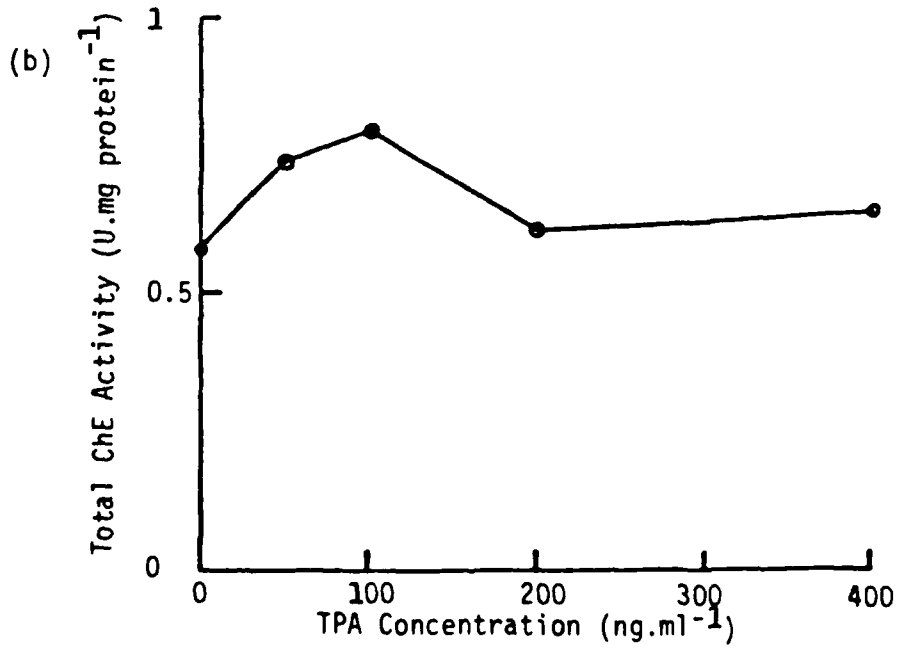


FIGURE 8

AChE Levels in IMR-32 Cells After 10 Days in Culture

Confluent IMR-32 cells in Linbro 24-well plates were incubated in medium containing various levels of inducer for 10 days with fresh medium plus inducer every 2-3 days. The cells were harvested, washed and assayed as described in the methodology section. The assay mix containing 28.4 μM lysivane.

(a) Sodium butyrate
(b) 5'-Bromodeoxyuridine (5-BrdU)
(c) Dimethylsulphoxide (DMSO)

FIGURE 8 (continued)

AChE Levels in IMR-32 Cells After 10 Days in Culture

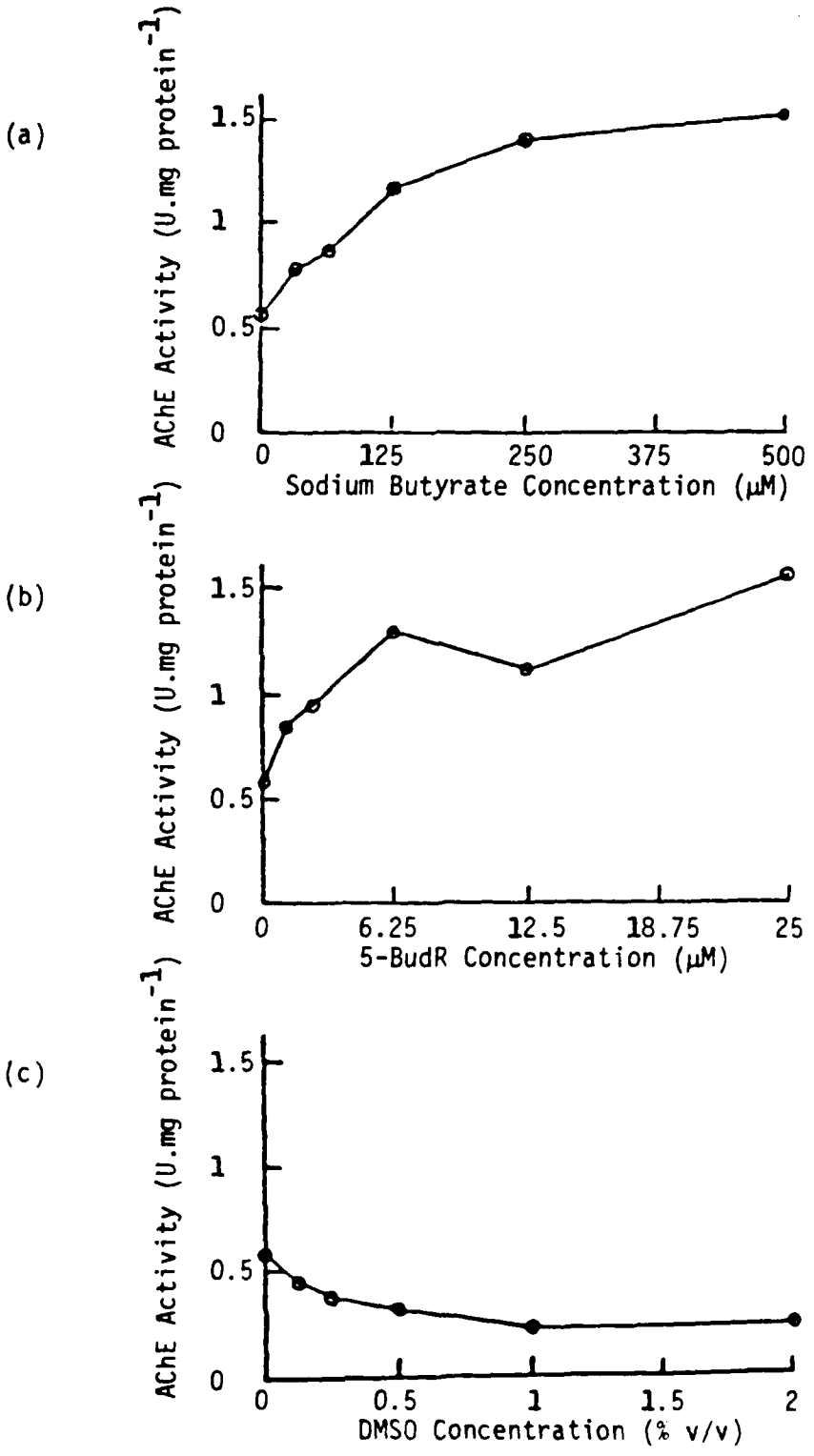


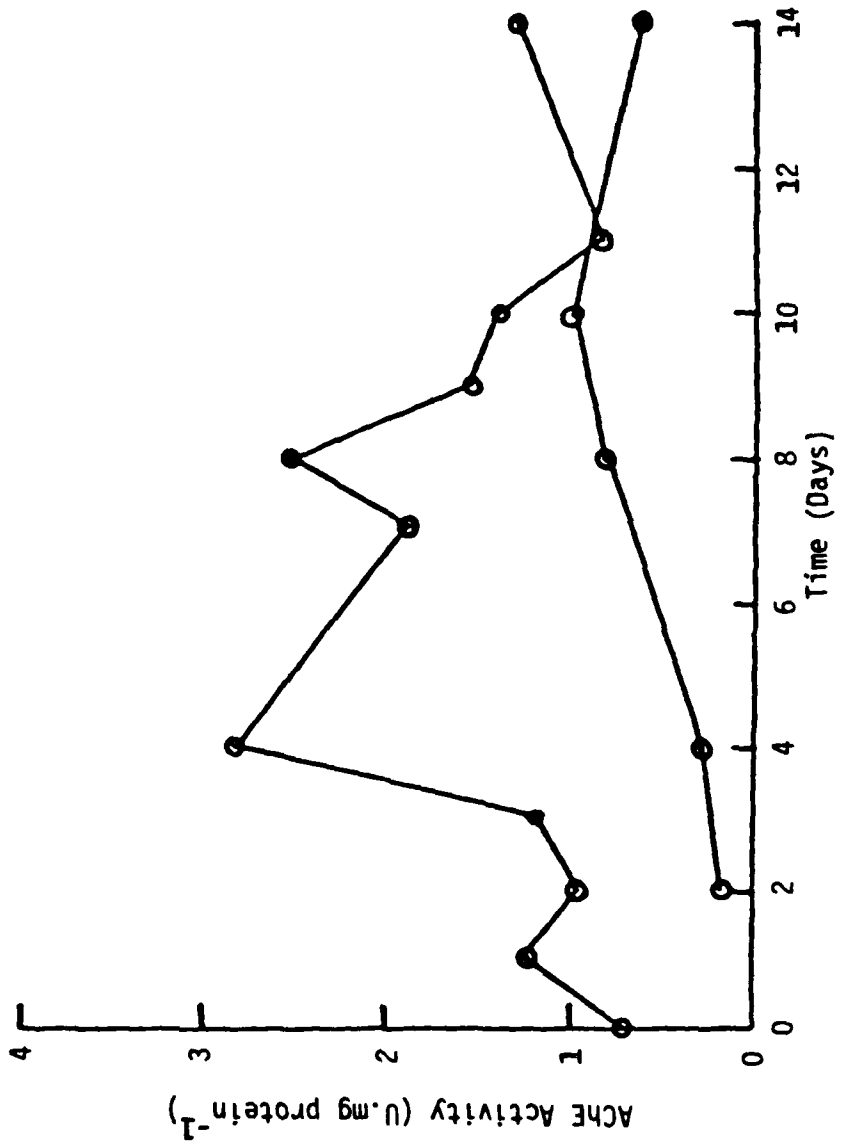
FIGURE 9

Time Course of the Action of Sodium Butyrate on ChE Levels in Neuroblastoma Cells

Confluent IMR-32 cells in a Linbro 24-well plate were induced by 0.625 mM sodium butyrate on day 0 and maintained on this supplemented medium for a total of 14 days with fresh medium every 2-3 days. At regular intervals (usually daily), 2 wells from the plate were harvested, washed and stored in the vapour phase of a liquid N_2 deep freeze. Control cells were treated identically except that no butyrate was added to the medium. Cells were assayed in a 96-well microtitre plate and lysivane was included at 28.4 µM in all assays.

FIGURE 9

Time Course of the Action of Sodium Butyrate on ChE Levels in Neuroblastoma Cells



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