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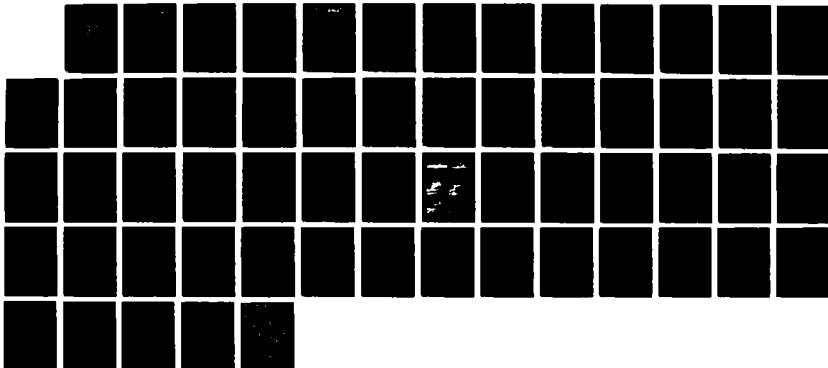
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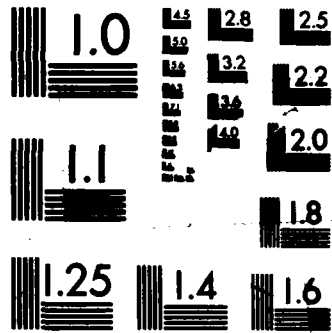
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Report No. 3

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**MOLECULAR CLONING OF THE HUMAN GENES(S)  
DIRECTING THE SYNTHESIS OF NERVOUS SYSTEM CHOLINESTERASES (U)**

ANNUAL REPORT

Submitted by  
HERMONA SOREQ, Ph.D.

DECEMBER 1985

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

Contract No. DAMD17-85-C-5025

Department of Neurobiology,  
The Weizmann Institute of Science,  
Rehovot 76100, Israel

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SUMMARY

The toxic effects of organophosphorous poisons are mainly attributed to their blocking of the catalytic activity of acetylcholinesterase (AChE or acetylcholine acetyl hydrolase, EC 3.1.1.7) within neuromuscular junctions and cholinergic synapses. However, ample evidence suggests that a considerable part of the human enzyme is involved in functions other than cholinergic transmission. In addition, human AChE is a highly polymorphic protein with an intricate regulation pattern, and it is unknown yet what steps are included in the pathway for AChE biosynthesis and/or regulation. To tackle this complicated research problem, it is important to distinguish between the different forms of AChE. However, it is not clear yet whether these forms are produced from discrete genes or by post-transcriptional and post-translational processing. In addition, the amino acid sequence of the various AChE forms has not been revealed. This issue is being approached in our research group by simultaneous experiments at the levels of DNA, of mRNA, and of the active brain enzyme. In the following report, we present evidence suggesting that both post-transcriptional and post-translational regulation events contribute to the complex expression pattern of AChE in the developing and the mature human brain.

(1) cDNA clones of human fetal brain and liver origins were selected out of lambda-gt10 libraries by synthetic oligodeoxynucleotides coding for the organophosphate binding site of cholinesterases (ChEs) and are being characterized in detail, sequenced and expressed to yield AChE-like proteins within bacterial cells.

(2) Human genomic DNA fragments were selected out of Charon 4a library by synthetic oligodeoxynucleotides coding for the N-terminal peptide of erythrocyte AChE and are being studied.

(3) The bioassay which we previously developed for the translation of ChE mRNA has been improved and refined for studying the post-translational processing of nascent AChE molecules.

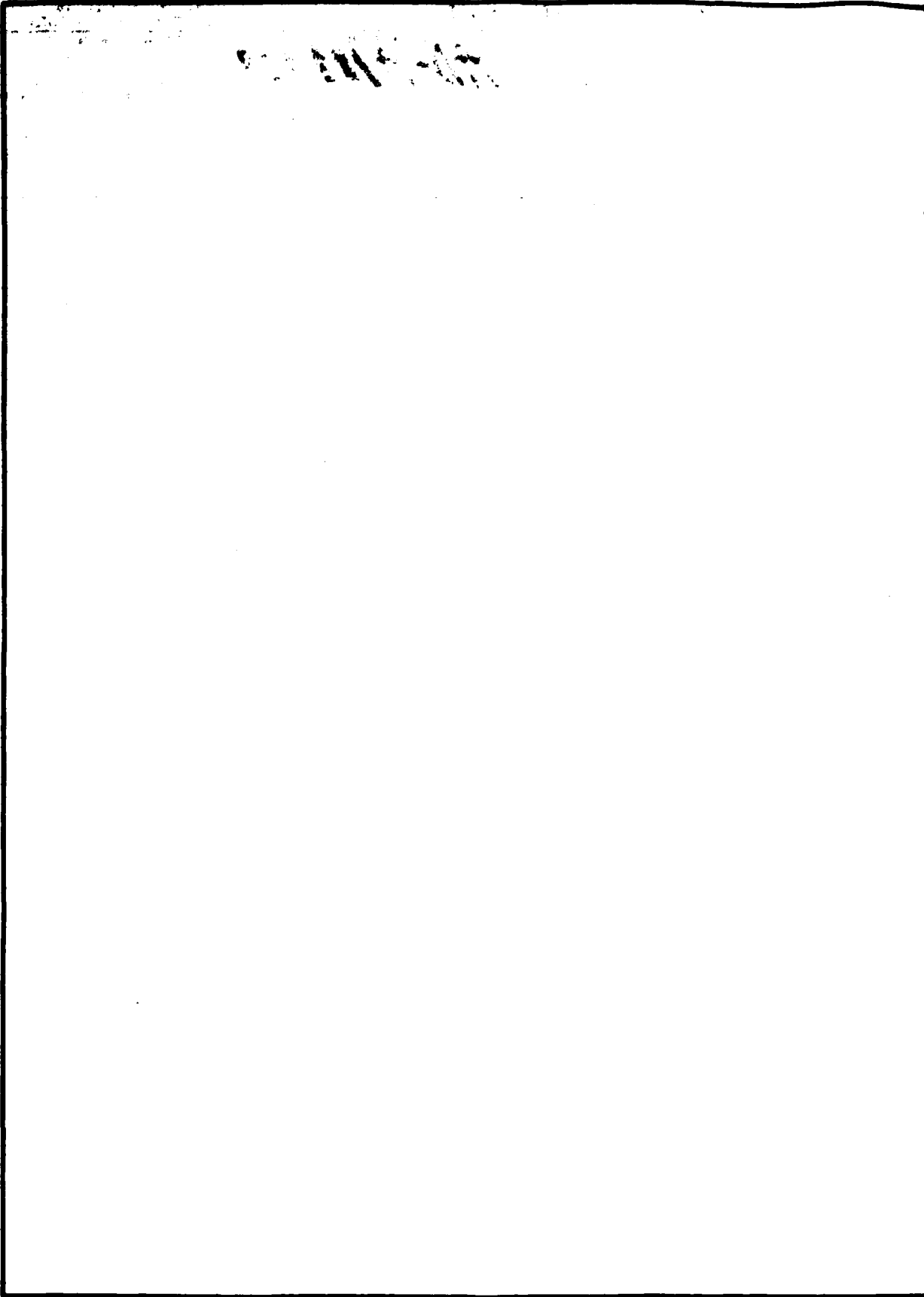
(4) The polymorphism of fetal brain AChE was studied in discrete brain regions, aiming to correlate at a later stage particular AChE DNA sequences to their descendant mRNA species and protein products.

(5) Autoimmune antibodies against human muscle AChE were discovered and characterized in a case of severe neuromuscular dysfunction. It is not clear yet whether AChE was the primary target for the immunological reaction or whether the anti-AChE antibodies are anti-idiotypes to natural antibodies directed against organophosphorous insecticides. This issue is being examined. In both cases, however, our findings imply that antibodies against muscle membrane AChE may play a major role in eliciting neuromuscular dysfunction, neuropathy, and muscle atrophy.

Altogether, this research aims to reveal how the neurotransmitter hydrolyzing enzyme AChE can be produced in ample quantities from cloned human DNA to be employed for further analyses and for therapeutic purposes against organophosphate poisoning.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)  (cDNA) and genomic DNA clones coding for human cholinesterase (ChE) were selected from phage DNA libraries by synthetic oligodeoxynucleotides coding for the organophosphate binding site of ChE and were characterized, sequenced and expres- sed within bacterial cells to yield AChE-like proteins. The <u>Xenopus oocytes</u> microinjection bioassay for translation of ChEmRNA has been improved and refined for studying the post-translational processing of nascent ChE molecules. ChE polymorphism and auto-immune anti-ChE antibodies were studied. <i>Keywords:</i>			

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FOREWORD

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

2. The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplement.

3. For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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## 1. STATEMENT OF THE PROBLEM

### 1.1 The Basic Research Problem

The route to the biosynthesis and regulation of brain cholinesterases (ChEs) is unknown yet, which leaves open several questions of considerable importance:

1. Are the various ChE forms produced from discrete genes, or is their biosynthesis regulated by post-transcriptional and/or post-translational processing?

2. Are the multisubunit forms of brain acetylcholinesterase (AChE) composed of similar polypeptides with identical amino acid sequences, or of different polypeptides with distinct domains which distinguish particular subunits from others?

3. What is the biosynthetic origin of ChEs in different subcellular locations within the brain tissue? Was the soluble fraction of brain AChE originally destined to be cytoplasmic and/or secreted, or has it been produced from membrane-associated amphipathic form(s) of the enzyme via enzymatic cleavage of their hydrophobic domains?

4. What are the biological roles of AChE in noncholinergic brain cells and of butyrylcholinesterase (pseudo-ChE or acylcholine acylhydrolase, EC 3.1.1.8) in the brain in general?

5. Is there an interrelationship between the regulation of AChE and of the muscarinic acetylcholine receptor in the mammalian brain?

### 1.2 Significance of Cholinesterase Research for Clinically Oriented Issues

In addition to its importance as a subject for basic research, the study of ChEs bears several implications for clinical purposes:

1. Genetic deficiencies in serum pseudo-ChE in humans (up to 0.05% of homozygotes in the caucasian population) result in prolonged apnea following the use of succinylcholine during anesthesia (1) This clinical complication could be diagnosed by a rapid method to detect such deficiencies or prevented by injecting, as a scavenger, active human pseudo-ChE, which would degrade the excess of drug. Purified human ChE can be used similarly to scavenge organophosphorous poisons, which act by blocking AChE (2) in cases of intoxication with these compounds (3). The use of synthetic preparations of the pure enzyme from cloned human genes would considerably decrease the expenses involved and promises to widen the use of this approach.

2. Tetrameric AChE is secreted into the amniotic fluid in cases of neural tube closure defects (4) but its detection is rather laborious (5). A simple radioimmunoassay, using specific antibodies for the tetrameric form of AChE, would be highly valuable for the routine detection of such defects.

3. The tetrameric form of AChE is selectively lost from particular regions in the brains of patients suffering from senile dementia of the

Alzheimer type (7). Antibodies directed against this form of the enzyme could hence be employed to develop a method for the clinical diagnosis of this disease.

Thus both for basic research and multiple clinical purposes, AChE is clearly an important protein to be studied extensively and, preferably, at all levels of the pathway for gene expression. In this report, we describe the simultaneous use of in vitro and in ovo translation systems for the synthesis of ChEs from human brain mRNA. When combined with several biochemical and immunochemical approaches for the characterization of the nascent ChEs produced, and with the use of molecularly cloned ChEs DNA sequences, these experiments strongly suggest that both post-transcriptional and post-translational processing events play important roles in the production of the polymorphic forms of AChE within the developing and the mature human brain.

### 1.3 Summary of the Research Done Under Previous Contract, DAMD 17-82-C-2145

In the research done under Contract DAMD17-82-C-2145, we studied the biosynthesis of mammalian ChEs simultaneously at three levels of gene expression.

1) At the level of DNA: A genomic fragment of human DNA directly related to AChE biosynthesis was isolated for the first time by its homology to Drosophila DNA from the vicinity of the Ace locus, which controls AChE expression.

To focus on the domain which codes for the active site in ChE genes, synthetic oligodeoxynucleotides were prepared according to the recently published consensus peptide sequence from the organophosphate binding region of ChEs. Two lambda-gt11-cloned complementary DNA chains from the AChE-rich mouse neuroblastoma cells were selected by their hybridization with these probes. Both were expressed in their host bacteria to yield AChE-like fusion proteins, immunoreactive with anti-AChE antibodies.

2) At the level of mRNA: We have developed and refined a novel bioassay to monitor the level of ChE mRNA using microinjected Xenopus oocytes, where catalytically active and immunoprecipitable ChEs are produced from such mRNAs. Using this sensitive assay, we studied the post-translational processing of nascent AChE molecules. Thus, we have shown that ChE mRNAs are polymorphic and are expressed in a tissue- and cell-type specific manner. We have also identified ChE DNA sequences by their ability to hybrid-select oocyte-translatable ChE mRNA chains.

3) At the level of protein: The polymorphic human ChEs were studied in various brain tumors and in discrete regions of the developing fetal brain. The parameters determined included sensitivity to selective inhibitors, sedimentation properties in sucrose gradients, interaction with nonionic detergents and other biochemical properties.

## 2. BACKGROUND

### 2.1 Regulation and Properties of Mammalian Cholinesterases: Research Importance, Advantages and Difficulties

#### 2.1.1. Acetylcholinesterase as a major cholinergic constituent

Within cholinergic synapses and neuromuscular junctions, the neurotransmitter acetylcholine is rapidly hydrolyzed by the serine esterase AChE (acetylcholine acetyl hydrolase, EC 3.1.1.7). AChE appears in numerous cell types, tissues, and organisms (8), albeit in extremely low concentrations and in multiple molecular forms (9). The various ChE forms differ in their substrate specificity and sensitivity to selective inhibitors (10), in their glycosylation patterns (11), in their hydrophobicity (12), and in their sedimentation properties (13). Genetic linkage analysis suggests the existence of allelic polymorphism for the human genes coding for particular ChEs (8), and molecular cloning studies indicate that various human ChEs are produced from cross-homologous DNA and mRNA sequences (14-16).

#### 2.1.2. Problems to be solved in the study of cholinesterases

In spite of the effort invested in the study of ChEs for many years by numerous research groups, it is not yet clear whether the different ChE forms are produced from different genes, or whether their biosynthesis is regulated by post-transcriptional and/or post-translational processing events. Furthermore, neither the amino acid sequence of ChEs nor the detailed structure of their catalytic site has yet been revealed. The biological role of these enzymes in tissues other than nerve or muscle is completely unknown, although several functions have been suggested (17,19). One of these suggestions is based on the demonstration of peptidergic activity for purified ChEs (20), which implies that these enzymes are biheaded proteins, with two unrelated active centers and peptide domains which are partially homologous to regions in other proteases and/or esterases.

Although AChE activity can be detected with an extremely high sensitivity (21-24), very little is known yet about its biological role(s) and mode of regulation. Even in a clearly cholinergic tissue such as the neuromuscular junction, where AChE does hydrolyze acetylcholine, it is not clear how its activity is controlled. Muscle denervation results in the disappearance of the tailed, asymmetric forms of AChE from the neuromuscular junction (8). This suggests the existence of a close relationship between the neuronal signal, the binding of acetylcholine to its receptor, and the way in which the expression of AChE is regulated. However, it is completely unknown which level of gene expression is affected by the neuronal signal so as to alter the expression of AChE or what detailed molecular mechanism leads to such alterations.

#### 2.1.3. Regulation of muscarinic binding sites and acetylcholinesterase in the human brain

Cholinergic mechanisms in the mature human brain may be interrupted both by ligands binding to muscarinic cholinergic receptors (mAChR) and by inhibitors of ChEs. Multiple brain functions, such as learning, memory, and even vegetative functions, are impaired by these agents (25), and

several neuronal disorders related to cholinergic mechanisms (for example, Huntington's Chorea) have been shown to be accompanied by decreases in mAChR (26) and in ChEs (27). This suggests that both mAChR and ChEs are essential for a wide range of brain functions in humans (see, for example, 28,29; and refs. therein); it is therefore of particular interest to study the regulation of mAChR and of ChEs in the human brain, and find out whether they are interrelated.

The expression of mAChR and of ChEs in the mature human brain has been studied by both biochemical and histochemical approaches, but very little is known about their regulation in the fetal brain. Binding of labeled muscarinic ligands to extracts (30) and sections (for a review, see 31) of post-mortem brain has revealed that mAChR are distributed throughout the brain, being mainly concentrated in areas known to be cholinergic (see, for example, 32). Hydrolysis of acetylcholine by brain extracts (33) and histochemical staining of brain sections by thiocholine complexes (34) showed that in the postnatal mammalian brain, ChEs as well are mainly localized in areas in which cholinergic neurons are concentrated (35). However, both ChEs and mAChR have also been detected in mammalian brain areas where the major neurotransmission is clearly non-cholinergic, such as the cerebellum (36-39), as well as in primary brain tumors of glial origin, which do not contain neurons at all (40,41). Thus, the expression of these cholinergic elements is, apparently, not limited to cholinergic or cholinceptive neurons.

The presence of mAChR in various cells and tissues has invariably been correlated to the regulation of cellular processes by acetylcholine (for a review see 42). The onset of their appearance in the fetal brain would hence be expected to be related to the development of cholinergic functions. In contrast, ChEs have been implicated with various roles unrelated to the hydrolysis of acetylcholine (for recent reviews, see 17-19), particularly in embryonic tissues (43-44). This implies that the two macromolecules should not necessarily be coregulated in the fetal brain. Since different human brain regions develop at various rates and gestational times (for recent studies, see 45,46), this issue should best be investigated in discrete brain regions and at several periods during fetal development and neuronal migration (48). During the period covered by the present report, we showed that the expression of mAChR, but not of ChEs, appears to be related to the development of cholinergic properties in various areas of the fetal human brain, and that transient, unrelated enhancement of ChE expression takes place at different time periods during the development of these brain regions.

#### 2.1.4. Biosynthesis of cholinesterases in human brain tissues

The ability of the brain to synthesize plasma proteins in general, and ChEs in particular, has recently been examined by various approaches. Certain plasma proteins have been demonstrated within cells in the fetal brain of several species including the human (47). These proteins include alpha-fetoprotein, albumin and transferrin, but there does not seem to have been any previous systematic attempt to discover how many of the wide range of known plasma proteins are present in the developing brain or whether the synthesis of particular proteins occurs at specific stages of brain development. The developing brain may turn out to contain as diverse a range of plasma proteins as it appears to contain peptides, many of which were originally found elsewhere in the body (49). It has been a

matter of some dispute whether the plasma proteins in fetal brain originate by uptake from the blood or by synthesis within the brain (see 50). At some stages of brain development there is now evidence for uptake of some plasma proteins from the cerebrospinal fluid into ventricular zone cells lining the cerebral ventricles (50-52). Initial studies of brain synthesis of plasma proteins involved methods that were relatively insensitive and the results were negative (e.g. 53). However, there now seems to be evidence for both amino acid incorporation into some plasma proteins (50,54) and for the presence of mRNA for angiotensinogen (55) and for transferrin (56) in adult rat brain.

To reveal whether the synthesis of plasma proteins takes place in the human brain, we combined the use of mRNA translation, crossed immunoelectrophoresis, and autoradiography. This approach gives a method of high sensitivity and low background. It has the advantage over immunohistochemical studies of using a polyvalent antiserum, so that a wide range of plasma proteins can be studied simultaneously. We have recently applied this approach to detect the biosynthesis of several plasma proteins in the developing rat brain (57): We used it to study the biosynthesis of plasma proteins in the developing human brain, and in some human cerebral tumors. The results show that several plasma protein-like polypeptides are synthesized both by normal fetal and adult brain and by tumor tissues. These observations confirm and extend previous immunocytochemical studies, in which the distribution of several plasma proteins was examined in the human fetal brain (58) and offer a new and powerful approach to study the biosynthesis and regulation of particular proteins in the human brain.

#### 2.1.5. The putative role of antibodies against acetylcholinesterase in neuromuscular dysfunction

In the neuromuscular junction, various types of conduction defects are known to induce muscle weakness. These include the impaired release of the neurotransmitter acetylcholine, inducing the Lambert-Eaton syndrome (59) and the disturbed interaction between acetylcholine and the nicotinic acetylcholine receptor occurring in myasthenia gravis (60). Severe muscular weakness can also be caused by excessive stimulation, resulting from the accumulation of acetylcholine within the synaptic cleft. This can happen because of inhibition of AChE, as is the case in organophosphate intoxication (61). Inhibition of AChE in neuromuscular junctions profoundly modifies neuromuscular transmission (7), as was shown by electrophysiological analyses (62), by studies of the muscle response to nerve stimulation (63) and by observations on spontaneous muscular activity *in vivo* (64). In principle, antibodies blocking the activity of AChE in neuromuscular junctions should have similar effects, distinguishable from those observed in the Lambert-Eaton syndrome and in myasthenia gravis.

Antibodies raised against AChE from particular tissue sources and species were reported to cross-react with AChE from other tissues and species (65,66). However, there were also reports on anti-AChE antibodies which distinguish between the detergent-extractable and buffer-soluble forms of from a single tissue (67). Altogether, this information implies that different forms of ChEs share common domains but may also contain distinct regions, specific to particular forms. In this report, we present evidence of the occurrence of anti-AChE antibodies with preferential affinity to muscle membrane AChE in the serum of a patient manifesting severe diffuse muscular weakness. Our findings indicate that the



autoimmune response to AChE played a major role in the etiology and the pathophysiology of his neuromuscular dysfunction.

### 3. THE EXPERIMENTAL APPROACHES

#### 3.1 Human Brain Tissues and mRNA Preparations

The experiments covered in this report were carried out using tissue extracts and purified poly(A)-containing RNA from several tissue sources of human brain origin. These include primary brain tumors of glial and meningeal origin (for detailed description of these tumor types see 40,68,69), discrete regions from fetal human brain (13), and dissected tissues from mature human brain (39,40). Several precautions were taken to ensure the intactness of the ChE mRNAs extracted from these tissues (14). To determine the migration properties of these mRNAs, poly(A)-containing RNA was denatured with dimethylsulfoxide and centrifuged in continuous sucrose gradients (14,69). Human epidermoid carcinoma, in which the levels of ChEs (38) and of ChE mRNA (69) are considerably lower than in the brain tissues, served as control.

#### 3.2 Translation Systems Employed for Cholinesterase Biosynthesis

The biosynthesis of human brain ChEs was examined using two different translation systems. In the *in vitro* system of rabbit reticulocyte lysate (70), poly(A)-containing ChE mRNA from human brain directs the incorporation of [<sup>35</sup>S]methionine into newly synthesized nascent polypeptides, which remain unprocessed and inactive (for detailed methodology, see 71). In the *in ovo* system of microinjected *Xenopus* oocytes (72), the <sup>35</sup>S-labeled ChE polypeptides also undergo post-translational processing and correct compartmentalization to yield the catalytically active enzyme in its natural subcellular localization (15,69,73).

#### 3.3. Immunochemical Analyzes of Newly Synthesized Cholinesterases

<sup>35</sup>S-labeled newly synthesized ChEs were purified and characterized by two independent immunochemical approaches. They were immunoprecipitated, following preadsorption of nonspecific precipitates, with the aid of anti-AChE monoclonal antibodies elicited against human erythrocyte AChE (AE-2, 65) see 15). Alternatively, they were identified by crossed immunoelectrophoresis (57), using rabbit antisera against human plasma proteins to precipitate pseudo-ChE, and against human erythrocyte membranes or against mammalian brain AChE to precipitate AChE (75). In both cases, identification was performed by autoradiography, and irrelevant antibodies served as controls.

Antisera against the following human plasma proteins were obtained from Dakopatts (Denmark): albumin, alpha-fetoprotein, alpha<sub>1</sub>-antitrypsin, inter alpha<sub>1</sub>-trypsin inhibitor, alpha<sub>2</sub>-macroglobulin, alpha<sub>2</sub>-plasminogen inhibitor, ceruloplasmin, fibrinogen, Gc globulin, haptoglobin, hemopexin, plasminogen, transferrin, and beta-lipoprotein. Antisera to alpha<sub>1</sub> easily precipitable protein and to alpha<sub>2</sub>-HS (Heremans and Schmid) were obtained from Hoechst (West Germany). Anti-rat AChE was kindly provided by Drs. M. Vigny and J. Massoulie; it has been shown to cross-react with human brain AChE (66). In order to identify individual proteins, the intermediate gel in the crossed immunoelectrophoresis plates contained one of the above antisera, elicited against a single purified plasma protein, and the top gel contained anti-adult serum. Two microliters of translation product and 5 microliters of carrier unlabelled plasma proteins (a mixture of human fetal plasma from fetuses of 18 and 30 weeks' gestation and adult

plasma, mixed 1:1:1 and diluted 1:20 in phosphate-buffered saline (PBS)) were placed in a well punched in a strip of buffered agarose gel; a voltage of 10 V/cm was applied for 60 min (first dimension). The gel strip was placed against antibody-containing gel and a voltage of 2 V/cm was applied overnight (second dimension) in a direction at right angles to that of the first dimension electrophoresis. The gel used in both dimensions was 1% (w/v) agarose (Litex) in Tris-barbitone buffer at pH 8.6. Plastic (Gel bond, Marine Colloids, Philadelphia, U.S.) or glass (10 x 10 cm) plates were used. After pressing and washing in the standard manner (75), the plates were dried and processed for autoradiography as described below. ChE activity was detected on dried unstained plates using the thiocholine method of Karnovsky and Roots (21).

Plates from each translation experiment were exposed together for several periods of time and the various autoradiograms of each plate were compared. Autoradiograms were then chosen, in which the intensity of all of the  $^{35}\text{S}$ -labeled immunoprecipitation curves continued to increase with longer exposures, indicating that within each of these autoradiograms and also between autoradiograms of a particular set, these curves reflect the relative amount of [ $^{35}\text{S}$ ]methionine incorporation into specific immunoprecipitable plasma proteins.

### 3.4 Cholinesterase Assays

Measurements of ChE activity were performed by hydrolysis of [ $^3\text{H}$ ]acetylcholine, according to Johnson and Russell (23) and in the presence of protease inhibitors, as detailed elsewhere (13). Alternatively, the hydrolysis of acetylthiocholine or butyrylthiocholine was measured spectrophotometrically by the technique of Ellman et al. (22) and as detailed (40). The selective inhibitors tetraisopropyl pyrophosphoramidate (iso-OMPA) and 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284C51) were used specifically to block the activities of pseudo-ChE and of AChE, respectively (10). Extraction with low salt buffer, or with salt + detergent, was employed to separate the hydrophilic fraction of active ChEs from the amphipathic part (13), and centrifugation in continuous sucrose gradients was used to separate the various molecular forms of the enzyme (40).

### 3.5 Oocyte Injection.

Mature (stage 6) oocytes from *Xenopus laevis* were injected with poly(A)-containing RNA together with lyophilized [ $^{35}\text{S}$ ]methionine. The procedure was as described by Burmeister et al. (76). Incubation medium, containing secreted protein, was separated. Oocytes were homogenized as described previously (69); the clear homogenates and incubation media were stored separately at  $-70^\circ\text{C}$  until they were assayed.

### 3.6 Liquid Scintillation

Trichloroacetic acid (TCA) precipitation of [ $^{35}\text{S}$ ]methionine-labeled proteins was carried out as described previously (77) with GFC/25 filters (Tamar, Rehovot). Filters were dried and scintillation fluid (4 ml of 90% xylene and 10% "xylofluor") was then added. The filters were counted in a Packard Model 3390 Tri-Carb liquid scintillation spectrometer.

### 3.7 The Synthesis and Use of Oligodeoxynucleotide Probes

Oligodeoxynucleotide probes were synthesized manually, essentially as described in detail in our Final Report for Contract DAMD17-82-C-2145 (January 1985). The probes synthesized included: (a) a series of synthetic oligodeoxynucleotides (OPSYN) all complementary to the organophosphate binding site of ChEs; (b) a series of second OPSYN probes, complementary to an additional hexapeptide from the active site peptide, as published by Lockridge (78); (c) two N-terminal probes, according to the different peptide sequences which were determined for the N-terminal region of human erythrocyte AChE by T. August and coworkers and by T. Rosenberry (79); and (d) a C-terminal probe, following the sequence published by Lockridge (78) for human serum pseudo-ChE. Further details regarding these probes and their use for screening of DNA libraries are included in the Results section (4.1.1.1).

### 3.8 Increase of Hybridization Selectivity by $(\text{CH}_3)_4\text{NCl}$ Washes

OPSYN probes were end-labeled by polynucleotide kinase as detailed in our last report (80). Hybridization and washing conditions were similar to the above, with the exception that 3 M tetramethylammonium chloride ( $(\text{CH}_3)_4\text{NCl}$ ),  $53^\circ\text{C}$ ) was employed to verify the stringency of hybridization in a base composition-independent manner (81). For this purpose, filters were first rinsed at  $37^\circ\text{C}$  with 3.0 M  $(\text{CH}_3)_4\text{NCl}$  solution, containing 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and sodium dodecyl sulfate (SDS) at 1 mg/ml.  $(\text{CH}_3)_4\text{NCl}$  was purchased from Aldrich (Milwaukee, WI), and made up as a nominal 5 M stock solution. Since  $(\text{CH}_3)_4\text{NCl}$  is hygroscopic, the actual molar concentration (C) was routinely determined from the refractive index (n) by the formula  $c = (n - 1.221)/0.018$  (81). The precise concentration was important, since Td varies with the  $(\text{CH}_3)_4\text{NCl}$  concentration. This rinse is necessary to substantially remove the NaCl/Cit solution and prevent its competition with  $(\text{CH}_3)_4\text{N}^+$  binding (81). Rinsed filters were washed twice in a shaking bath for 20 min with the same  $(\text{CH}_3)_4\text{NCl}$  solution at  $53^\circ\text{C} + 1^\circ\text{C}$ . Under these washing conditions, only 17- and perhaps some 16 base-pair matches are allowed. In this case, the expected number of random clones is only five for a complexity of  $1 \times 10^6$  in base pairs (or  $1 \times 10^5$  different clones of an average length of 1000 base pairs, a fair assumption for fetal brain) as probed with a pool of 128, 17-mers (80). In other words, our lambda-gt10 cDNA library from fetal brain origin should contain about six sequences with 16-17 base-pair matches to each of the OPSYN probes employed, and of these, only one or two should be the correct true positive. That this is the case is shown in the Results section (4.1.2.3.).

### 3.9 Schedule of Experiments Taken to Characterize Positive Clones

It should be noted that positively hybridizing clones, even when the hybridization signal is stable to 3 M  $(\text{CH}_3)_4\text{NCl}$  at  $53^\circ\text{C}$ , do not necessarily contain the pursued DNA sequence. First, they may contain only 16 of the 17 examined nucleotide bases (81); second, they may represent another cDNA or a genomic DNA species which happens to contain the searched-for hexapeptide (see other sections for further details regarding this issue). Finally, the structural properties of the DNA stretch also affect the stability of hybridization (see, for example, clone FBC4, below). Altogether, a positive clone selected under the conditions we used was calculated to have about a 15% chance to contain an AChE cDNA insert. To find out whether this is the case, we need to carry out several experiments in a particular order. During the period covered by this report, we

developed a general plan for such experiments, which is designed both to save time in case of a false positive and to gain the maximal amount of information regarding the selected clone in case it is a true one. Scheme I presents the schedule of such experiments for the detailed characterization of a positively hybridizing DNA clone.

Scheme I.Schedule of experiments for the detailed characterization  
of a positively hybridizing DNA clones

<u>Step No.</u>	<u>Research Material</u>	<u>Experiment Carried Out</u>
(1)	Confirmed positive clone, which hybridises with the oligodeoxynucleotide probe to yield a signal stable to 3 M $(\text{CH}_3)_4\text{NCl}$ at 53°C	(1)a Pick plaque, amplify, prepare DNA polyethylene glycol (precipitation, CsCl gradient, phenol extraction)
(2)	Phage DNA containing the hybridizing insert	(2)a Restrict with enzymes which cut the lambda-gt10 multi-linker sequence (EcoRI, Xho, Sst, SalI) to separate the entire insert of cDNA clones  b Restrict with enzymes which cut the M13 multi-linker sequence (Alu, RsaI, HaeIII, Sau3A, and PvuII) as a prerequisite to sequencing
(3)	Enzymatically restricted phage DNA + insert	(3)a Run an analytical agarose gel, blot onto nitrocellulose, hybridize with the employed labeled oligodeoxynucleotide probe, wash with $(\text{CH}_3)_4\text{NCl}$ 3 M, 65°C b Test for hybridization with an additional probe to confirm the identity of the examined insert and find out its size (for example, does it include the N-terminal sequence?)
(4)	Positively hybridizing restricted DNA fragments	(4)a Run a preparative gel, electroelute fragment, and insert into: b M13 single-stranded vectors for sequencing from both orientations c pEX bacterial expression vectors for production of protein in all three

## translation frames

- (5) M13 inserted positive
- (5)a Find out whether it contains the sequence coding for the pursued hexapeptide and, if possible, additional confirmatory amino acid sequences
- b If so, return to step (3) and insert all other insert fragments into M13 for sequencing. If not, end the experiment and proceed with another positive
- (6) pEX-inserted cDNA insert (test only for cDNA clones in which step (5) is positive, since this experiment by itself is not an ultimate proof)
- (6)a Induce plasmid expression, immunoblot versus anti-AChE antibodies
- b If positive test for binding of diisopropyl fluorophosphate (DFP) and for hydrolysis of [<sup>3</sup>H]-acetylcholine by the bacterial cDNA product.
- (7) Sequenced and expressible AChE DNA insert

### 3.10 Immunoblotting of Acetylcholinesterase from Crude Tissue Extracts

To immunoblot AChE from crude tissue extracts, various fetal tissues were homogenized in 5 volumes/weight of extraction buffer. This contained 1.2 M NaCl, 1% Triton X-100, 0.05 M Tris-HCl, pH 8.0, and 0.01 M EDTA. Homogenization in a Teflon-glass homogenizer was for 20 sec at room temperature and 1000 rpm, using a Heidolf motor. Homogenates were further incubated for 15 min at 37°C to ensure the complete release of AChE from membranes and were then dialyzed overnight against 1 liter of 0.10 M Tris-HCl, pH 8.0, and 0.1% Triton X-100. Protein concentration was determined in dialyzed homogenates, and ChE activities were radioactively determined prior to dialysis and as previously detailed (13). Samples of these homogenates were electrophoretically separated on 5-15% polyacrylamide/SDS gels, 3 hr at 100 V together with prestained molecular weight markers (Sigma, St. Louis, MO). Gels were electroblotted overnight at 50 mA onto nitrocellulose sheets, using 20 mM Tris-HCl, 150 mM glycine, 20% methanol, and 0.05% SDS, pH 8.3, as transfer buffer. The nitrocellulose was immediately transferred for shaking for 30' min at 37°C in PBS containing 5% non-fat dry milk powder (Carnation). It was then incubated for 90 min and shaken in the same solution, containing anti-AChE antiserum at 1:1000 dilution, and washed successively in 5% milk/PBS (5 min), in milk/PBS containing 0.05% NP-40 (10 min x 2), and in milk/PBS (5 min), 200 ml each. Incubation with second antibody (Donkey F(ab)<sub>2</sub> anti-rabbit immunoglobulin G (IgG), coupled to horseradish peroxidase (Amersham), 1:50 in milk/PBS) was for 90-min shake at room temperature. This was followed by

the same order of washes as before. Washed filters were rinsed for 2 min in 100 mM Tris-HCl, pH 7.6, prewarmed to 37°C, and then transferred to peroxidase substrate solution (25 mg of diaminobenzidine (Aldrich, Milwaukee, WI) dissolved in 50 ml of warm 0.1 M Tris-HCl, pH 7.6, for 20 min at 37°C, to which 150 ul of 3% H<sub>2</sub>O<sub>2</sub> was added). Red-brown bands of immunoreactive proteins develop within 10-20 min, and 20-40 ng of AChE are easily visible either in a pure form or in the crude extract gels. Processed filters are rinsed in tap water, dried and photographed.

### 3.11 Use of pEX Bacterial Expression Vector to Produce cDNA-derived Proteins

The bacterial expression vectors pEX1-3 were recently constructed by Stanley and Luzio (82) in the European Molecular Biology Laboratory in Heidelberg. The pEX vectors are derived from a crolac2 gene fusion plasmid, which expresses large quantities (up to 40% of total protein) of the fusion protein. This is produced under the control of the P<sub>R</sub> promoter of bacteriophage lambda. The pEX vectors were engineered to contain a polylinker in the 3'-end of the lac2 gene in all three translational reading frames, as well as stop signals for transcription and translation. Thus, any open reading frame DNA may be expressed in these vectors as a hybrid-beta-galactosidase protein, with the cro-beta-galactosidase part being of 117,000 dalton molecular weight. The hybrid fusion proteins accumulate in the bacterial cells as heavy low solubility precipitates, which allows a rapid isolation of these proteins for immunoblot analysis and protects them from proteolytic degradation.

Recombinant pEX plasmids are amplified at 30°C in an *Escherichia coli* strain containing the cIts857 repressor, and then transient expression is induced by shifting to 42°C. Hybrid proteins are extracted with SDS and dithiothreitol and the translational reading frame is randomized by using all three pEX vectors for subcloning of the pursued insert.

The pEX vectors are not appropriate for our needs as cloning vectors, since their cloning efficiency is not as high as that of the lambda-gt10 vector and since we cannot use their expression properties for colony screening because small cDNA inserts would be better expressed and yield stronger signals than large ones (82). However, the combination of lambda-gt10 as a cloning vector with pEX as an expression vector easy to subclone in that it bears important advantages for our project. Thus, we employ synthetic oligodeoxynucleotide probes for the initial screening and test the expression of positive clones as subcloned into pEX vectors by immunoblotting (see sections 4.1.1.-4.1.2.2. for further details).



#### 4. RESEARCH OBSERVATIONS

##### 4.1 Level of DNA

##### 4.1.1. Use of Synthetic oligodeoxynucleotides to select ChE DNA sequences

##### 4.1.1.1. OPSYN probes

Synthetic oligodeoxyribonucleotides have been shown to hybridize specifically to cDNA sequences (81). Under appropriate hybridization conditions, only well base-paired oligonucleotide xDNA duplexes will form (83); duplexes containing many mismatched base pairs will not be stable. This hybridization specificity has led to the development of a general method for using synthetic oligonucleotides as specific probes to identify cloned DNAs coding for proteins of interest. This technique has been applied to the successful isolation of several cDNA clones for proteins with known amino acid sequences, for example, those coding for human beta<sub>2</sub>-microglobulin (84), for murine transplantation antigen (85), and for human apolipoprotein CII (86).

The amino acid sequence of AChE is not known. However, it has recently been found that the same six amino acids are included in the organophosphorous binding site of both Torpedo AChE tetramers (87) and human pseudo-ChE tetramers (78). The same hexapeptide was previously found in electric eel AChE. This was determined by peptide sequencing of proteolytic degradation products of the purified ChEs, accompanied by identification of the organophosphate-binding serine residue using <sup>3</sup>H-DFP. Therefore, one may predict that some sequence homology, at least at the level of amino acid sequence, would exist even between ChEs from sources which are genetically remote, such as Torpedo and human.

The consensus hexapeptide sequence from the organophosphate-binding site of ChEs (designated OPSYN) is Phe-Gly-Glu-Ser-Ala-Gly. Because of the ambiguity in the genetic code, this peptide could be encoded by one of 384 different oligonucleotides, as follows:

5'	Gly	Ala	Ser	Glu	Gly	Phe	3'
			C			G	
	CC	NGC	NGA	TC	NCC	AA	
		A	T			A	
		CT					
		C					

where N equals all four possibilities (C, G, T, or A).

It appears that different species display certain preferences for the use of specific codons (88). However, we could not take advantage of this phenomenon, since (a) the number of cloned and sequenced human genes is still very limited, and (b) the genes coding for ChEs may not behave as species-specific genes, since they represent proteins which have been conserved through evolution. Consequently, we decided to prepare the entire collection of the OPSYN sequences. However, it would have been impractical to use a mixture of 384 different sequences, of which only one 17-nucleotide-long oligomer would have the right sequence, since the specific activity of the correct probe would inevitably be very low and since there would be considerable chances for false positive hybridization

signals. To overcome this difficulty, we prepared, in collaboration with Dr. O. Goldberg from the Dept. of Organic Chemistry, three mixtures of 128 different oligonucleotides each.

SCHEME II  
OPSYN CHOLINESTERASE PROBES

The following are all of the probes synthesized and used in searching for AChE organophosphate binding sites. All probes are equivalent to cDNA sequences homologous to the mRNA encoding the described amino acid sequence. All go from 5'---->3'.

OPSYN I, II, and III (both true and pseudo-ChE)

OPSYN I, II, and III encode for a hexapeptide including the active site serine. The amino acid sequence that they encode is as follows:

Phe-Gly-Glu-Ser-Ala-Gly

OPSYN I	AA <sup>A</sup> <sub>G</sub>	A CC <sup>C</sup> <sub>G</sub> T	CT <sup>C</sup> <sub>T</sub>	TC <sup>G</sup> <sub>A</sub> ---	CG <sup>C</sup> <sub>G</sub>	CC				
OPSYN II	AA <sup>A</sup> <sub>G</sub>	A CC <sup>C</sup> <sub>G</sub> T	CT <sup>C</sup> <sub>T</sub>	AG <sup>G</sup> <sub>C</sub> ---	CG <sup>C</sup> <sub>G</sub>	CC				
OPSYN III	AA <sup>A</sup> <sub>G</sub>	A CC <sup>C</sup> <sub>G</sub> T	CT <sup>C</sup> <sub>T</sub>	AG <sup>A</sup> <sub>T</sub> ---	CG <sup>C</sup> <sub>G</sub>	CC				
OPSYNO	AA <sup>A</sup> <sub>G</sub>	CCI	CT <sup>C</sup> <sub>T</sub>	TC <sup>A</sup> <sub>G</sub> AGI	CGI	CCI	LGI	CGI	TC <sup>A</sup> <sub>G</sub>	CA

To increase the certainty of our search, we prepared additional oligodeoxynucleotide probes. The first of these was an additional probe from the vicinity of the organophosphate binding site. In constructing this probe, we used the published sequence of the organophosphate binding site of human serum pseudo-ChE (78). The selected hexapeptide and the putative oligodeoxynucleotide coding for it were:

5'	Pro	Ser	Leu	Leu	His	Phe	3'
	NGA	NAG	NAG	A	A		
	GG	A	T	T	TG	AA	
		CT	AA	AA	G	G	

G C C

To limit the complexity of probes employed to 128, we prepared three mixtures of these probes. These were designated OPSYN IV, V, and VI and are described Scheme III:

Scheme III  
Additional oligodeoxynucleotide probes for  
the organophosphate binding site.

OPSYN IV, V, and VI encode for a hexapeptide from the vicinity of the organophosphate binding site of human serum pseudo-ChE, as published by O. Lockridge (78). The amino acid sequences that they encode are as above. The nucleotide sequences are:

	A	NAG	NAG	A A
OPSYN IV	GG GA			TG AA
	T	C	C	G G
		AA	AA	
		T	T	
	C	NAG	NAG	A A
OPSYN V	GG GA			TG AA
	G	C	C	G G
		AA	AA	
		T	T	
	A	NAG	NAG	A A
OPSYN VI	GG CT			TG AA
	G	C	C	G G
		AA	AA	
		T	T	

The OPSYN probes IV, V, and VI were tested with all of our positively hybridizing clones, but did not hybridize with any of them (see following sections). In a recent preprint which was mailed to me by Dr. Lockridge, the sequence surrounding the organophosphate binding site differs by four amino acids from the previously published one. Dr. Lockridge does not mention her 1984 publication (78), or the apparent changes in the determined sequence, in her new manuscript. One of these changes involves the removal of the Phe residue from the hexapeptide sequence which we employed to construct the OPSYN IV, V, and VI probes. This means that these probes were erroneous and that true ChE cDNA clones should not hybridize with any of these probes.

#### 4.1.1.2. C-terminal and N-terminal probes

Another oligodeoxynucleotide probe which was synthesized according to Lockridge's results from 1984 was based on the C-terminal hexapeptide of human serum pseudo-ChE. This probe is detailed in Scheme IV:

Scheme IV

C-terminal probe  
(pseudo-cholinesterase, (78))

The amino acid sequence is as follows: Glu-Ser-Cys-Val-Gly-Leu. The probe sequence is:

```

      A
      C G NGA C
AGCC AC CA TC
      G A G T
      T CT
      A
  
```

Finally, we designed two oligodeoxynucleotide probes according to two independent sequences which were reported for the N-terminal region of "true" AChE purified from human erythrocytes. The first of these, designated NTES, was derived by Dr. T. August and coworkers and was received as a personal communication from Dr. E. Schmill, (personal communication). It is based on peptide sequencing of the erythrocyte enzyme as purified on a column to which monoclonal antibodies against human AChE were bound (AE-2, 65). The second probe, designated NTTR, was synthesized following the sequence as reported by T. Rosenberry (12,79). In this case, the purification of the enzyme from human erythrocytes was carried by affinity chromatography, and a completely different sequence was determined. A positive sign for this probe being correct is that 20 out of 35 amino acid residues in this sequence are identical to those which were found in true AChE from fetal bovine serum. (B.P. Doctor, personal communication). The N-terminal probes are presented in Scheme V:

Scheme V

Oligodeoxynucleotide probes for the N-terminal peptides  
determined for human erythrocyte AChE  
(1) (NTES)

The amino acid sequence is as follows: Lys-Asn-Asn-Val-Lys-Ala-Trp-Gly  
The probe sequence is:

```

          J   A A C
CCCCAGGCGG TTCAC TT TT TT
          I   G G I
  
```

N-terminal probe  
(2) (NTTR)

The amino acid sequence is as follows: Glu-Gly-Pro-Glu-Asp-Pro. The probe sequence is:

```

          A A
          A C C C C
GG TC TC GG CC TC
          G T G G T
          I I
  
```

#### 4.1.2. Screening results of cDNA libraries

##### 4.1.2.1. Screening for OPSYN-containing cDNA sequences from cDNA libraries in lambda-gt10 vectors

As mentioned before, the amino acid sequence of human AChE is not known yet, with the exception of several fragments. One of these is the consensus OPSYN sequence. This sequence has been determined for human pseudo-ChE (78). It should, therefore, be included in the human gene coding for ChE mRNA. Since the same organophosphate-binding sequence has been determined in Torpedo AChE (76) and in eel AChE (78), it is very likely to be a common one for ChEs in general. For this reason, we used the OPSYN probes I, II, and III for our initial screening, whereas the other oligodeoxynucleotide probes were primarily used to test positive clones.

The cDNA libraries which we screened were prepared in the lambda gt10 vector. lambda vectors for cDNA cloning offer a major advantage over the plasmid vectors of the previous generation, such as pBR322, in that they can be used (this particularly applies to the Lambda-gt10 vector) to prepare very large cDNA libraries, containing on the order of  $10^5$  to  $10^7$  recombinants. This is necessary to isolate cDNA clones for rare mRNAs (89).

Since fetal brain contains considerably more AChE activity than fetal liver, we searched for AChE cDNA sequences in a cDNA library prepared in the lambda-gt10 vector from fetal brain mRNA (22 weeks gestation, poly(A)<sup>+</sup>RNA). In one case, we have also used a cDNA library prepared from fetal liver mRNA of the same gestational age.

In general, screening of these cDNA libraries was performed as previously described (see our Summary Report for Contract DAMD17-82-C-2145 for further details). An additional step was the wash with 3 M  $(\text{CH}_3)_4\text{NCl}$ , 53°C, which we employed to reduce the number of false positives (81; see Experimental Approaches section for details regarding this procedure). Table I summarizes the results of the cDNA-screening experiments with the various OPSYN probes.

Table I

Results of cDNA screening with the various OPSYN probes

Probes	Lambda-gt10 library source	No. of phages screened	No. of positive clones*
OPSYN I	Fetal brain	$1.5 \times 10^6$	1 (FBC4)
OPSYN II	Fetal brain	$1.5 \times 10^6$	2 (FB24, FB7B)
	Fetal liver	$1.0 \times 10^6$	1 ( FL20 = FB24)
OPSYN III	Fetal brain	$1.5 \times 10^6$	None

#### 4.1.2.2. Characterization of positive cDNA clones by restriction analysis, blot hybridization, and DNA sequencing

##### 1. FB24 = FL20

This cDNA clone consists of a 3.2-b insert and was selected by the OPSYN II oligodeoxynucleotide probe as detailed above, with the same insert size both from fetal brain and from fetal liver libraries. Sequencing of both cDNAs revealed an identical sequence, containing all 17 nucleotides of the OPSYN II probe in the correct order, but with a single additional thymidine at position 10, which makes it a false positive. The sequence data presenting this evidence are included in the following:

OPSYN II clone from fetal brain and fetal liver.  
The sequencing was accomplished by use of the M13 sequencing method.  
This is the m13 sequence=cDNA if it is a true positive only.  
THE NUCLEOTIDE SEQUENCE (FROM 5'-->3' IF IT'S a true positive) IS:

---

```
TAAGACAAATCCTGACGFATATATATGCTCCTTTAAAGGA
AATTCCTCCIGCCGACATCCCCAAAAATAAAAACTTCTA
ACFGATGGCTATGCTTG'
```

---

##### 24--OPSYN II INVERSED AND HOMOLGY

This is=mRNA if it is a true positive  
THE NUCLEOTIDE SEQUENCE IS:

```
0          10          20          30          40          50          60
CAAGCATAGC CATCAGTTAG AAGTTTITTA TTTTGGGGA TGTCGGCAGG AGGAATTTCC
0          70          80          90
TTTAAAGGAG CATATATATA CGTCAGGATT TGTCCTA
```

---

##### THE TRANSLATED SEQUENCE PHASED FROM POSITION 1 OF INVERSE HOMOLGY

```
0          30          60
CAA GCA TAG CCA TCA GTT AGA AGT TTT TTA TTT TTG GGG ATG TCG GCA GGA GGA ATT TCG
GLN ALA END PRO SER VAL ARG SER PHE LEU PHE LEU GLY MET SER ALA GLY GLY ILE SER
0          90
TTT AAA GGA GCA TAT ATA TAC GTC AGG ATT TGT CTT
PHE LYS GLY ALA TYR ILE TYR VAL ARG ILE CYS LEU
```

---

##### THE TRANSLATED SEQUENCE PHASED FROM POSITION 2 OF INVERSE HOMOLGY

```
0          31          61
AAG CAT AGC CAT CAG TTA GAA GTT TTT TAT TTT TCG GCA TGT CCG CAG GAG GAA TTT CTT
LYS HIS SER HIS GLN LEU GLU VAL PHE TYR PHE TRP GLY CYS ARG GLN GLU GLU PHE PRO
0          31
TTA AAG GAG CAT ATA TAT ACG TCA GGA TTT GTC TTA
LEU LYS GLU HIS ILE TYR THR SER GLY PHE VAL LEU
```

---

##### THE TRANSLATED SEQUENCE PHASED FROM POSITION 3 OF INVERSE HOMOLGY

```
0          32          62
AGC ATA GCC ATC AGT TAG AAG TTT TTT ATT TTT GGG CAT GTC GGC AGG AGG AAT TTC CTT
SER ILE ALA ILE SER END LYS PHE PHE ILE PHE GLY ASP VAL GLY ARG ARG ASN PHE LEU
0          32
TAA AGG AGC ATA TAT ATA CGT CAG CAT TTG TCT
END ARG SER ILE TYR ILE ARG GLN ASP LEU SER
```

2. FBC4

This cDNA clone consists of a 1.2-kb insert and was selected from the fetal brain cDNA library by the OPSYN I oligodeoxynucleotide probe as detailed above. Sequencing of the cDNA fragment which hybridized with the labeled probe revealed an interesting phenomenon. Five of six amino acids in the active site hexapeptide were found to be correctly encoded, with the last one being lysine instead of phenylalanine (i.e., a "permissive" change in terms of the protein properties). Furthermore, two amino acids adjacent to those of the active site peptide were also found to be identical to those included in the active site peptide as reported recently by Lockridge and LaDu (90). This implies that the protein encoded by this cDNA is clearly close in its properties to those of the human ChEs, but is probably not our true positive either. The information showing these data is included below, and additional positive cDNA clones (see section 4.1.2.2. above) are sequenced as well.

THE NUCLEOTIDE SEQUENCE IS:

```

-
-
0          10          20          30          40          50          60
+
CCCCCTCCT GTTTCCTGGG CTTTCTCCTT TTCCIGIGAA ATCTCCGAGG AGAAGAAAGA
0          70          80          90
+
ATGATGGACA GTTATCCTTC ACTGCCACAA GGGGG
  
```

THE TRANSLATED SEQUENCE PHASED FROM POSITION 1

```

0
0
0          30          60
CCC CCT TGT GGC AGT GAA GGA TAA CTG TCC ATC ATT CTT TCT TCT CCT CGG AGA TTT CAC
PRO PRO CYS GLY SER GLU GLY END LEU SER ILE ILE LEU SER SER PRO ARG ARG PHE HIS
0
0          30
AGG AAA AGG AGA AAG CGC AGG AAA CAG GAG GGG
ARG LYS ARG ARG LYS ARG ARG LYS GLN GLU GLY
1
-
  
```

THE TRANSLATED SEQUENCE PHASED FROM POSITION 2

```

0
0
0          31          61
CCC CTT GTG GCA GTG AAG GAT AAC TGT CCA TCA TTC TTT CTT CTC CTC GGA GAT TTC ACA
PRO LEU VAL ALA VAL LYS ASP ASN CYS PRO SER PHE PHE LEU LEU LEU GLY ASP PHE THR
0
0          31
GGA AAA GGA GAA AGC GCA GGA AAC AGG AGG GGG
GLY LYS GLY GLU SER ALA GLY ASN ARG ARG GLY
1
-
  
```

THE TRANSLATED SEQUENCE PHASED FROM POSITION 3

```

0
0
0          32          62
CCC TTG TGG CAG TGA AGG ATA ACT GTC CAT CAT TCT TTC TTC TCC TCG GAG ATT TCA CAG
PRO LEU TRP GLN END ARG ILE THR VAL HIS HIS SER PHE PHE SER SER SER GLU ILE SER GLN
0
0          32
GAA AAG GAG AAA GCG CAG GAA ACA GGA GGG GGG
GLU LYS GLU LYS ALA GLN GLU THR GLY GLY GLY
1
-
  
```

#### 4.1.2.3. Screening results of human genomic DNA library (N-terminal probes)

The Charon 4a DNA library was the same one on which we reported before, prepared by partial EcoRI digestion from peripheral blood cell DNA (14,15). The probes used were: (a) a mix of eight 26-mers designated NTES complementary to mRNA coding for amino acids 7-15 from the N-terminal peptide sequenced by T. August and coworkers (personal communication), with the four nucleotides at positions 6, 9, and 15 being replaced by G, G, and C, respectively, and (b) a mix of 128 17-mers designated NTTR, complementary to mRNA coding for amino acids 1-6 from the N-terminal peptide as sequenced by T. Rosenberry and coworkers (12). Three genome equivalents ( $7.5 \times 10^5$  phages) were screened, using 25 petri dishes of  $15 \text{ cm}^2$  for the first screen. Hybridization solution contained 6 x SSC, 5 x Denhardt, 10 mM EDTA, 100  $\mu\text{g/ml}$  salmon sperm DNA, denatured, and 20  $\mu\text{g/ml}$  of E.coli-denatured DNA. Probes were end-labeled with polynucleotide kinase to a specific activity of  $1.8 \times 10^6$  cpm of Cherenkov/pmol and  $80 \times 10^6$  cpm were included in 80 ml of hybridization mixture with 50 duplicate filters. Washes were 4 x 40 min at room temperature followed by 1 hr at  $52^\circ\text{C}$  in 3 x SSC.

The results of these screens were as follows:

Table II  
Results of cDNA screening with N-terminal probes

Probe	No. of positives			Sequenced clones
	1st Screen	2nd Screen	3rd Screen	
NTES	18	5	5	1 (4 mismatches) of 1 (1 deletion, 1 addition, and 1 mismatch) 26 nucleotides 3 are studied at present
NTTR	7	4	4	Currently carried out

In summary, finding the human cDNA clones appears to be more difficult than we originally perceived. So far, we have screened the fetal brain library once with each probe, and the liver library only once. These screens resulted in the finding of four positive clones, of which three were found to be false positives and one remains to be sequenced. This is in complete agreement with Wood *et al.*, (81), who expect the ratio between false positives and true ones to be about 5:1 (see previous sections for details). Also, it is possible that we did not yet cover the entire repertoire of the brain library for the following reasons:

- The sequence complexity of brain mRNA is exceptionally high;
- Part of the cDNA chains may be incomplete, which implies that they do not necessarily include the OPSYN regio;
- There is a possibility of selection against phages which carry AChE cDNA insert. This could have happened during the amplification of the cDNA library (for example, because of their large size) and would decrease the number of AChE cDNA clones below their rate of abundance.



To overcome these difficulties, we need to cover a larger number of phages in each screen to find the true positive ones, which is what we have just started. In addition, we are currently preparing overlapping probes for the OPSYN and the N-terminal regions; positive hybridization with both probes will take place only with true positives and will increase the certainty of our future screening efforts.

#### 4.2. Level of mRNA

##### 4.2.1. Crossed immunoelectrophoresis as used to detect nascent cholinesterases

Labeled translation products directed by human brain mRNA preparations from different brain regions and fetal development stages were analyzed by crossed immunoelectrophoresis followed by autoradiography. Labeled proteins from translation experiments in which human mRNA was not added were analyzed in parallel, as controls. The labeled immunoprecipitates appeared in the crossed immunoelectrophoresis plates as sharp, thin arcs of [<sup>35</sup>S]methionine-labeled polypeptides, with rather intense radioactivity and high resolution. Plates were loaded with only 2  $\mu$ l of reticulocyte lysate translation mixture or oocyte homogenates. These volumes contained about 10<sup>4</sup> cpm of TCA-insoluble proteins, a much lower amount of labeled protein mixture than is routinely used for immunoprecipitation (see 76, for example). This is because in this technique the immunoprecipitate is mostly retained within the dried agarose network, whereas careful washes of the plates ensure low levels of background irradiation and increase the sensitivity of detection.

The use of the combination of crossed immunoelectrophoresis and *in vitro* translation of mRNAs extracted from human brain tissue for the identification of individual mRNA products was illustrated using Coomassie blue-stained gels which contained plasma standard (see The Experimental Approaches) as carrier in order to indicate the range of possible plasma proteins whose mRNAs might be present in the product from the *in vitro* reticulocyte translation system. The middle gel contained monospecific antibodies and showed the arc for the corresponding protein which was missing from the top gel (containing antiserum against whole serum). This staining thus emphasized the monospecificity of the sera employed. The autoradiograms prepared from these plates showed which of the precipitation arcs produced by plasma proteins were also labeled by [<sup>35</sup>S]methionine and were synthesized by the reticulocyte lysate system in the presence of mRNA extracted from human brain tissue. Autoradiograms from plates prepared from control experiments in which no mRNA was added to the translation system indicate that there is a considerable amount of radioactivity associated with albumin and a broad  $\alpha_1$ -lipoprotein peak in the upper part of the intermediate gel. There was only very faint radioactivity associated with any of the other peaks and in the intermediate gels there was no radioactivity corresponding to the two middle plates. This is important evidence for the presence of mRNAs for plasma protein-like polypeptides in the brain extract. This method was used to identify a number of plasma protein mRNAs in human fetal and adult brain and in brain tumor extracts. The results are summarized in Table III.

##### 4.2.2. Fetal brain

Poly(A)<sup>+</sup>RNA was extracted from a series of human fetal brains of 10-18 weeks' gestation. The mRNA was translated as described (in The

Experimental Approaches) and the products were run in crossed immunoelectrophoretic plates against whole human antiserum using a mixture of fetal and adult plasma as a carrier. There was clear evidence from these experiments of the presence of mRNAs for several plasma proteins in the human fetal brain samples. The total TCA-precipitable counts applied to each plate were similar at each fetal age, indicating that the level of mRNA activity in fetal brain at 10 to 18 weeks was similar. At 10 weeks gestation transferrin, Gc globulin, and alpha<sub>1</sub>-antitrypsin-like polypeptides were clearly present. A faint peak for alpha-fetoprotein was also present at 10 weeks. In the alpha-beta region there were several flat and indistinct peaks that were difficult to identify, even when analyzed in the transparent autoradiograms. By 14-16 weeks gestation, the peak for transferrin was larger, with a higher level of radioactivity, and the peak for alpha<sub>1</sub>-antitrypsin was about the same. However, several new peaks were now clearly apparent. These were identified as alpha<sub>2</sub>-macroglobulin, fibrinogen, and appeared to be more radioactive, especially those of transferrin, haptoglobin, and fibrinogen; an immunoprecipitation arc for ChE was identified by its coprecipitated enzyme activity, using the method of Karnovsky and Roots (21). The control (no mRNA added to the translation system) showed [<sup>35</sup>S]-labeling of albumin and an alpha-lipoprotein, probably due to nonspecific adhesion of [<sup>35</sup>S]methionine itself or of labeled translation products. Alternatively, some of the material synthesized by the rabbit reticulocyte lysate system may have cross-reacted with the anti-albumin and anti-alpha<sub>1</sub>-lipoprotein antiserum. From the reticulocyte lysate experiments, it was therefore not possible to distinguish whether the fetal brain samples contained mRNA for these proteins.

#### 4.2.3. Postnatal brain

Comparison of the fetal brain mRNA data with results obtained from three different regions of postnatal human brain shows some similarities but also interesting differences. In all three regions at each postnatal age, the intensity of labeling of the peaks was less than at 14 to 18 weeks' gestation.

The basal ganglia at 5 years showed all of the mRNA peaks that were present in the fetal material except for alpha-fetoprotein. With the exception of the peak for ChE, the mRNA levels at 5 years in this brain region were clearly less than those in the whole fetal brain at 14-18 weeks' gestation. In the postnatal parietal cortex, there were fewer mRNAs detectable and their level was much lower than in the postnatal basal ganglia or in the whole fetal brain. Only transferrin and alpha<sub>2</sub>-macroglobulin-like polypeptides were seen at 5 years, and alpha<sub>2</sub>-macroglobulin and haptoglobin at 35 years. The pattern of mRNAs for hypothalamus from 35 and 4 years was similar to that for the basal ganglia, but the mRNA level for transferrin and haptoglobin seemed higher, whereas the levels for ChE and Gc globulin were lower.

#### 4.2.4. Brain tumors

Translation products from samples of several different meningiomas and gliomas (see 29 and 69 for details regarding these tumor tissues) were run in crossed immunoelectrophoretic plates against whole human serum antiserum with plasma standard as carrier. As in fetal and adult brain samples, mRNAs for albumin and alpha<sub>2</sub>-macroglobulin, Gc globulin, and haptoglobin. In addition, the mRNA for ceruloplasmin was identified in meningioma and that for alpha<sub>1</sub> easily precipitable protein was found in glioma tissue.

These results are summarized and compared with those from normal fetal and adult brain tissue in Table III.

TABLE III  
IDENTIFICATION OF mRNA TRANSLATION PRODUCTS FOR PLASMA PROTEINS IN SIX FETAL (10-22 WEEKS GESTATION) AND FIVE ADULT HUMAN BRAINS AND IN TWO SAMPLES EACH OF HUMAN MENINGIOMAS AND GLIOMAS<sup>a</sup>

Protein	Fetal brain (weeks)				Adult brain	Meningioma	Glioma
	10	14	16	18			
$\alpha$ -Fetoprotein	+	+	+	+	-	-	-
$\alpha_1$ -Antitrypsin	+	+	+	+	+	±	-
Inter- $\alpha_1$ -TI	-	-	-	-	n.t.	-	n.t.
$\alpha_2$ -Macroglobulin	-	+	+	+	+	+	+
Ceruloplasmin	n.t.	-	n.t.	n.t.	-	+	-
Fibrinogen	-	+	+	+	+	±	+
Gc Globulin	+	+	+	+	+	+	+
Haptoglobulin	-	+	+	+	+	+	+
Hemopexin <sup>b</sup>	-	-	-	-	-	-	-
Plasminogen	-	-	-	-	-	-	-
Transferrin	+	+	+	+	+	-	-
$\beta$ -lipoprotein	+	+	+	+	+	+	+
$\alpha_1$ -EPP	-	-	n.t.	n.t.	+	-	+
$\alpha_2$ -HS <sup>c</sup>	-	-	-	-	n.t.	-	n.t.
Cholinesterase <sup>d</sup>	+	+	+	+	+	+ <sup>d</sup>	+

Note. +, Positive identification; -, not detected; ±, positive for some samples, negative for others. For fetal brain samples the age(s) at which positive identification was obtained is indicated: 10, 14, 16, and 18 weeks gestation. n.t. = not tested.

<sup>a</sup> Identification was made as described under Materials and Methods and as illustrated in Fig. 1; monospecific antibody to each protein was included in the intermediate or the top gel of crossed immunoelectrophoretic plates.

<sup>b</sup> Positive in oocyte injection experiments. Inter- $\alpha$ -TI = inter- $\alpha$ -trypsin inhibitor,  $\alpha_1$ -EPP =  $\alpha_1$ -easily precipitable protein,  $\alpha_2$ -HS =  $\alpha_2$ -Heremans and Schmid. Samples of meningioma were negative for retinal binding protein,  $\alpha_2$ -plasminogen inhibitor, C-reactive protein, transcortin, and thyroglobulin.

<sup>c</sup>  $\alpha_2$ -HS contains only one methionine residue thus the translation product might not have been labeled; positive in oocyte injection.

<sup>d</sup> Confirmed by mRNA injection into *Xenopus* oocytes and assay of esterase activity.

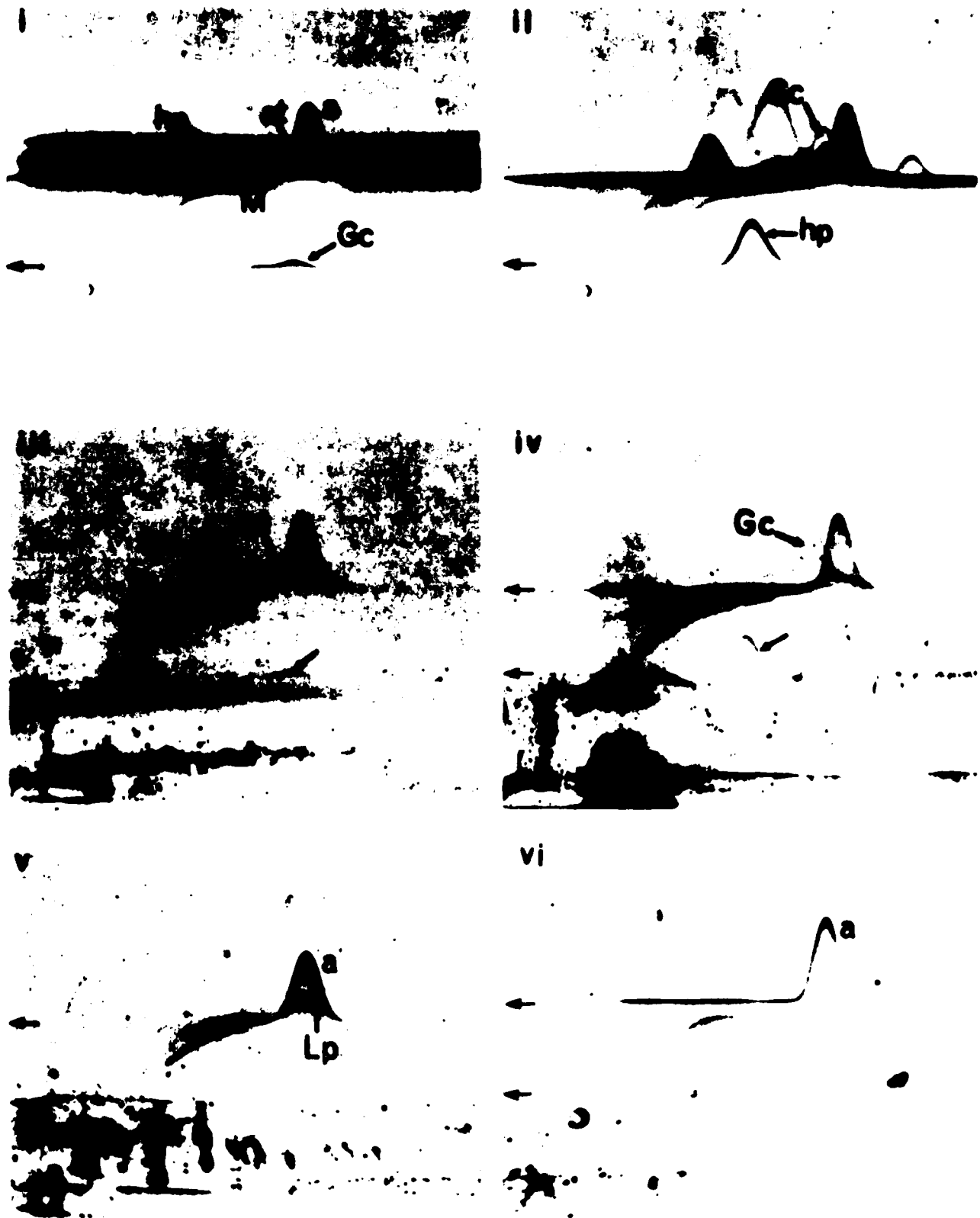


FIG. 1. Identification of plasma protein translation products from mRNA of human brain tumor. mRNA was extracted and translated as described under Materials and Methods. 2  $\mu$ l of translation product together with 3  $\mu$ l of mixed fetal-adult plasma carrier was applied to the well of each plate for the first dimension. For the second dimension the intermediate gel contained anti-Gc globulin (left plates) or anti-

There was considerable variation in the mRNA pattern from primary tumors of the same tissue type, and not enough tumor samples have been examined for it to be clear whether there are any tissue-specific differences in the plasma protein mRNAs present in the different types of tumors.

Additional information about plasma proteins in cerebral tumors was obtained by running extracts of various brain tumors in cross immunoelectrophoresis against anti-human serum. In many tumor extracts, either haptoglobin or hemopexin showed a particularly prominent peak. In some tumors both were prominent. Since mRNA for haptoglobin was identified in some tumors using translation in reticulocyte lysate, the haptoglobin in the tumor extracts was presumably mainly due to local synthesis of this protein. mRNA for hemopexin was identified by using oocyte injection (see below).

#### 4.2.5. Synthesis of plasma proteins in microinjected oocytes

Antibodies elicited against purified mature proteins often do not interact with the nascent, unprocessed polypeptide forms of the same proteins. Since no post-translational processing takes place *in vitro*, in reticulocyte lysate, we have used microinjected *Xenopus* oocytes as an additional *in vivo* translation system. When microinjected into oocytes together with [<sup>35</sup>S]methionine, mammalian mRNA efficiently directs the biosynthesis of [<sup>35</sup>S]-labeled polypeptides (see, for example, 76). These then undergo various post-translational processing events, and secretory proteins are correctly transported into the oocytes' incubation medium (for a recent review see 73). Therefore, we used the oocytes to examine whether particular plasma proteins are produced from brain mRNA and to find out whether they are destined to be secreted in the brain.

Plasma proteins produced in *Xenopus* oocytes microinjected with fetal brain poly(A)-containing RNA together with [<sup>35</sup>S]-methionine were compared with the translation products directed *in vitro* by fetal brain. Most of the proteins which were detected in the autoradiograms of the *in vitro* products could also be observed in autoradiograms of oocyte homogenates. An exception was Gc globulin which created a clear precipitate in the crossed immunoelectrophoresis plates loaded with *in vitro* synthesized products, but not in plates for the oocytes. In contrast, hemopexin, which appeared to be one of the major products in the oocyte homogenate, was not observed among the *in vitro* products. Different levels of mRNA competition on rough endoplasmic polyosomes and the fact that post-translational processing takes place in the oocytes but not in reticulocyte lysate (73) may explain these differences.

Albumin and alpha-lipoprotein peaks were clearly visible in the oocyte extract following mRNA injection but the controls were negative. Thus, it seems that mRNA for these proteins is present in fetal brain.

Most of the plasma proteins were retained within the oocytes, suggesting that they are normally synthesized in the brain as cytoplasmic proteins. An exception was ChE; earlier experiments involving the injection of brain mRNA into oocytes have shown that most of the synthesized ChE is secreted (38,69). It is also known that *in vivo* ChE is secreted into the cerebrospinal fluid (7).

#### 4.2.6. Human brain mRNA is translated into multiple nascent cholinesterase polypeptides

Different ChE mRNAs (69) could all be translated into a single polypeptide, as could the mRNAs for kininogen (91) and glucagon (92). Alternatively, they could each code for a distinct nascent ChE polypeptide. To distinguish between these immunoprecipitated the [ $^{35}\text{S}$ ]-labeled nascent ChE polypeptides produced. Since the concentration of ChE mRNA appeared to be very low (38), we took special precautions to reduce to a minimum the amount of immunoglobulins employed for the immunoprecipitation reaction and thus to prevent nonspecific background precipitation (93). Under the conditions employed, the anti-AChE AE-2 monoclonal antibodies (65), but not an irrelevant monoclonal antibody, specifically immunoprecipitated several nascent polypeptides, with clear differences in their electrophoretic migration properties in a gradient polyacrylamide gel. Since size is the major criterion for separation in such gels, this implies that the various ChE mRNAs in the fetal human brain give rise to several nascent polypeptides of different lengths, all of which contain the peptide domain which immunoreacts with anti-AChE antibodies. It should be noted that these may include polypeptides other than AChE polypeptides, if these contain the same immunoreactive domain.

To reveal whether the nascent polypeptides leading to the production of AChE differ from those which eventually yield pseudo-ChE, we employed crossed immunoelectrophoresis in gels in which selective antibodies against either AChE or pseudo-ChE were encased. Our observations indicated the existence of non-cross-reactive immunoprecipitates for AChE and pseudo-ChE (93), which further expands the variety of polypeptides translated from mRNAs.

#### 4.2.7. Post-translational modifications contribute to the heterogeneity of human brain cholinesterases

To examine the possibility that post-translational modifications also contribute to the heterogeneity of human brain ChEs, we employed the *in vitro* translation system of microinjected *Xenopus* oocytes, in which such modifications are performed. Oocytes were injected with poly(A)-containing RNA from fetal human brain together with [ $^{35}\text{S}$ ]methionine, and oocyte homogenates and incubation medium were subjected to crossed immunoelectrophoresis and autoradiography, using rabbit antisera against AChE and pseudo-ChE, respectively. The electrophoretic separation in agarose gels is mainly based on charge differences (see 57 for further details). Using this technique, we detected the appearance of polymorphic ChE immunoreactive polypeptides with distinguishable migration coordinates in both the oocyte homogenates and incubation medium. This indicates that in addition to the size and sequence differences inherent to the nascent ChEs which were observed in the *in vitro* immunoprecipitated polypeptides, post-translational modifications alter the charge of the newly produced ChEs. The ChEs observed in the incubation medium displayed limited heterogeneity and faster migration, as compared with the intracellular forms of the enzyme. This may indicate that a hydrophobic part of an intracellular nascent polypeptide had to be separated to enable the release of the enzyme into the incubation medium in a soluble, hydrophilic form. A similar mechanism with the same effect on the electrophoretic migration of the enzyme has been demonstrated *in vitro* for purified human erythrocyte AChE (94).

It is not yet clear from our findings whether intracellular, amphipathic forms of the enzyme are precursors of the secreted, soluble forms

or whether each was produced from a different nascent polypeptide chain. Molecular cloning and DNA sequencing experiments will be necessary to reveal whether the secretory enzyme is formed in one step or whether it represents enzyme molecules which were released from their association with the membrane by natural enzymatic reactions.

Hybridization-selection experiments were employed to confirm that the various ChE mRNAs bear sequence homologies. Different ChE cDNAs were used to hybrid-select fetal brain ChE mRNAs; the hybrid-selected mRNAs were microinjected together with [<sup>35</sup>S]methionine into *Xenopus* oocytes and oocyte extracts and incubation medium were subjected to autoradiography. Multiple, polymorphic AChE and pseudo-ChE catalytically active (14) and immunoreactive (93) proteins were detected. This experiment revealed that although AChE and pseudo-ChE display distinct immunological domains both the mRNA species producing these enzyme hybridize to several ChE cDNA clones. Also, the various forms of AChE, which exhibit different migration coordinates on the crossed immunoelectrophoretic gels, were indicated by this experiment to be produced by cross-homologous mRNA sequences. Table IV summarizes the evidence we accumulated for the existence of various cross-homologous ChE mRNAs which code for several distinct nascent polypeptides.

Table IV

Different cross-homologous cholinesterase mRNAs contribute to the polymorphism of fetal brain cholinesterases.

Experimental indications	Reference No.
a. Size separation of fetal brain mRNAs translatable into various ChEs in oocytes	69
b. Production of various fetal brain cDNAs, all homologous to a single AChE gene	15
c. <i>In vitro</i> translation of fetal brain mRNA into distinguishable polypeptides immunoreactive with anti-AChE or anti-pseudo-ChE polyclonal antibodies	93

In various regions of the fetal brain, AChE activity was ca. 35-50% low salt-soluble and 45-65% detergent-soluble throughout brain development (13). This implies, in agreement with Gennari and Brodbeck (95), that a considerable part of fetal brain AChE contains a hydrophobic domain. This observation could explain the slow electrophoretic migration of part of the mRNA-directed brain AChE produced in microinjected oocytes and analyzed by crossed immunoelectrophoresis.

#### 4.3 Level of the Active Enzyme Protein

##### 4.3.1 Cholinesterases in adult, fetal, and neoplastic brain tissues

The activities, molecular forms, and membrane association of ChEs were studied in dissected regions of adult, postmortem human brain, in parallel

regions of developing fetal brain, and in a collection of primary brain tumors consisting of primarily gliomas and meningiomas. All of these tissue sources contained substantial amounts of ChE activities, with several tissue samples expressing exceptionally high levels. In normal mature and fetal brain and in meningiomas, AChE accounted for almost all of the ChE activity, but in almost all gliomas, elevated levels of pseudo-ChE could be detected. Two major forms of AChE, sedimenting as 10-11S and 4-5S, respectively, were detected on sucrose gradients in most of the mature and fetal brain regions examined (13) and in gliomas. In meningiomas, a light (4-5S) form was the principal component (40). The 10S and the 4S; forms of brain AChE appeared to possess similar catalytic properties, as indicated by their individual  $K_m$  values toward [ $^3\text{H}$ ]acetylcholine (ca.  $4 \times 10^{-2}$  M) and their interaction with selective inhibitors.

#### 4.3.2 Divergent regeneration of acetylcholinesterase and muscarinic cholinergic receptors

To examine whether AChE activity in the brain is coregulated with other cholinceptive properties, the muscarinic binding activity in these brain tissues was examined, using the muscarinic antagonist [ $^3\text{H}$ ]N-methyl-4-piperidyl benzilate ([ $^3\text{H}$ ]4NMPB). Individual data for ChE activity were divided by the corresponding values for the density of [ $^3\text{H}$ ]4NMPB binding sites, and average values of these arbitrary ratios were calculated for the different tissue types. In most stages of brain development, the average arbitrary ratios showed a tendency to decrease with maturation. The average values and variability ranges in the fetal brain were  $83 \pm 50$  and  $19 \pm 19$  at 14 and 24 weeks' gestation, respectively. The parallel value calculated for the collection of brain tumors composed of undifferentiated brain cells was  $86 \pm 66$ , and in mature post-mortem brain samples it was as low as  $11 \pm 8$ , as calculated from the data described by Gurwitz *et al.*, (41) and Egozi *et al.*, (39). These observations suggest that if a correlation exists between muscarinic receptors and ChE levels in glial tumors, it differs from that of the nonmalignant mature brain tissue. In addition, the developmental decrease of the ChE:muscarinic receptors ratio indicates that AChE is also involved in functions other than cholinergic transmission in the developing human brain.

#### 4.3.3. Human antibodies against muscle membrane acetylcholinesterase

Serum from a patient suffering from neuromuscular dysfunction was examined for its ability to hydrolyze [ $^3\text{H}$ ]acetylcholine. The level of [ $^3\text{H}$ ]acetate released was below 0.10 nmol/hr/ $\mu\text{l}$  of serum; under the same conditions, normal human serum releases about 50 nmol of [ $^3\text{H}$ ]acetate/hr/ $\mu\text{l}$ . To examine whether the absence of ChE activity in the tested serum was due to the presence of anticholinesterase antibodies, we subjected human AChE to indirect immunoprecipitation. The enzyme was extracted from fetal brain tissue with salt and non-ionic detergent and its specific activity was examined by measuring [ $^3\text{H}$ ]acetylcholine hydrolysis in the presence of  $10^{-5}$  M iso-OMPA, which selectively blocks pseudo-ChE activity (10), and of protease inhibitors. The tested serum and normal human serum, which served as control, were diluted by 1:10 and AChE activity was determined separately in the supernatants and in the redissolved precipitated pellets. Under these conditions, the enzymatic activity of the brain AChE in the AChE in the supernatant was reproducibly reduced by about 40%.



Anti-AChE antibodies do not necessarily inhibit the enzymatic activity of AChE. However, in this case, the AChE activity was considerably reduced (by up to 80%) in the immunoprecipitated pellet. This decrease could not be due to proteolytic degradation of AChE, since we included a potent mixture of protease inhibitors in the reactions. This implied that the tested serum contained one or more types of anti-AChE antibodies, at least some of which inhibit the catalytic activity of the enzyme.

#### 4.3.4. Direct immunoprecipitation of human acetylcholinesterase

Purified AChE from human erythrocytes, received from Dr. U. Brodbeck, was radiolabeled with  $^{125}\text{I}$  and incubated with the examined serum in 1:10,000 dilution or with normal human serum for control. Under these conditions, the tested serum specifically immunoprecipitated 60% of the radioactively labeled enzyme. Since most of the clinical symptoms observed were directly related to neuromuscular dysfunction and muscle atrophy, we were interested to find out whether the tested serum would also display the ability to immunoprecipitate the membrane-associated fraction of muscle AChE. Direct immunoprecipitation of fetal muscle enzyme, extracted with salt and detergent, revealed that the antibodies interacted efficiently with this enzyme as well, reducing the activity of the enzyme by 20 and 35% in 1:500 and 1:10,000 dilutions, respectively.

To confirm the immunological nature of the process leading to this pronounced decrease in the enzymatic activity, we subjected both the tested and the control sera to preadsorption of serum immunoglobulins onto goat antibodies against the human Fab determinant, bound to Sepharose beads. This treatment completely abolished the immunoprecipitation of AChE activity.

#### 4.3.5. Preferential precipitation of salt-and detergent-extractable acetylcholinesterase from fetal muscle

In these experiments, we compared the precipitation of buffer-soluble muscle AChE to that of the salt- and detergent-extractable muscle enzyme. The latter fraction contains the membrane-associated forms of AChE and is particularly enriched with the collagen-tailed forms of AChE that disappear from the neuromuscular junctions of denervated, atrophied muscles (9). Precipitation was determined by assaying the activity in the supernatant after centrifugation. This activity presents a maximum at intermediate serum dilutions, since precipitation does not occur if dilution is too high or too low. The optimal dilution for immunoprecipitation of the salt- and detergent-extractable enzyme ranged around 1:100,000, whereas the buffer-soluble muscle enzyme precipitated best at serum dilution of about 1:2000. Furthermore, the efficiency of precipitation of the salt- and detergent-extractable AChE appeared to be significantly higher than that of the buffer-soluble enzyme (43% vs. 32% at optimal serum dilutions).

When direct immunoprecipitation of AChE was examined with salt and detergent extracts from several other fetal tissues, the preferential immunoprecipitation of the membrane-associated muscle enzyme was also found to be tissue-specific.

Salt- and detergent-extractable AChE from brain, liver, heart, and adrenal could be immunoprecipitated best at serum dilutions ranging between 1:2000 and 1:8000, which is much more concentrated than the 1:500,000

dilution of serum required for the optimal precipitation of the membrane-associated enzyme from striated muscle.

## 5. DISCUSSION

### 5.1 Biosynthesis of Plasma Proteins in the Human Brain

This report presents evidence for the presence of mRNAs for ChEs, as well as for a number of other plasma proteins, in human brain tissue from several different sources. This confirms and extends previous studies showing the presence of mRNA for transferrin in postnatal and adult rat brain (56), for ChEs in the human brain (69), and for several plasma proteins in postnatal rat cerebellum (57). Thus, it is possible that in non-cholinergic neurons and in other cell types in the brain, ChEs are expressed as plasma protein(s) rather than as neurotransmitter-hydrolyzing enzymes. The present observations may explain the presence of several plasma proteins in human fetal brain, demonstrated by immunoperoxidase histochemistry proteins in human fetal brain, demonstrated by immunoperoxidase histochemistry (58). The combination of in vitro and in ovo translation with crossed immunoelectrophoresis and autoradiography, which we used in this study, has been estimated to be one to two orders of magnitude more sensitive than methods previously used for immunoprecipitation of translation products (see 57). This may explain why we have been successful in obtaining evidence for mRNAs for plasma proteins in fetal brain when others have not (53,96). Possible problems which may be associated with the present method have been discussed in detail in 57. The controls, in which no mRNA has been added to the translation system, are particularly important, since they show substantial radioactivity attached to albumin and  $\alpha_1$ -lipoprotein and occasionally weakly attached to other usually unidentifiable proteins. In the presence of such positive controls, it is not possible to be sure whether or not mRNA for these proteins was present in the experimental samples. However, the results of the oocyte injection experiments suggest that mRNA for both of these proteins was present in the developing brain. Some mRNAs may not have been detected, either because the translation conditions were not appropriate (97) or because post-translational modifications do not generally occur in the reticulocyte lysate used. Also, the physiological state of the brain samples used in this study was likely to have been suboptimal; it is possible that mRNAs for some proteins might be more affected than others by deterioration postmortem. Thus, a negative result is inconclusive.

Møllgaard and Jacobsen (58) described the distribution of several plasma proteins in developing human fetal brain. Of those which they studied, we now have evidence that mRNAs for  $\alpha$ -fetoprotein,  $\alpha_1$ -antitrypsin, and transferrin are present in the human fetal brain.

In addition, there is evidence from in vitro amino acid incorporation experiments that  $\alpha$ -fetoprotein, albumin, and transferrin are synthesized by developing brain (48,52). The demonstration of mRNAs for other plasma proteins, such as Gc globulin and haptoglobin, suggests that these products may also be demonstrable within the developing brain by immunohistochemical techniques.

Secretory proteins are generally produced from mRNAs which include a region coding for an N-terminal signal peptide, to be cleaved prior to the secretion process (98). In mRNA-microinjected oocytes, secretory proteins

will appear in the incubation medium (73). Our microinjection results imply that in the brain, most of the immunoprecipitable plasma proteins are mostly secreted. It should be noted that this observation corroborates previous observations, in which activity was mostly detected in the incubation medium of oocytes injected with brain mRNA (38,69).

The significance of the presence of these plasma proteins within cells in the developing and mature brain is not known. Most functions ascribed to plasma proteins relate to their presence in plasma Serum, particularly fetal serum is commonly used for the culture of many cell types and different tissues. Certain individual plasma proteins have been described as having growth-promoting effects in cell culture systems. For example, transferrin is a constituent, in most "defined media" (99); alpha<sub>2</sub>-macroglobulin (100) and fetuin (101) have been described as having various growth-promoting effects on cells and tissues in culture. However, it is the intracellular production and localization of plasma proteins within cells in the nervous system that seem intriguing. Immunocytochemical studies of human fetuses and fetuses of other species (for reference, see 102) show that the plasma proteins have an intracellular and not an extracellular distribution. It is not likely that any extracellular plasma protein would have been lost, since the cerebrospinal fluid usually stains strongly for individual proteins. The only plasma protein with an intracellular localization that has so far been implicated in neuron function is transferrin and sciatin have been shown to have trophic effects on myoblasts in culture (103). It is possible that some plasma proteins are extracellular members of families of proteins that also have an intracellular distribution. Individual proteins may have small differences in their amino acid sequences that are not detectable by polyclonal antisera. Serum transferrin and sciatin may be members of a transferrin family (103). Another example may be albumin and beta-acitinin. The latter is found in muscle and appears to be indistinguishable from albumin by physicochemical and immunological criteria (104). Another suggestive observation is that of Mollgard *et al.*, (48), who showed that in fetal sheep neocortex, most of the cells in the early cortical plate contain fetuin but the innervating fibers on either side of the cortical plate contain transferrin. These fibers are thought to originate from brain stem and midbrain nuclei, where there are many transferrin positive fetuin-positive perikaria in the cortical plate is known to be an area of intense synaptogenesis (105); thus, it is possible that some plasma proteins are involved in this process or in some other interactions between different populations of neurons.

It is not clear from the present study which cell types contained the extracted mRNA. It is possible that haptoglobin and hemopexin are present in extramedullary hemopoietic cells which have been described in fetal brain (106). The observation that mRNA for transferrin was present in fetal and adult brain but not in the samples of either glioma or meningioma suggests that the transferrin may originate from neurons. This conclusion is supported by immunocytochemical findings (e.g. 58). Further immunohistochemical studies are required to identify the cells of origin of the various plasma protein mRNAs identified in this study. The findings presented in his report also demonstrate that ChEs and muscarinic binding sites are expressed in various regions of the fetal human brain with different developmental patterns, dependent both on the cell type composition and on the gestational age of the examined regions. This strongly suggests the existence of divergent regulation for ChEs and for

muscarinic acetylcholine binding sites in the developing human brain.

### 5.2 Divergent Regulation of Cholinesterases and Muscarinic Receptors in the Human Brain

The analysis of enzymatic activities and receptor binding assays is rather complicated in postmortem fetal brain tissues because of the incomplete knowledge regarding the biological background and the exact maturity stage of the fetuses. We therefore took several precautions in order to minimize these difficulties. Experiments were performed only in brain samples which appeared to be representative in terms of brain region size and protein concentration (107). Various inhibitors were added to the tissue homogenates to prevent destructive changes in ChEs during the prolonged incubations, and each analysis was carried out in parallel on several dissected brain areas from various developmental stages. The considerable [<sup>3</sup>H]4NMPB binding and enzymatic activities in these homogenates, the clear developmental patterns of the density of muscarinic binding sites and the general similarity of our findings with the reported properties of muscarinic receptors (25) and of ChEs (40) from postnatal human brain (see also 108) and the brains of other species suggest that these findings are reflective of the true *in vivo* composition of cholinergic constituents in the fetal human brain. It should be noted, however, that the amount of postmortem change that has occurred in each tissue sample remains unknown. This issue may be of significance for the developmental profiles of muscarinic receptor density and AChE activities. Thus, the decrease in the density of muscarinic receptors in several of the 22-week samples has been seen as a single observation on an otherwise continually, more smoothly changing profile for several regions. Likewise, in the cortical regions there is an abrupt increase in the 18-week samples. While these nondevelopmental, postmortem changes could play a significant role.

In previous studies, we observed that primary human brain tumors of gliogenous origin display both ChE activities (69, 40) and muscarinic binding properties (39), suggesting that both may be expressed also by nonneuronal cells. Furthermore, the ratio between ChE activity and the density of muscarinic receptors was higher by about 9-fold in dedifferentiated glioblastoma tumors present findings strongly suggest that high ratios between the levels of ChEs and muscarinic binding sites are not limited to the malignant state, but are common to nondifferentiated brain tissues in general. A survey of the literature shows that the regional distribution of muscarinic binding sites remains unchanged in the brain of Alzheimer's type dementia patients, in spite of the extensive loss in ChE (6). This is also observed, although to a limited extent, in the brains of elderly patients (109) and was confirmed by morphological analysis of senile degeneration areas in the brain (32). Altogether, this information implies that in brain areas where the cholinergic circuits are damaged, the ratio of ChE:mAChR should be considerably lower than that observed in the mature, healthy brain. Thus, ChE:mAChR ratios can be indicative both of the differentiation state and of the integrity of cholinergic functioning of an examined brain area.

It is clear that the excess ChE activity in nondifferentiated brain tissue is not required for operation in cholinergic circuits. This is not surprising in view of the accumulating evidence which suggests that a substantial portion of this enzyme in the brain is probably involved in processes unrelated to the hydrolysis of acetylcholine. Activities of ChEs

have recently been shown to be unaffected by blocking the cholinergic site with selective inhibitors. Of these, the most substantiated is the peptidergic activity on specific neuropeptides (for example, substance P and Met- and Leu-Enkephalins) (see 19). Thus, it is possible that in differentiating brain cells, ChEs are also involved in unrelated pathways, such as the specific hydrolysis of certain peptides, perhaps growth-related ones. It should be noted that growth-related functions have already been suggested for ChEs in a number of embryonic tissues of the thalamus in the primate brain (45). In the developing chicken brain, the transient appearance of ChEs in particular cell layers has been correlated with cell migration and tissue reorganization (44). In WRL-10A mouse fibroblasts (111) and in megakaryocytes (112, 113), ChEs appear to be related to cell division and growth. In addition, considerable levels of ChEs were detected in various neoplastic tissues, such as ovarian carcinomas (43) and brain tumors of glial and mesenchymal origin (40).

The detailed processes leading to the development of connectivities in the human brain in general and of its cholinergic circuits in particular are still largely obscure. Our present observations deepen the understanding of some aspects in this process and show that different molecules which participate in the functioning of cholinergic circuits appear at different rates, with various onset times and under divergent regulatory mechanisms in discrete areas of the fetal human brain.

### 5.3. Antiacetylcholinesterase Antibodies as Involved in Neuromuscular Dysfunction

The history, clinical course, laboratory tests, and bedside examinations of the patient described in this report were all indicative of defective transmission in neuromuscular junctions, with symptoms that were clearly distinguishable from those observed in all of the familiar disease categories with chronic muscle weakness and fluctuating course (114). However, the disease had features common with those reported for intoxication with anti-AChE agents in general (115, 116) and with organophosphorous poisons in particular (61, 62, 63). Furthermore, minute quantities of serum from the examined patient inhibited and precipitated the activity of AChE from various human tissues, with an efficiency similar to that reported by Marsh *et al.*, (66) for rabbit antibodies raised against purified rat brain AChE, and with special preference toward the membrane-associated fraction of muscle AChE. This inhibition depended on the presence of immunoglobulins and was not affected by protease inhibitors, confirming that it involved an immunological interaction. Altogether, these observations implied that AChE in neuromuscular junctions was the target for the primary lesion in this disease, and that antibodies against this enzyme played a major role in the etiology and the pathophysiology of the chronic neuromuscular dysfunction in this patient.

Inhibition of neuromuscular AChE by antibodies, as by other anti-AChE compounds, would lead to accumulation of acetylcholine in the synaptic cleft. This would cause a persistent depolarization and subsequent desensitization of the postsynaptic membrane, inducing muscle paresis (115, 116). Other mechanisms may also cause muscle and nerve injury, either directly, as was exhibited by the application of AChE-blocking agents (115, 116), or by secondary adaptation phenomena, as was demonstrated in numerous neuromuscular disorders (117-122). An example of the extensive damage which can be caused by antibodies to neuromuscular functioning is

the effect elicited by antibodies against the acetylcholine-synthesizing enzyme, choline acetyl transferase (123). All of these forms of lesions join to create the impairment of neuromuscular junctions which is manifested in the examined patient. The histological was consistent both with neuropathy and with myasthenic-like lesions (119), in agreement with the role attributed to anti-AChE antibodies in this case. An important conclusion to be drawn from this research is that both histological analysis of muscle biopsies and electrodiagnostic measurements may indicate the existence of a neuromuscular disease, but are not sufficient to diagnose conclusively which of the components of the neuromuscular junction was subject to the primary injury.

Anti-AChE antibodies may recognize an epitope which is common to all of the forms of ChEs in various tissue and cell types (8). In this case, the specific activity of both serum ChE and red blood cell AChE would be considerably reduced, as was the case. When most of the ChE activity is inhibited, complete block of the remaining minute amounts of functioning enzyme by an anti-AChE drug such as edrophonium would lead to loss of muscle control and add autonomic symptoms to the clinical picture, in contrast with the improvement effect which this drug has on myasthenic patients. However, the blood-brain barrier would prevent the anti-AChE antibodies from blocking the brain enzyme, in agreement with the lack of higher function impairment which was observed in the examined patient. Our experiments demonstrated that the antibodies detected in the examined serum were capable of inhibiting and immunoprecipitating membrane-associated AChE from various human tissues, with preferential interaction toward the membrane-associated AChE from muscle. This would be expected from antibodies against an immunogenic epitope common to multiple ChE forms, but preferentially exposed in the membrane-associated muscle enzyme. Alternatively, or in addition, there could be different types of anti-AChE antibodies in the diseased serum. Some of these would be directed against common epitopes, but would display weak binding, while other anti-AChE antibodies in the same serum would exclusively interact with the membrane-associated forms of muscle AChE with high efficiency (for example, these could recognize the collagen-like "tail" specific to these forms).

Two cases in which the reported symptoms were close to those of the examined patient are both related to defects in AChE expression. These are a congenital defect specific to the  $\alpha$ -tailed form of muscle AChE (122) and the development of anti-AChE antibodies in a myasthenic patient who suffered from thymoma (124). It is difficult to postulate, based on our observations, what caused the appearance of anti-AChE antibodies in the presently described case. These could be primarily elicited against the enzyme itself, either from an external or from an autologous origin, in the form of an autoimmune disease. Additional regulatory mechanisms, such as anti-idiotypic antibodies against these primary anti-AChE ones, may then complicate the course of the disease and induce fluctuations in the intensity of inhibition. The presence of anti-idiotypic antibodies in myasthenia gravis has already been demonstrated (125-127).

Another putative origin for this disease could be a complex immunological response to prolonged, subacute intoxication by organophosphate poisons. The increased use of these compounds as insecticides in Israel is reflected in a number of recent cases of chronic subacute poisoning (128), and it is possible that in other cases anti-organophosphate antibodies would be elicited. If these act also as antigens, anti-idiotypic

antibodies would appear as well. These, in turn, could interact with the organophosphate binding site which is common to many ChEs (17) and inhibit their enzymatic activity. Furthermore, the diseases being primarily due to an anti-idiotypic reaction would also be compatible with the fluctuating nature of the clinical symptoms. Further experiments should be carried out to distinguish between these alternatives.

Several questions of considerable importance to this research remain open, partially since the only material available for our experiments was a very limited amount of the patient's serum. For example: (a) It is not clear whether the appearance of anti-AChE antibodies is an exceptional phenomenon or whether it is a relatively prevalent one, which has not been studied before because of inhibitory anti-AChE antibodies would also block the acetylcholine-binding site in the acetylcholine receptor; testing for interference with the binding of  $^{125}\text{I}$ -alpha-bungarotoxin would not exclude this possibility. (c) The postulated neuromuscular defects related to cholinergic constituents, should be tested. Finally, the putative role of organophosphate poisons as inducers of immunological response and neuromuscular diseases is of considerable ecological importance.

## 6. CONCLUSIONS

1. In cholinergic synapses and neuromuscular junctions, organophosphate poisons (OPs) bind to the acetylcholine-hydrolyzing enzyme, AChE, and block its catalytic activity. To study this phenomenon in detail, we aim to isolate and characterize the human AChE gene and use the cloned gene to produce AChE in large quantities and test its protective value against OP.

2. In different species and cell types, there are polymorphic ChEs, with different properties. However, all of these hydrolyze acetylcholine. To study the nucleic acids producing AChE, we first extracted mRNA from ChE-expressing tissues, size-fractionated it and microinjected it into oocytes of *Xenopus laevis* frogs. Differently sized human brain mRNAs induced the biosynthesis of catalytically active AChE in oocytes, indicating that ChE polymorphism extends to the level of mRNA as well.

3. We have then isolated a fragment of the human AChE gene by its homology to DNA from the AChE-regulating Ace locus in the fruit fly, *Drosophila melanogaster*. To focus on the DNA sequence which actively codes for the ChE protein, we synthesized oligodeoxynucleotides according to the consensus sequence of the OP-binding hexapeptide as determined in several laboratories by peptide sequencing. Using these synthetic oligodeoxynucleotides, we have isolated two partial cDNA clones (730 and 1250 nucleotides long) which contain this sequence and which are expressible in their bacterial hosts into proteins immunoreactive with anti-AChE antibodies. The products of the partial cDNAs do not bind OPs or hydrolyze acetylcholine. We therefore isolated longer cDNAs (up to 3300 nucleotides) from human fetal brain, using the same approach. These cDNAs and their bacterial-expressed proteins are currently characterized in detail.

4. To achieve, eventually, the expression of AChE cDNA into the active human enzyme, we also studied the post-translational processing of nascent AChE molecules within microinjected oocytes. Crossed immunoelectrophoresis of  $^{35}\text{S}$ -labeled oocyte proteins against anti-ChE sera indicated that additional, tissue-specific proteins take part in these processes. This

suggests that we may need an expression system of mammalian origin to produce mature AChE from cloned AChE cDNA.

5. To use such a clone-originated AChE for therapeutic purposes, it will be important to prevent immunological reaction against the injected enzyme. For this reason, we searched for the appearance of anti-AChE antibodies in humans.

We discovered autoimmune antibodies against human muscle membrane AChE in a case of severe neuromuscular dysfunction. The prevalence and characteristics of this phenomenon are currently being studied.



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