PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 33/573, 33/68

A1

(11) International Publication Number: WO 93/05398

(43) International Publication Date: 18 March 1993 (18.03.93)

(21) International Application Number: PCT/GB92/01650

(22) International Filing Date: 9 September 1992 (09.09.92)

(30) Priority data:
9119240.1 9 September 1991 (09.09.91) GB

(71) Applicant *(for all designated States except US)*: KODAK CLINICAL DIAGNOSTICS LIMITED [GB/GB]; Mandeville House, 62 The Broadway, Amersham, Buckinghamshire HP7 0HJ (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : BRENNAND, David, Mark [GB/GB]; 45 Bradford Road, Coombe Down, Bath, Avon BA2 5BW (GB).

(74) Agents: WOODS, Geoffrey, Corlett et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

(81) Designated States: AU, CA, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: ENZYME CAPTURE ASSAY

(57) Abstract

A method of determining human secretory acetylcholinesterase (sAChE) in a test sample, which method comprises contacting a ligand which is capable of binding sAChE and which is not an antibody with the sample and determining wether human sAChE has bound to the ligand; and a method of determining an enzyme in a test sample, which method comprises: i) bringing into contact the sample and a ligand which is immobilised on the surface of a solid or matrix support, which ligand is: a) a substrate or substrate analogue of the enzyme; b) an effector or effector analogue of the enzyme; c) a co-factor or co-factor analogue of the enzyme; d) a co-enzyme or a co-enzyme analogue of the enzyme; or e) a prosthetic group or prosthetic group analogue of the enzyme; and ii) determining whether the enzyme has bound to the ligand.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland			SE	Sweden
CI	Côte d'Ivoire	KR	Republic of Korea	SK	Slovak Republic
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CS	Czechoslovakia	LK	Sri Lanka	SU	Soviet Union
CZ	Czech Republic	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America

ENZYME CAPTURE ASSAY

This invention relates to a method and a test kit for determining an enzyme in a sample, especially human secretory acetylcholinesterase. The determination of human secretory acetylcholinesterase enables Neural Tube Defects or Alzheimer's disease to be detected.

Neural Tube Defects (NTDs) are a number of structural anomalies of the central nervous system (CNS) of the human fetus. They result from the failure of the neural tube to close properly during embryogenesis. There are three major types of NTD:

(1) Anencephaly: where the closure defect can occur at the top of the neural tube. It results in babies being stillborn.

(2) Encephalocele: is relatively uncommon, with the closure defect occurring a little lower down the neuroaxis. Babies do survive with little paralysis, and they usually grow up spastic, severely retarded and often blind.

(3) Spina bifida: where the closure defect occurs lower still resulting in babies being born with a portion of the spinal cord exposed. This results in paralysis of the legs, incontinence and hip, knee and feet deformities. About 90% of these babies will die if not operated on.

NTD incidence varies considerably throughout the world. In Europe, the prevalence is 6-13 per 10,000 births. Currently NTD's are diagnosed by:

(1) Genetic counselling: by checking the family history to find out if a couple is in a risk group, i.e. if they have a previously affected child or parent or close relative. A drawback of this method is that over 95% of all NTDs occur in couples not previously considered at risk from this disorder.

(2) Ultra-sound scanning (US): has proved very useful in identifying many fetal abnormalities. However,

there are some drawbacks:

(a) US has 100% accuracy in detecting anencephaly but only 80% for spina bifida.

(b) It relies on the experience of the operator of the scanner.

(c) Misdiagnosis occurs if scanning is done at a very early gestational age.

(3) Alpha-fetoprotein (AFP): is a glycoprotein with a molecular weight of 70,000 Daltons. It is usually secreted by the embryo yolk sac and later by the fetal liver. It diffuses into the Amniotic Fluid (AF) and then into the maternal circulation. AFP levels were found to be elevated in maternal serum of women with NTD fetuses. Currently, its measurement by an immunoassay is the main diagnostic test, in conjunction with US, for NTD.

Normally, AFP is measured in maternal serum. An elevated value is followed by US scanning, then by amniocentesis. The amniotic fluid (AF) is then tested for AFP. However, there are problems with AFP:

(a) It requires a precise knowledge of gestational age since the normal levels of AFP vary in maternal serum considerably with gestational age.

(b) AFP levels in AF also vary with gestational age. Furthermore, a major problem with assaying AFP in AF is the likelihood of a false positive due to contamination of the AF samples with fetal blood and/or maternal blood.

A U.K. collaborative study has found that only 80% of NTD are detected by AFP measurements.

(4) Neural-specific acetyl cholinesterase (AChE): since the main defect in NTD is the incomplete closure of the neural tube, many of the neurally-derived proteins and other components are expected to be found in the AF and to diffuse into maternal serum. These could, therefore, be potential markers for NTD. In 1979, Smith et al. (Lancet, i,685,1979) proposed the analysis of AChE in AF as a supplementary test to improve the diagnosis of NTD. Cholinesterases are a group of enzymes that hydrolyse cholinesters into choline. Pseudo-cholinesterases, which are present in serum, muscle and brain, have a substrate

preference to butyryl cholines. Acetyl cholinesterases (AChE) have a substrate preference to acetyl cholines. The two types are different immunologically and biochemically. AChEs are of multiple forms, globular and asymmetric forms. The latter are found predominantly in muscle tissue. Globular forms are dominant in the central nervous system and red blood cells (RBC). They exist as a monomer, dimer, or tetramer. Globular AChEs are membrane-bound or soluble.

In human brain, about 70% of AChE is membrane-bound and 100% of red blood cells AChE is membrane-bound. The central nervous system soluble AChE is in itself of multi-forms. The bulk of it is believed to be derived from the membrane-bound AChE as is shown by its behaviour in the presence of detergents.

One type of the neural soluble AChE is known as the secretory AChE (sAChE). It is secreted by neural cells, has a distinct electrophoretic mobility, is the only form of AChE found in mammalian cerebrospinal fluid (CSF), is not influenced by detergents and is the only form that binds selectively to the AChE inhibitor edrophonium chloride when this is immobilised on Sepharose (Trade Mark)-4B gel. The isolation and partial characterisation of the secretory form of human brain AChE has been described (Gardner et al., Biochem.Soc.Trans., 14,1234-1235,1986).

In amniotic fluid (AF), there are three forms of AChE present in addition to pseudo-ChE. A monomer (4.0 S), a dimer (5.5 S), and a tetramer (10.3 S). The origin of the first two is not clear. However, the dimer is very much similar to the dimer membrane-bound AChE found in red blood cells (RBC). The tetramer form is the neural-specific form, the secretory AChE (sAChE). The amount of this form goes up to 62-fold in NTD.

AChE is currently detected in AF in three ways. These are:

(a) Enzymatically: by using the standard Ellman test for assaying esterases. The test requires the use of highly specific inhibitors in order to be able to distinguish between AChE and pseudo-ChE. It cannot distinguish between the different forms of AChE. Hence, it is useless in cases where the AF samples are contaminated by fetal or maternal blood.

A recent modification to this test involves a prior separation of the different molecular forms by sucrose density gradient centrifugation followed by the Ellman test. This makes the test complicated requiring ultra-centrifuges, and overnight spins. Furthermore, it does not distinguish between the sAChE and other brain-derived AChE with similar molecular weight.

(b) Gel electrophoresis and histochemical staining: this is the standard procedure currently employed in clinical laboratories. It relies on the electrophoretic mobility of the sAChE. AF samples are electrophoresed on 6% acrylamide gels which are subsequently stained for esterase activity. Specific AChE activity is identified by using a specific inhibitor. A U.K. collaborative study has shown this test to be very useful, finding that AChE levels do not vary with gestational age as in the case with AFP. Therefore, precise knowledge of fetal age is not necessary.

There are a number of drawbacks with this procedure, though:

(i) It is a qualitative test.

(ii) The test is useless if AF samples are contaminated with fetal or maternal blood because serum pseudo-ChE and RBC AChE mask the specific sAChE band on the gel.

(iii) The test is time-consuming as it requires electro-phoresis and histochemical staining.

(iv) The test requires the collection of amniotic fluid in a procedure which carries a risk of causing a miscarriage of the pregnancy.

(c) Immunological testing: many different antibodies, both polyclonal and monoclonal, have been raised against different forms of AChE. Many of these do not cross react with pseudo-ChE. Zaneta et al (FEBS Lett.129,293,1981) raised a rabbit antiserum against rat brain membrane AChE, which had only low cross reactivity with soluble AChE. Gennari and Brodbeck (J. Neurochem. 44,697,1985) reported an antiserum raised to detergent soluble AChE from human brain which showed a moderate preference for detergent soluble versus the water soluble enzyme. An antibody raised to rabbit AChE binds the membrane-bound form of brain AChE better than the soluble form (Rakonczay and Brimijoin, BBA 832, 127,1985).

Immunological differences between AChEs of different tissues are also coming to light. Most antibodies raised to AChE show equivalent affinity for brain and erythrocyte enzymes from the same species. Rasmussen et al (Clin.Chim. Acta,166,17,1987) evaluated 11 monoclonal antibodies raised to human RBC AChE with respect to reactivity with AChE from both RBC and brain. They claimed that one of their monoclonals actually showed preference for membrane-bound AChE purified from human brain, but all their monoclonal antibodies reacted with RBC AChE.

There are few reports on the use of immunoassays of AChE in the diagnosis of NTD. The main problems are the difficulty of obtaining specificity to the neural ~~and~~ more exactly, the secretory AChE. Reported assays rely on the use of either monoclonal or polyclonal antisera raised to human brain AChE. The assays are of two main types.

In the first type of assay, the antigen, AChE, is sandwiched between two monoclonal or one monoclonal and one polyclonal antibodies (Brimijoin et al., J. Neurochem.49, 555,1987). The second type is also of the antigen-capture type but relies on the AChE's own enzyme activity. Monoclonal anti-RBC AChE antibodies are immobilised followed by incubation with test sample. Bound AChE is detected by using the Ellman test (Norgaard Pedersen et al, Clin.Chim.29,1061,1983). This second approach does not require pure AChE. Varying degrees of success have been reported for these assays when used to diagnose NTD (Norgaard et al.,1983; Brock and Bader,Clin.Chim.Acta 127, 419,1983; Brimijoin et al, Fed.Proc.46,2557,1987; Brock et al., Lancet i,5,1985; Sorensen et al., Prenat.Diag 7,75, 1987; Wald et al, Prenatal Diagnosis 9, 813-829, 1989; Rasmussen et al Prenatal Diagnosis 10, 449-459, 1990). These assays do not work properly however when red blood cell esterase is present, even in relatively low quantities.

It is possible that the raised levels of sAChE in the amniotic fluid of a pregnancy affected with abnormality may manifest themselves as a rise in the circulating maternal serum. Measurement in maternal serum has numerous advantages over the measurement in amniotic fluid, since the collection of the latter causes a significant risk of miscarriage. Measurement of this material has not until recently been described on account of the difficulties in devising a suitably specific assay.

Recent work by Jehanli, McBride and Brennand (WO91/08302) showed how antibodies specific for sAChE can be used to detect sAChE in samples even where red blood cell esterase is also present i.e human serum and amniotic fluid.

AChE from the electropax of Electrophorus electricus (eel) has been shown to bind to both arginine-Sepharose as well as edrophonium-Sepharose (Small et al, Neuroscience 21, 991-996, 1987). There is also evidence that AChEs purified from some sources (eel electropax organ and foetal bovine serum) possess protease activities (Small et al, 1987 and Small, 1989 Neuroscience Lett. 95, 307-312, 1989). This work has led to suggestions that some AChEs may be zymogens (i.e. inactive precursors) of proteases (Small, D.H., Neuroscience 29, 241-249, 1989).

Previous molecular modelling of peptidyl-lysine and peptidyl-arginine structures has demonstrated the possible similarities in orientation of the C-terminal peptide bond to the ester bond in acetylcholine, with respect to a centre of positive charge. From this a model by which a C-terminal arginine or lysine residue might fit into the same active site pocket of AChE as that used for the hydrolysis of acetylcholine has been suggested for some AChEs (Small 1990

Trends Biochem. Sci 15, 213-216). The author of this work has however recognised the considerable structural polymorphism of AChEs with their many different forms varying in molecular weight, solubility and amino-acid sequence. The above model cannot therefore be considered generally applicable.

Normal cleavage of the transmembrane amyloid precursor protein (APP) occurs at or near lysine 16 of the βA4 sequence of the protein (Esch, F.S., Kein, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D., and Ward, P.J. Science 248, 1122-1124, 1990). This normal cleavage would prevent formation of the βA4 fragment which is amyloidogenic thus preventing the occurrence of the amyloid deposits found in the brains of patients with Alzheimer's disease. The protease necessary for this cleavage although previously not identified, has been referred to as "APP secretase".

Small, D.H., Moir, R.D., Fuller, S.J., Michaelson, S., Bush, A.I., Qiao-Xin Li, Milward, E., Hilbich, C., Weidemann, A., Beyreuther, K., and Masters, C.L. in Biochemistry 30 10795-10799, 1991 purified sAChE from human brain by a method using using an edrophonium-Sepharose column. They called this purified material AChE-associated protease (AChE-AP) and showed that it has the function of the missing "APP secretase". They showed that the human AChE-AP so purified cleaved the βA4 protein sequence adjacent to lysine 16 of the βA4 sequence.

This work also shows that peptidyl-lysine is a substrate for the enzyme sAChE. It also shows how in Alzheimer's disease loss of sAChE activity or a deficiency in levels of the enzyme may result in localised loss of ability to cleave APP within the βA4 sequence. This itself would lead to an increased concentration of the full length form of APP so increasing the likelihood of amyloid deposits forming. Proteolysis of APP results in the secretion of a 100-110K ectodomain which may hold a Kunitz type protease which may be able to inhibit the sAChE activity itself. This may be an alternative route by which sAChE activity is lowered in localised areas of the brain. Alternation of sAChE, perhaps by proteolytic cleavage to an anomalous form, may also explain a loss of activity. Recently, an anomalous molecular form of AChE has been demonstrated in the cerebrospinal fluid of patients with Alzheimer's disease (Navaratnam, D.S., Priddle, J.D., McDonald B., Esiri, M.M., Robinson, J.R., and Smith, A.D. Lancet 337, 447-450, 1991.

No *in vitro* clinical diagnostic test is currently available for the diagnosis of Alzheimer's disease. Such a test will form an invaluable tool for clinicians in the management of dementias.

According to the present invention, there is provided a method of determining human secretory acetylcholinesterase (sAChE) in a test sample, which method comprises contacting a ligand which is capable of binding sAChE and which is not an antibody with the sample and determining whether human sAChE has bound to the ligand.

There is also provided a method of determining an enzyme in a test sample, which method comprises:

i) bringing into contact the sample and a ligand which is immobilised on the surface of a solid or matrix support, which ligand is:

a) a substrate or substrate analogue of the enzyme;

b) an effector or effector analogue of the enzyme;

c) a co-factor or co-factor analogue of the enzyme;

d) a co-enzyme or co-enzyme analogue of the enzyme; or

e) a prosthetic group or prosthetic group analogue of the enzyme; and

ii) determining whether the enzyme has bound to the ligand.

There is additionally provided a method for determining antibodies to sAChE in a test sample, which method comprises:

a) contacting the sample with sAChE which is prebound to a ligand; and

b) measuring bound antibody.

This may be a test to, for example, measure antibody in serum or monoclonal antibody secreted by a cell line. The ligand in this test method may be, for example, any of the ligands used in the previous methods.

The term "antibody" in the above methods includes immunoreactive antibody fragments such as Fab fragments.

An enzyme capture assay according to the invention can be used to measure levels of an enzyme, especially human sAChE, in biological fluids and other samples. The assay includes, as its first step, the coupling or binding onto a solid or matrix support of the ligand to which the enzyme, e.g. sAChE, is able to actively bind. This enables the enzyme to be actively bound from a solution, or biological fluid. By inclusion of a further step consisting of a detection stage (i.e. via an antibody capable of binding to the enzyme, for an Enzyme Capture ELISA), an assay is produced which is able to measure quantitatively levels of the enzyme, e.g. sAChE, in a sample, solution or biological fluid.

The method according to the present invention when applied to sAChE can be used in the diagnosis of NTDs and, in particular, anencephaly, encephalocele and Spina bifida. The method can also be used in the diagnosis of omphalocele, gastroschisis, intrauterine death, esophageal atresia, twin pregnancy with acardiac fetus, normal infant, teratoma, ascites, cystic hygroma, hypoplasia of heart and lungs, cloacal exstrophy, hydrocele, epidermolysis bullosa-dystrophica, and aplasia cutis congenita: as well as other neurodegenerative disorders such as senile dementia, Parkinson's disease, and Alzheimer's disease. The method may also be used in the diagnosis of Down's Syndrome. In each case, diagnosis may depend upon detecting and measuring levels of sAChE. For the diagnosis of these conditions the ligand must bind AChE either the foetal form or the adult form or both.

The method of the present invention may, for example, be used to diagnose Neural Tube Defects in a fetus. The presence of Neural Tube Defects is indicated by, for example, increased sAChE in the amniotic fluid, maternal serum or cerebrospinal fluid. The method may also, for example, be used to diagnose Alzheimer's disease in an adult. The presence of Alzheimer's disease is indicated by, for example, decreased sAChE in human serum and abnormal levels of sAChE in cerebrospinal fluid.

The present method can be used as a screen to be used while raising monoclonal antibodies specific for the enzyme determined, e.g. human sAChE.

An enzyme such as adult or fetal human sAChE can therefore be assayed using the present invention. A sample suspected of containing the enzyme is contacted with the ligand. The sample may be a biological fluid such as cerebrospinal fluid, or amniotic fluid or human serum. The sample may be diluted in a buffer such as phosphate buffered saline (PBS). A detergent may be present such as Tween.

The sample may be mixed with high ionic strength buffer in order to increase the ability of the assay to distinguish between samples containing raised levels of the enzyme, e.g. sAChE, and normal samples. The sample may therefore be mixed with a solution having a higher ionic strength than that of physiological saline. The conductivity of the solution may be greater than that of physiological saline. PBS having an ionic strength at least as great as that of 0.15M NaCl may be used. The ionic strength may be that of 0.15 to 1M, preferably 0.15 to 0.5M and more preferably from 0.2 to 0.4M, NaCl. If a Tris (trade mark) buffer is employed, preferably the buffer is at least 25mM Tris buffer.

An assay may be performed qualitatively, semi-quantitatively or quantitatively. A variety of assay formats may be employed. These may be simultaneous or sequential assay formats. The ligand can be used to capture the enzyme, e.g. adult or fetal human sAChE, from solution selectively onto a surface separable from the solution such as a solid surface or matrix to label selectively this enzyme or both to capture and to label this protein. The ligand also may be used in a variety of homogeneous assay formats in which the enzyme is detected in solution with no separation of phases.

The types of assay in which the ligand is used to capture the enzyme from solution involve immobilization of the ligand onto a surface. This surface should be capable of being washed. The types of surfaces which may be used include polymers of various types (moulded into Microtiter (trade mark) wells; beads; dipsticks; aspiration tips; electrodes; and optical devices) a capillary fill device, carbon discs (for an electrochemical enzyme immunoassay); particles (for example latex; stabilized red blood cells (erythrocytes); bacterial or fungal cells; star-burst dendrimers; spores; gold or other metallic or metal-containing sols; organic sols; and proteinaceous colloids; with the usual size of the particle being from 0.005 to 5, for example from 0.1 to 5, microns), membranes (for example of nitrocellulose; paper; cellulose acetate; chemically-activated membranes such as Millipore Immobilon (Trade Mark) or Pall Biodyne (Trade Mark); and high porosity/high surface area membranes of an organic or inorganic material).

The attachment of the ligand to the surfaces can be by passive adsorption from a solution which may include surfactants, solvents, salts and/or chaotropes; or by active chemical bonding. Active bonding may be through a variety of reactive or activatable functional groups which may be exposed on the surface (for example condensing agents; active esters; acid halides; anhydrides; amino, hydroxyl, or carboxyl groups; sulphydryl groups; carbonyl groups; diazo groups; epoxy groups; unsaturated groups).

The ligand used for binding to the enzyme in the general assay method of the invention is a substrate or substrate analogue, an effector (e.g. an inhibitor (competitive or non-competitive) or activator of, for example, competitive, allostearic or isostearic sites) or effector analogue, a co-factor or co-factor analogue, a co-enzyme or co-enzyme analogue or a prosthetic group or prosthetic group analogue of the enzyme. The analogues may be, for example, synthetic analogues.

The ligand which can be used to determine human sAChE or antibodies to sAChE is typically a positively charged compound, or a positively chargeable compound, capable of binding to an anionic locus of human sAChE. The ligand may, for example, be a substrate, effector, co-factor, co-enzyme or prosthetic group, or an analogue thereof, as mentioned above, for human sAChE, especially a substrate, inhibitor or activator for human sAChE. An inhibitor of esterase activity such as edrophonium chloride can be employed. Alternatively the ligand may comprise an amino acid such as arginine, histidine, lysine or ornithine which is capable of being positively charged. The ligand may comprise two or more such amino acids, for example as a dimer or other polymer. The amino acid may be in D- or L-form.

A useful ligand is a polymer, typically a homopolymer, of an amino acid capable of being positively charged. A ligand may therefore be selected from poly-L-arginine, poly-L-histidine, poly-D-lysine, poly-L-lysine or poly-L-ornithine. A mixture of two or more such ligands may be employed. Such polyamino acids may have a molecular weight of from 350 to 1,000,000, for example 4,000 to 300,000. Polyamino acids adsorb easily onto plastic surfaces to facilitate presentation of the ligand. Further, multiple copies of the ligand (i.e. the amino acid) are simultaneously presented for sAChE to bind to.

After contacting (reacting) the surface bearing the ligand with a test sample, allowing time for reaction and, where necessary, separating or removing the excess of the sample by any of a variety of means (washing, centrifugation, filtration, application of a magnetic field, capillary action), the captured enzyme is detected by any means which will give a detectable signal. For example, this may be achieved by use of a molecule, in particular monoclonal or polyclonal antibody, or particle as described above which will react with the captured enzyme. The antibody is capable of binding to, and typically is specific for the enzyme, e.g. human sAChE. The molecule or particle may be labelled or may be capable of being labelled. For example, a first unlabelled antibody capable of binding to the enzyme, e.g. human sAChE, may be used followed by a second labelled antibody which is capable of binding to the first antibody. Alternatively, the activity of the immobilised enzyme, e.g. AChE, can be assayed.

The monoclonal antibody may be a monoclonal antibody which is specific for the enzyme but which shows no cross-reactivity with other types of enzyme, for example one which is specific for human sAChE but which shows no cross-reactivity with other types of human AChE according to WO 91/08302. The monoclonal antibody may therefore show no reactivity with human red blood cell AChE, and preferably no cross-reactivity with human serum AChE, human membrane-bound neuronal AChE, human muscle AChE and neuronal-soluble non-secretory AChE. The antibody does not cross-react either with human serum pseudo-ChE. The monoclonal antibody may be specific for fetal human sAChE or adult human sAChE. The monoclonal antibody may be IgA, IgD, IgE, IgG or IgM. It may be a monoclonal antibody of any animal species, for example a mammalian monoclonal antibody such as a rat, mouse or human monoclonal antibody. A polyclonal antibody specific for human sAChE may be employed instead of a monoclonal antibody. The polyclonal antibody may be raised in an appropriate animal, typically mammal, for example mouse, rat, sheep, goat or rabbit.

The detectable signal may be optical or radio-active or physico-chemical and may be provided either directly by labelling the molecule or particle, especially antibody, referred to with for example a dye, radiolabel, electroactive species, magnetically resonant species or fluorophore, or indirectly by labelling the molecule or particle with an enzyme itself capable of giving rise to a measurable change of any sort. Alternatively the detectable signal may be due to, for example, agglutination, diffraction effect or birefringent effect occurring if any of the surfaces referred to is in the form of particles.

A preferred assay format is the sandwich assay. A test sample may be brought into contact with a ligand which is capable of binding to the enzyme, e.g. fetal human sAChE, and which is immobilised on a support separable from the sample, for example on a solid support. The ligand captures the enzyme in the test sample onto the support and an antibody capable of binding to the enzyme labels the captured enzyme. The antibody may be a monoclonal antibody or a polyclonal antibody. The capturing and labelling operations may be performed in any order or simultaneously. The antibody may be labelled with an enzyme such as an alkaline phosphatase or peroxidase or ß-galactosidase.

A useful sandwich assay comprises:

(i) bringing into contact the sample and a said ligand which is immobilised on the surface of a solid support;

(ii) washing the surface of the solid support;

(iii) contacting the washed surface with a first unlabelled antibody which is capable of binding to the enzyme, e.g. human sAChE;

(iv) further washing the surface of the solid support;

(v) contacting the further washed surface with a second antibody which is labelled and which is capable of binding to the first antibody;

(vi) yet further washing the surface of the solid support; and

(vii) detecting the presence or absence of the label of the second antibody on the yet further washed surface.

Another useful sandwich assay format involves contacting a test sample with enzyme-labelled antibody, capturing the resulting immune complex onto a solid surface using a ligand capable of binding to the enzyme, e.g. human sAChE, removing any excess labelled antibody and adding a substrate for the enzyme label in the enzyme-labelled antibody. The presence of the enzyme, e.g. the human sAChE sample, is thus revealed.

Other formats which may be employed are any of those suitable for assays including (1) agglutination with the ligand adsorbed onto particles of, for example, polystyrene latex; (2) an enzyme-linked immuno-adsorbant assay (ELISA) carried out in microtitre plates, (3) a dipstick ELISA with a ligand-coated dipstick, and (4) sandwich assays using small magnetic particles coated with ligand together with the antibody or ligand labelled either with coloured particles, or particles with the potential of colour development, or with an enzyme or a fluorescent moiety.

Test kits suitable for use in determining an enzyme, e.g. human sAChE, in a sample comprise the ligand as defined above and means for detecting the enzymes, e.g. human sAChE, when bound to the ligand. Means are therefore provided for determining whether the ligand, in use, binds to the enzyme, e.g. human sAChE. Specific components of the kits may be as described above. The kits may also comprise one or more additional components including one or more control, one or more buffer and one or more diluent. A standard curve may be provided.

A kit for use in an enzyme-immunoassay typically includes an enzyme-labelled reagent and a substrate for the enzyme label. The enzyme label may either be bound to ligand which can bind to captured enzyme, e.g. human sAChE, or be bound to polyclonal or monoclonal antibody capable of binding to the captured enzyme, e.g. human sAChE. Thus, the means for detecting enzyme, e.g. human sAChE, bound to the ligand may comprise an enzyme-labelled antibody which is capable of binding to the enzyme, e.g. human sAChE, and a substrate for the enzyme label in the enzyme-labelled antibody. Alternatively the means for detecting the enzyme, e.g. human sAChE, bound to the ligand may comprise a first unlabelled antibody which is capable of binding to the enzyme, e.g. human sAChE, a second enzyme-labelled antibody which is capable of binding to the first antibody and a substrate for the enzyme label in the second enzyme-labelled antibody.

The following Examples illustrate the invention. Two Reference Examples are also provided. In the accompanying drawings:

Figure 1 shows the results of the Enzyme Capture ELISA of Example 4 in which concentration of added sodium chloride (M) (x-axis) is plotted against optical density at 405nm, • denotes cerebrospinal fluid (CSF), ♦ denotes spina bifida serum A, * denotes anencephalic serum C, ▲ denotes normal serum E, ■ denotes normal serum D and ▼ denotes lysed normal serum.

Figure 2 shows a typical cerebrospinal fluid reference curve employed in Example 6 in which sAChE units (x-axis) are plotted against optical density at 405nm.

Figure 3, shows sAChE levels in normal pregnancy (Southampton samples). The results are expressed as mean values ± twice the standard deviation. The numbers of samples in each group are given in brackets. The number of weeks gestation (x-axis) is plotted against sAChE units (y-axis).

Figure 4 shows sAChE (upper line) and AFP (lower line) results in NTD cases expressed as multiples of the median, ▪ denoting anencephaly and • denoting spina bifida.

Figure 5 shows sAChE levels in units for NTD pregnancies relative to the upper limit of normal (mean + twice the standard deviation, or 97.5 centile) of equal to 43.5 units, the symbols being the same as for Figure 4.

REFERENCE EXAMPLE 1: Isolation and Partial Characterisation of Human Adult and Fetal sAChE

1. EXPERIMENTAL PROCEDURES

Materials

All chemicals were of analytical grade and purchased from Sigma Co., Poole, U.K.

Biological material

Cerebrospinal fluid (CSF) and human adult brain were obtained from the Department of Clinical Chemistry, Southmead Hospital, Bristol, U.K. Human fetal brains were dissected from 12-18 week old aborted fetuses. In both cases tissues were frozen at -70°C within 1h of dissection.

Preparation of tissue homogenate

Extraction of soluble AChE was carried out by thawing the brain tissue and homogenising in 10 volumes (adult brain) or 5 volumes (fetal brain) per weight of ice-cold 0.3M sucrose containing 2 mM EDTA, in a Waring blender (5 x 30s) at 4°C. The homogenate was subjected to two cycles of freezing and thawing to disrupt the membranes and then centrifuged at 100,000 x g for 90 min at 4°C. The supernatant was removed, aliquoted and stored at -70°C until required.

Gel filtration of tissue supernatant

Gel filtration was carried out on Sephacryl (Trade Mark) S-200 (Pharmacia, Uppsala, Sweden) at 4°C. Supernatant samples (40 ml) were applied to a 2.5 cm x 100 cm column equilibrated in phosphate buffered saline, pH 7.3 (phosphate buffered saline (PBS), 142 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.47 mM KH_2PO_4) containing 0.02% NaN_3. Elution was by upward flow at a rate of 50 ml/h. Fractions (5 ml) were collected and monitored for (i) protein content by measuring the optical density at 280 nm and (ii) acetylcholinesterase activity by the Ellman assay (see below). The column was calibrated with a Pharmacia High Molecular Weight kit.

Affinity purification of sAChE

sAChE in the gel filtration eluant was purified further by affinity chromatography on a column of edrophonium chloride-epoxy-Sepharose gel (Pharmacia) prepared according to Hodgson and Chubb (J.Neurochem.,41,654-662,1983). Samples (25 ml) were recycled through a 1 cm x 4 cm column equilibrated in PBS, at a flow rate of 15 ml/h for 24h at 4°C. Unbound material was eluted with PBS containing 0.5 M NaCl (40 ml). The bound material was then eluted with the same buffer containing 12 mM edrophonium chloride (25 ml). Edrophonium chloride was removed by gel filtration on a Sephadex (Trade Mark) -G50 fine (Pharmacia) column (2.5 cm x 50 cm) equilibrated in ammonium acetate solution (50 mM). Fractions containing AChE activity as determined by the Ellman assay were pooled, lyophilised, reconstituted in PBS (2 ml) and aliquots frozen at -20°C until required.

Enzyme assays

Cholinesterase activity was measured at 30°C by the method of Ellman et al. (Biochem.Pharm.,7,88-95,1961).

Enzyme activity is expressed as IU (micromoles product formed per minute). Acetylthiocholine iodide (1 mM) was used as substrate. AChE activity was taken as that activity which was inhibited by 1,5-bis-(4-allyldimethyl-ammonium phenyl)-pentane-3-one dibromide (BW 284 C51; 1.5 μM), while pseudocholinesterase (PsChE) activity was estimated as the difference between two reaction mixtures, one containing eserine (20 μM), the other BW 284 C51 (Hodgson and Chubb, 1983).

Gel electrophoresis analysis of AChE activity

AChE activity in different samples was located on 6% polyacrylamide slab gels (8 cm x 8 cm) by the histochemical method of Coupland and Holmes (Q.J.Microsc.Sci.,98,327-330, 1957), using acetylthiocholine iodide (4 mM) as substrate. The specificity of AChE activity was established by inhibition with BW 284 C51 (1.5 x 10^{-4}M).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially according to Laemmli (Nature,227,680-685,1970) with 8% polyacrylamide slab gels (16 cm x 18 cm). Protein was stained with silver (Nielsen and Brown,Anal.Biochem.,141,311-315,1984). The molecular weight reference standards used were: ß-galactosidase (120,000), phosphorylase b (97,000), human IgM μ chain (74,000), bovine serum albumin (65,000), human IgG chain (50,000), aldolase (40,000), carbonic anhydrase (29,000) and human light chain (23,000).

Sucrose density gradient centrifugation

The sedimentation coefficient of sAChE (300 μl) was estimated in 4-20% (w/v) sucrose gradients (5 ml) in 50 mM sodium phosphate buffer, pH 8.0, using ß-galactosidase (15.9 S), catalase (11.3 S) and glucose oxidase (7.9 S), as sedimentation markers. Centrifugation was performed at 4°C in a Beckman L5-50 ultracentrifuge with a SW50 rotor (15h at 150,000 xg). Fractions (250 μl) were recovered from the gradient and assayed for AChE activity by the Ellman assay.

Isoelectric focusing (IEF)

Analytical isoelectric focusing was carried out at 10°C on 5% polyacrylamide - 0.5% agarose cylindrical gels (0.4 cm x 8 cm) as described by Wrigley (Meth.Enzymol.,22, 559-564,1971). Ampholytes used were LKB, pH range 3 - 10 and 4 - 6. Following electrofocusing, gels were histochemically stained for enzyme activity as described above. The pI of the enzyme was determined from a standard plot constructed by electrofocusing a number of marker proteins of known pI (IEF calibration kit, Sigma Co.)

Lectin Binding

The interaction of sAChE with lectins was investigated by affinity chromatography on Agarose-bound lectins. The following resins were used (Sigma Co.):Agarose-Concanavalin A type III-A, 10 mg lectin/ml gel; Agarose-Lens culinaris 2.5 mg lectin/ml gel and Agarose-ricinus communis, 2.5 mg lectin/ml gel. The gel (100 μl) was washed by centrifugation (three times, 1 ml each) with 50 mM sodium acetate buffer, pH 7.0, containing 0.3M NaCl and 2.5 mM of each of the following salts, $CaCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$. sAChE (10 m unit) was added in the same buffer (400 μl) and the suspension was incubated for 4h at 4°C with gentle mixing. The gel was washed by centrifugation (three times, 0.5 ml each), and the enzyme activity in the unbound material was measured by the Ellman assay. Non-specific binding was determined by using Agarose-bound goat immunoglobulin G gel (Sigma Co.).

Protein Estimation

Protein concentration was determined by using a modified Lowry assay (Markwell et al., Anal.Biochem.,87,206-210, 1978). Bovine serum albumin was used as a standard.

2. RESULTS

Extraction of soluble AChE

20 - 30% of the total AChE activity in adult and fetal brains was recovered in the high speed supernatant.

Enzyme activities and protein contents are shown in Table 1.

Table 1 AChE and protein content of human brain high-speed supernatant

Supernatant	Protein conc. mg/ml	AChE activity Unit/l	AChE activity Unit/mg	ChE Unit/l	Specific activity Unit/mg
Adult brain	1.4	43.0	0.031	11.0	0.008
Fetal brain	2.1	42.6	0.020	34.40	0.016

AChE and ChE activities were measured by the Ellman assay using acetylthiocholine iodine (1 mm) as a substrate and BW 284 C51 (1.5 μm) as AChE inhibitor. Protein concentration was estimated by Lowry. Protein concentrations and enzyme activity varied by <20% between different preparations.

Analysis of the enzyme activity by PAGE showed that both supernatants contained three bands of esterase activity: a slow moving PsChE band which was not inhibited by BW 284 C51, an intermediate and a fast moving AChE band, both of which were inhibited by BW 284 C51.

Gel Filtration

Brain supernatant was fractionated by gel filtration on a Sephacryl S-200 column. Three major protein peaks were obtained, eluting at molecular weights, ≥250,000 (void volume), 50,000 - 60,000 and <10,000, respectively. AChE activity in both supernatants was fractionated into two well-resolved peaks, P1 and P2, with elution volumes corresponding to approximate molecular weights of 220,000-240,000 and 45,000-55,000 daltons, respectively. Fractions corresponding to each peak were pooled. In the adult brain supernatant, the applied AChE activity was equally divided into the two peaks, while >70% of the fetal supernatant AChE activity appeared in peak P1. Analysis of esterase activity by PAGE showed that P1 contained most of the slow moving

AChE (which corresponds to sAChE in CSF) as well as PsChE and some of the fast moving AChE. P2 contained some sAChE, a negligible amount of PsChE and most of the fast moving AChE.

Affinity chromatography of AChE

Adult and fetal P1 and P2 were subjected to affinity chromatography on edrophonium-Sepharose gels. AChE activity in the applied samples, unbound fraction and the edrophonium chloride eluted fraction was determined by the Ellman assay. Between 60-80% of the AChE activity in P1 bound to the affinity column, 40-50% of which could be subsequently eluted with edrophonium chloride, while less than 3% AChE activity in P2 bound. Analysis of the edrophonium chloride-eluted fraction by PAGE showed the presence of one band of enzyme activity which was completely inhibited by BW 284 C51 and had an electro-phoretic mobility corresponding to that of sAChE present in CSF. Protein staining of PAGE showed the presence of one band in the edrophonium chloride fraction corresponding to the position of the AChE band. The edrophonium chloride fraction will thereafter be termed sAChE. The specific activity of the purified enzyme following desalting on the Sephadex G50 column was 500-800 units/mg, and 900-1500 units/mg for adult and fetal sAChE, respectively. However, both preparations lost activity rapidly. Hence, following freeze-drying and reconstitution in PBS, the specific activity decreased to 50-100 units/mg. The drop in activity continued even on storage at -20°C. A similar inactivation of the enzyme upon purification was reported for fetal bovine serum sAChE (Chubb et al., Neuroscience, 10(4),1369-1377,1983), and brain AChE (Gordon et al., Biochem.J.,157,69-76,1976).

A summary of the activities of AChE and PsChE in various human biological samples is shown in Table 2. This Table also shows the ability of edrophonium-Sepharose to bind to the various AChE preparations.

Table 2
Activities of the various antigen solutions and percentage of AChE bound by edrophonium-Sepharose determined using the Ellman assay

Antigen	AChE activity (m units/ml)	PsChE activity (m units/ml)	Edrophonium Sepharose column
Adult brain homogenate	43.0	11.0	-
Fetal brain homogenate	42.6	34.0	61% AChE
CSF	120.0	50.0	85% AChE
Detergent-soluble AChE (foetal)	820.0	48.2	0% AChE
Human serum PsChE	224.0	1970.0	0% AChE
Human red blood cell AChE	1000.0	120.0	0% AChE
Normal human serum	53.0	658.6	0% AChE
Amniotic fluid	62.2	87.5	-

SDS-PAGE

Analysis of adult and fetal sAChE by SDS-PAGE under reducing conditions showed the presence of a predominant band at M_r 66,000. There was no difference in molecular weight between the adult and fetal enzymes.

Sucrose-density gradient centrifugation

Adult sAChE revealed two major forms sedimenting at 15.3 S_{4W} and 10.4 S_{4W}. The former is probably aggregated material. A minor peak with a sedimentation constant of 6.25 S_{4W} is also apparent. Fetal sAChE also revealed three forms of AChE activity, though with different relative distribution, at 15.0 S_{4W}, 10.5 S_{4W} and 6.1 S_{4W}. The 6.1-6.2 S form in both cases increased on storage, even at -20°C. The differences in sedimentation coefficients between the adult and fetal forms of AChE were not significant.

Isoelectric focusing of sAChE

Electrofocusing of purified sAChE was performed using ampholytes with pH ranges of 3-10 and 4-6. Enzyme staining gels revealed one band of activity which was completely inhibited by BW 284 C51. The pI values obtained (n = 10, mean ± S.D.) were: adult sAChE 5.47 ± 0.22, and fetal sAChE 5.47 ± 0.17.

Lectin binding

Adult or fetal sAChE (10m units of each) were mixed with the lectin agarose for 4h at 4°C. After washing, the amount of unbound enzyme in the eluant was determined by the Ellman assay. The results are shown in Table 3. Values are the average of five experiments with the range in brackets.

Table 3 Binding of sAChE to Lectin columns

		% sAChE bound	
Lectin	specificity	adult sAChE	fetal sAChE
Con-A	mannose and glucose	92 (85-98)	95 (84-97)
Lentil	mannose and glucose	97 (83-99)	98 (95-99)
Ricinus	galactose	82 (75-89)	88 (80-90)
IgG	nil	6 (3-8)	5 (3-9)

Table 3 shows that over 80% of both adult and fetal sAChE was absorbed onto the lectin resins used. This interaction was specific as shown by the lack of binding to Agarose - IgG resin. The binding of sAChE to the lectins was very strong and no more than 50% of the bound enzyme could be desorbed with the corresponding ligand (0.5M α-methyl glucoside, 0.5M α-methyl mannoside and 0.25M ß-methyl galactoside).

REFERENCE EXAMPLE 2

1. MATERIALS

Microtiter plates were "Immunoplate II" from Gibco (U.K.) Monoclonal anti-Rabbit Immunoglobulins Alkaline Phosphatase Conjugate Clone RG-16 was from Sigma Chemical Co. Ltd. (U.K.)
Casein (light white soluble) was from B.D.H..
All other chemicals were of analytical grade and purchased from Sigma Chemical Co. Ltd. (U.K.), F.S.A. (U.K.), or B.D.H.(U.K.).

2. METHODS

Production of polyclonal antisera: A rabbit (Rb35) was immunised by intramuscular injection with sAChE (50 μg) in Freunds Complete Adjuvant (FCA). After 4 weeks, the rabbit was boosted by injection with sAChE (50 μg) in Freunds Incomplete Adjuvant and bled 5 days later. The antiserum was prepared and stored aliquoted at -20°C.
Normal rabbit serum was from an unimmunised rabbit.
ELISA To Detect Antibodies Against sAChE
Where Tween 20 (polyoxyethylenesorbitan monolaurate) was used it was at a concentration of 0.05%. In all incubation steps 100 μl aliquots of solutions per well were used. Wash steps used 150μl aliquots per well. Wells were quickly shaken dry in between all wash and incubation stages.
Microtiter wells were coated overnight at room temperature with sAChE (500ng/ml) in 0.05M bicarbonate/carbonate buffer pH 9.6. Wells were next washed with PBS/Tween a total of three times, the final wash being left at 37°C for 30 minutes. Rabbit antisera, at a dilution of 1/1000 in PBS/Tween/1% casein were incubated in wells for 2 hours at 37°C. Wells were washed five times with PBS/Tween.
Monoclonal anti-rabbit immunoglobulin-alkaline phosphatase conjugate at a dilution of 1/1000 in PBS/Tween/1%casein was incubated in wells for 1.5 hours at 37°C. Wells were washed five times with PBS/Tween. Substrate solution

(p-nitrophenyl phosphate 2mg/ml in 0.05M bicarbonate/carbonate buffer pH 9.6) was incubated for 30 minutes. The substrate reaction was stopped by the addition of 50μl of 1M sodium hydroxide, and optical density readings were measured at 405nm using a Titertek Multiscan MCC plate reader.

3. RESULTS

An ELISA was set up using sAChE as the coating antigen to test the reactivity of the anti-sAChE antiserum and compare it with normal rabbit serum. The results are presented in Table 4. Table 4 shows that serum from the rabbit immunised with sAChE contains antibodies capable of binding to sAChE in a direct ELISA and that normal rabbit serum does not.

Table 4

	Optical density 1	Optical density 2	Average optical density
Normal Rabbit Serum	Zero	Zero	Zero
Anti-sAChE Rabbit serum	0.308	0.299	0.304

EXAMPLE 1

1. MATERIALS

Microtiter plates were "Immunoplate II" from Gibco (U.K.)
Poly amino acids were from Sigma Chemical Co. Ltd. (U.K.)
Monoclonal anti-Rabbit Immunoglobulins Alkaline Phosphatase Conjugate Clone RG-16 was from Sigma Chemicals Co. Ltd. (U.K.)
Casein (light white soluble) was from B.D.H..
All other chemicals were of analytical grade and purchased from Sigma Chemical Co. Ltd (U.K.), F.S.A.(U.K.), or B.D.H. (U.K.).

Samples

Human Serum Samples and Cerebrospinal Fluids (CSF) were obtained from: the Department of Clinical Chemistry, Southmead General Hospital, Bristol (B1), the Department of Endocrinology, Southampton General Hospital (S1), Wessex Regional Immunology, Southampton General Hospital (S2), or the Department of Microbiology, Southampton General Hospital (S3).

2. METHODS

Purification of human foetal sAChE: described in Reference Example 1.

Production of polyclonal antisera: Rabbit sera described in Reference Example 2.

Samples

Spina bifida serum A was a pool of 4 sera, from source B1, in equal ratios from mothers carrying Spina bifida positive foetuses. Spina bifida serum B was a pool of 5 sera, from source S1, in equal ratios from mothers carrying Spina bifida positive foetuses. Anencephalic serum C was a pool of 5 sera, from source S1, in equal ratios from mothers carrying Anencephalic positive foetuses. Normal human sera D and E were pools of 5 sera each, from source S3, in equal ratios from a normal pool of pregnant mothers. CSF was a pool of 20 CSF samples, from source S2, in equal ratios.

Enzyme Capture ELISA:

Where Tween 20 (polyoxyethylenesorbitan monolaurate) was used it was at a concentration of 0.05%. In all incubation steps 100μl aliquots of solutions per well were used. Wash steps used 150μl aliquots per well. Wells were quickly shaken dry in between all wash and incubation stages. Microtiter wells were coated overnight at 4°C with poly-L-lysine solution (50μg/ml poly-L-lysine, Molecular weight 150,000-300,000, in PBS). Control wells were incubated with PBS alone. Wells were next washed with PBS/Tween a total of five times, the final wash being left at 37°C for 30 minutes. Human serum samples or cerebrospinal fluid (CSF), at an appropriate dilution in PBS/Tween were incubated in wells for 2 hours at 37°C. Wells were washed five times with PBS. Rabbit anti-sAChE serum at a dilution of 1/1000 in PBS/1% casein was incubated in wells for 2 hours at 37°C. Wells were washed five times with PBS. Monoclonal anti-rabbit immunoglobulin - alkaline phosphatase conjugate at a dilution of 1/1000 in PBS/1% casein was incubated in wells for 1.5 hours at 37°C. Wells were washed five times with PBS. Substrate solution (p-nitrophenyl phosphate 2mg/ml in 0.05M bicarbonate/carbonate buffer pH 9.6) was incubated for 6 minutes. The substrate reaction was stopped by the addition of 50μl of 1M sodium hydroxide, and optical density readings were measured at 405nm using a Titertek Multiscan MCC plate reader.

3. RESULTS

An Enzyme Capture ELISA was set up using poly-L-lysine as capturing agent and rabbit anti-sAChE antisera as the detecting agent.

The results are presented in Table 5. Table 5 shows that where no poly-L-lysine was used to coat the microtiter plate wells, resulting optical density readings at the end of the assay were negligible. This shows that in such a case wells did not have the ability to specifically adsorb the sAChE from the samples, and therefore no detection was observed. Where poly-L-lysine was used to coat the wells significant positive signals were obtained for the three disease positive sera A, B and C, together with CSF, and relatively low signals were observed for the normal sera. This shows that with poly-L-lysine as capture agent the assay is able to distinguish between samples where sAChE levels are higher (i.e. CSF or sera from mothers carrying foetuses with Spina bifida, or anencephaly) and samples where sAChE levels are lower or negligible (i.e. sera from a pool of mothers with normal pregnancies).

Where PBS/Tween controls were used in place of the test sample the resultant signals were negligible. This result shows that the pre-block step using PBS/Tween, and the subsequent use of PBS/1% casein in the detecting antibody stages is sufficient to block any significant non-specific binding by these reagents.

TABLE 5

Enzyme Capture ELISA Results

Sample and dilution	Poly-L-lysine coated wells Optical density 450nm		Ave. OD.	No poly-L-lysine on wells Optical density 450nm		Ave. OD.
Anencephaly C 1/30	0.766	0.783	0.775	0.093	0.136	0.115
Spina bifida A 1/30	0.889	0.885	0.887	0.09	0.104	0.097
Spina bifida B 1/30	0.835	0.808	0.822	0.093	0.088	0.091
Normal serum D 1/30	0.207	0.207	0.207	0.106	0.078	0.092
Normal serum E 1/30	0.279	0.278	0.279	0.135	0.125	0.13
CSF 1/3	0.571	0.553	0.562	0.088	0.1	0.094
PBS/Tween control	0.009	0.049	0.029	Blank	Blank	Blank

EXAMPLE 2

1. MATERIALS

Described in Example 1.

2. METHODS

Purification of human foetal sAChE: described in Reference Example 1.

Production of polyclonal antisera: described in Reference Example 2.

Samples: described in Example 1.

Enzyme Capture ELISA:

Where Tween 20 (polyoxyethylenesorbitan monolaurate) was used it was at a concentration of 0.05%. In all incubation steps 100μl aliquots of solutions per well were used. Wash steps used 150μl aliquots per well. Wells were quickly shaken dry in between all wash and incubation stages. Microtiter wells were coated overnight at 4°C with poly-L-lysine solution (50μg/ml poly-L-lysine, 150,000-300,000, in PBS). Wells were next washed with PBS a total of three times and blocked with PBS/Tween at 37°C for 30 minutes. Wells were washed with PBS three times. Human serum samples or cerebrospinal fluid (CSF), at an appropriate dilution in one of four solutions PBS, PBS/Tween, PBS plus 0.35M sodium chloride or PBS/Tween plus 0.35M sodium chloride) were incubated in wells for 2 hours at 37°C. Wells were washed five times with PBS. Rabbit anti-sAChE serum at a dilution of 1/1000 in PBS/1% casein was incubated in wells for 110 minutes at 37°C. Wells were washed five time with PBS. Monoclonal anti-rabbit immunoglobulins alkaline phosphatase conjugate at a dilution of 1/1000 in PBS/1% casein was incubated in wells for 80 minutes at 37°C. Wells were washed fives times with PBS. Substrate solution (p-nitrophenyl phosphate 2mg/ml in 0.05M bicarbonate/carbonate buffer pH 9.6) was incubated for 5 minutes. The substrate reaction was stopped by the addition of 50μl of 1M sodium hydroxide, and optical density readings were measured at 405nm using a Titertek Multiscan MCC plate reader.

3. RESULTS

An Enzyme Capture ELISA was set up using poly-L-lysine as capturing agent and rabbit anti-sAChE antisera as the detecting agent.

The results are presented in Table 6. From Table 6 it can be seen that when the samples under test are loaded in any of the four various solutions (PBS, PBS/Tween, PBS plus 0.35M sodium chloride or PBS/Tween plus 0.35M sodium chloride) a clear distinction can be seen between the resulting signals obtained from different samples. Thus in the assay higher optical density values are observed for samples high in levels of sAChE (samples A, B, C and CSF) and much lower optical density values are obtained for samples low in levels of sAChE (Normal sera).

PBS/Tween/0.35M sodium chloride appears to be the best solution in which to load the sample as it gives negligible optical density signals for normal sera whereas CSF and sera from mothers carrying foetuses with either Spina bifida or Anencephaly show positive signals.

EXAMPLE 3

1. MATERIALS:

Described in Example I.

2. METHODS

Purification of human foetal sAChE: described in Reference Example 1.

Production of polyclonal antisera: described in Reference Example 2.

Samples:

CSF was as described in Example 1.

Enzyme Capture ELISA:

TABLE 6

ENZYME CAPTURE ELISA RESULTS

Sample and Dilution	Sample in PBS OD(405nm)		AveOD	Sample in PBS/Tween OD(405nm)		AveOD	Sample in PBS/0.35M NaCl OD(405nm)		AveOD	Sample in PBS/Tween/ 0.35M NaCl OD(405nm)		AveOD
Anencephic c 1/100	0.501	0.576	0.539	0.482	0.407	0.445	0.379	0.325	0.352	0.195	0.212	0.204
Spina bifida A 1/100	0.646	0.611	0.629	0.538	0.515	0.527	0.637	0.600	0.619	0.454	0.417	0.436
Spina bifida B 1/100	0.571	0.519	0.545	0.470	0.498	0.484	0.567	0.506	0.537	0.471	0.434	0.453
Normal Serum D 1/100	0.253	0.250	0.252	0.164	0.233	0.199	0.149	0.118	0.134	0.015	0.000	0.008
Normal Serum E 1/100	0.315	0.333	0.324	0.289	0.234	0.262	0.136	0.159	0.148	0.059	0.020	0.040
CSF 1/3	0.548	0.452	0.500	0.445	0.458	0.452	0.471	0.485	0.478	0.369	0.396	0.383

Where Tween 20 (polyoxyethylenesorbitan monolaurate) was used it was at a concentration of 0.05%.

In all incubation steps 100µl aliquots of solutions per well were used. Wash steps used 150µl aliquots per well. Wells were quickly shaken dry in between all wash and incubation stages.

Microtiter wells were coated overnight at 4°C with poly-L-lysine solution (50µg/ml poly-L-lysine, 150,000-300,000, in PBS). Wells were next washed with PBS a total of three times and blocked at 37°C for 30 minutes with PBS/Tween. Wells were washed five times with PBS/Tween. CSF at a dilution of 1/6 in PBS/Tween/0.35M sodium chloride was used at the sample stage as the source of sAChE and was incubated in all wells for 2 hours at 37°C. Wells were washed five times with PBS/Tween. Normal rabbit serum or rabbit anti-sAChE serum at a dilution of 1/1000 in PBS/1%casein was incubated in wells for 2 hours at 37°C. Wells were washed five times with PBS/Tween. Monoclonal anti-rabbit immunoglobulin - alkaline phosphatase conjugate at a dilution of 1/1000 in PBS/1% casein was incubated in wells for 1.5 hours at 37°C. Wells were washed five times with PBS/Tween. Substrate solution (p-nitrophenyl phosphate 2mg/ml in 0.05M bicarbonate/carbonate buffer pH 9.6) was incubated for 5 minutes. The substrate reaction was stopped by the addition of 50µl of 1M sodium hydroxide, and optical density readings were measured at 405nm using a Titertek Multiscan MCC plate reader.

3. RESULTS

An Enzyme Capture ELISA was set up using poly-L-lysine as capturing agent and rabbit anti-sAChE antisera as the detecting agent.

The results are presented in Table 7. From Table 7 it can be seen that normal rabbit serum is unable to recognise and bind to the sAChE from CSF which has been captured by the poly-L-lysine on the plate, the end assay optical density being only 0.020 at a 1/400 dilution of serum. The Anti-sAChE serum is however able to bind specifically to the captured sAChE and hence a positive signal in the Enzyme Capture ELISA is obtained, an end of assay optical density of 0.349 being seen at a dilution of 1/3600.

Table 7

Optical density values shown at 405nm.

	Dilution of Rabbit Serum			
Serum Type	1/400	1/1200	1/3600	1/10800
Normal Rabbit Serum	0.020	0.009	0.005	Blank
Rabbit Anti-sAChE	1.117	0.688	0.349	0.147

Example 4

1. MATERIALS: described in Example I

2. METHODS

Purification of human foetal sAChE: described in Reference Example 1.

Production of polyclonal antisera: described in Reference Example 2.

Samples:

Described in Example 1.

Lysed normal human serum was from a nonpregnant woman.

Enzyme Capture ELISA:

Where Tween 20 (polyoxyethylenesorbitan monolaurate) was used it was at a concentration of 0.05%.

In all incubation steps 100µl aliquots of solutions per well were used. Wash steps used 150µl aliquots per well. Wells were quickly shaken dry in between all wash and incubation stages.

Microtiter wells were coated for 48 hours at 4°C with poly-L-lysine solution (50µg/ml poly-L-lysine, 150,000-300,000, in PBS). Wells were next washed with PBS-Tween a total of three times, the final wash being left at 37°C for 30 minutes. Two more washes with PBS/Tween were then performed. Human serum samples at 1/100 dilution or cerebrospinal fluid (CSF) at a dilution of 1/3, in PBS/Tween plus various additional concentrations of sodium chloride were incubated in wells for 2 hours at 37°C. Wells were washed five times with PBS/Tween. Rabbit anti-sAChE serum at a dilution of 1/1000 in PBS/Tween/1%casein was incubated in wells for 2 hours at 37°C. Wells were washed five times with PBS/Tween. Monoclonal anti-rabbit immunoglobulins alkaline phosphatase conjugate at a dilution of 1/1000 in PBS/Tween/1%casein was incubated in wells for 1.5 hours at 37°C. Wells were washed five times with PBS/Tween. Substrate solution (p-nitrophenyl phosphate 2mg/ml in 0.05M bicarbonate/carbonate buffer pH 9.6) was incubated for 15 minutes. The substrate reaction was stopped by the addition of 50μl of 1M sodium hydroxide, and optical density readings were measured at 405nm using a Titertek Multiscan MCC plate reader.

3. <u>RESULTS</u>

An Enzyme Capture ELISA was set up using poly-L-lysine as capturing agent and rabbit anti-sAChE antisera as the detecting agent.

The results are presented in Fig1. From Fig1 it can be seen that throughout the range of added sodium chloride concentration the CSF and disease positive sera give significantly higher optical density values than the normal sera. As the concentration of sodium chloride is increased however, there is a decrease in the ratio of optical density values for the normal sera relative to the signals given by disease positive sera. Therefore the addition of sodium chloride up to 0.4M extra, to the PBS/Tween for the sample buffer increases further the ability of the assay to distinguish between disease positive sera and normal sera.

Example 5

1. MATERIALS: part described in Example 1.
Poly-L-lysine was of molecular weight 150,000-300,000 (300K), and 4,000-15,000 (15K).
Poly-D-lysine was of molecular weight 150,000-300,000.
Poly-L-asparagine was of molecular weight 5,000-15,000.
Poly-L-glutamate was of molecular weight 50,000-100,000.
Poly-L-histidine was of molecular weight 15,000-50,000.
Poly-L-ornithine was of molecular weight 100,000-200,000.
Poly-L-arginine was of molecular weight 70,000-150,000.
Poly-L-aspartate was of molecular weight 15,000-50,000.

2. METHODS

Purification of human foetal sAChE: described in Reference Example 1.
Production of polyclonal antisera: rabbit anti-sAChE was as described in Reference Example 2.
Samples:
The CSF used in the study was produced by pooling 20 CSF samples obtained from Wessex Regional Immunology, Southampton General Hospital.
Enzyme Capture ELISA:
Where Tween 20 (polyoxyethylenesorbitan monolaurate) was used it was at a concentration of 0.05%.
In all incubation steps 100μl aliquots of solutions per well were used. Wash steps used 150μl aliquots per well. Wells were quickly shaken dry in between all wash and incubation stages.
Microtiter wells were coated overnight at 4°C with poly-amino acid solutions (50μg/ml poly-amino acid in PBS).
Wells were next washed with PBS/Tween a total of five times, the final wash being left at 37°C for 1 hour. Dilutions of CSF of 1/4, 1/16, or 1/64 (in PBS/Tween plus 0.35M sodium chloride) were incubated in wells for 2 hours at 37°C.
Wells were washed five times with PBS/Tween. Rabbit anti-sAChE serum at a dilution of 1/1000 in PBS/Tween/1%casein was incubated in wells for 2 hours at

37°C. Wells were washed five times with PBS/Tween. Monoclonal anti-rabbit immunoglobulin - alkaline phosphatase conjugate at a dilution of 1/1000 in PBS/Tween/1%casein was incubated in wells for 1.5 hours at 37°C. Wells were washed five times with PBS/Tween. Substrate solution (p-nitrophenyl phosphate 2mg/ml in 0.05M bicarbonate/carbonate buffer pH 9.6) was incubated for 15 minutes. The substrate reaction was stopped by the addition of 50µl of 1M sodium hydroxide, and optical density readings were measured at 405nm using a Titertek Multiscan MCC plate reader.

3. <u>RESULTS</u>

An Enzyme Capture ELISA was set up using different poly-amino acids as capturing agents, and rabbit anti-sAChE antisera as the detecting agent. The results are presented in Table 8. From Table 8 it can be seen that poly-L-lysine (300K), poly-L-lysine (15K), poly-D-lysine, poly-L-histidine, poly-L-ornithine, and poly-L-arginine are all able to act as ligands for sAChE to bind to giving positive optical density readings in the Enzyme Capture ELISA. Poly-L-asparagine, poly-L-glutamate, and poly-L-aspartate, however, only give final optical density values equating with those where PBS was used in place of the poly- amino acids (i.e. control wells), and were therefore unable to be bound by the sAChE.

TABLE 8

Coating Ligand	Average ODs at 405nm Sample			
	CSF 1/4	CSF 1/16	CSF 1/64	BUFFER
Poly-L-lysine (300K)	0.961	0.381	0.103	BLANK
Poly-L-lysine (15K)	0.402	0.160	0.045	0.003
Poly-D-lysine	0.942	0.381	0.086	0.010
Poly-L-histidine	1.315	0.923	0.422	BLANK
Poly-L-ornithine	1.204	0.585	0.163	0.002
Poly-L-arginine	0.563	0.225	0.137	BLANK
Poly-L-asparagine	0.194	0.058	0.005	BLANK
Poly-L-glutamate	0.178	0.052	0.005	BLANK
Poly-L-aspartate	0.176	0.043	Zero	BLANK
PBS only	0.174	0.039	BLANK	BLANK

EXAMPLE 6

1. MATERIALS: described in Example 1

2. METHODS

Production of polyclonal antisera: rabbit anti-sAChE was as described in Example 1.

Serum Samples and CSF Standards

Serum samples were obtained from the Department of Endocrinology, Southampton General Hospital, and were transported whilst frozen, and were stored at -20°C. Serum samples were all treated the same way, with the minimum number of freeze/thaw cycles. Table 9 summarises the samples used in the study.

TABLE 9

SOURCES OF SAMPLES

SOUTHAMPTON GENERAL HOSPITAL(1)	
Normal pregnancies with known weeks gestation	83
Anencephaly	4
Open spina bifida	17
Other pregnancy complications	15
Raised AFP/normal outcome	13

The CSF standard used in the study was produced by pooling 20 CSF samples obtained from Wessex Regional Immunology, Southampton General Hospital.

Enzyme Capture ELISA:

Where Tween 20 (polyoxyethylene sorbitan monolaurate) was used it was at a concentration of 0.05%.

In all incubation steps 100μl aliquots of solutions per well were used. Wash steps used 150μl aliquots per well. Wells were quickly shaken dry in between all wash and incubation stages.

Microtiter wells were coated overnight at 4°C with poly-L-lysine solution (50μg/ml poly-L-lysine, 150,000-300,000, in PBS). Wells were next washed with PBS/Tween, a total of five times, the final wash being left at 37°C for 1 hour. Human serum samples were used at a dilution of 1/300 and assays were performed in triplicate for every sample. On every plate a dilution curve of CSF standard was included, each dilution being assayed in duplicate. Three internal standard sera were included in duplicate on each plate. These standard sera included one high positive serum one medium positive serum and one low normal serum. All serum samples and CSF were diluted in PBS/Tween/0.35M sodium chloride. Wells were incubated with the appropriate test sera, standard curve dilutions, or internal standards for 2 hours at 37°C. Wells were washed five times with PBS/Tween. Rabbit anti-sAChE serum at a dilution of 1/1000 in PBS/Tween/1%casein was incubated in wells for 2 hours at 37°C. Wells were washed five times with PBS/Tween. Monoclonal anti-rabbit immunoglobulin-alkaline phosphatase conjugate at a dilution of 1/1000 in PBS/Tween/1%casein was incubated in wells for 1.5 hours at 37°C. Wells were washed five times with PBS/Tween. Substrate solution (p-nitrophenyl phosphate 2mg/ml in 0.05M bicarbonate/carbonate buffer pH 9.6) was incubated for 15 minutes. The substrate reaction was stopped by the addition of 50μl of 1M sodium hydroxide, and optical density readings were measured at 405nm using a Titertek Multiscan MCC plate reader.

Expression of results

Levels of sAChE determined by the Enzyme Capture ELISA are expressed in arbitrary units relative to the standard curve reference CSF performed in each case. The units are defined thus:

Dilution of Reference Standard CSF	Defined Units
1/3	200.00
1/6	100.00
1/12	50.00
1/24	25.00
1/48	12.50
1/96	6.25
1/192	3.13

Following initial testing on a plate where a 1/6 dilution of CSF was the highest reference standard, any serum which had a value greater than 100 Units was reassayed on a plate where CSF at 1/3 dilution (i.e. 200 Units) was the highest reference value. A typical CSF reference standard curve is shown in Fig. 2.

3. RESULTS

A. sAChE levels between the 14 and 20 weeks gestation

A total of 83 samples from normal pregnancies of known gestational age were analysed. The results (Table 10 and Figure 3) showed a relatively constant mean from 15-20 weeks gestation. The five results for week 14 were significantly higher than week 15-20 levels and those levels were not included in the overall reference range. Results for 78 samples from 15-20 weeks gestation were combined to give an overall reference range. This had a median of 26 (2 MOM = 52), mean of 27.67% and 97.5 centile upper limit of normal (mean + 2SD) of 43.5%.

Results for the Southampton samples from abnormal pregnancies were then assessed in terms of two different criteria:-

i) Results greater than 2 x the median (52)

ii) Results greater than mean + 2SD (43.5)

TABLE 10

Normal Pregnancy Samples (Southampton)

		SAChE (UNITS)		
Weeks gestation	N	Mean	Median	Mean+2SD
14*	5	40.1	39.4	52.6
15	7	28.8	29.6	40.4
15.5	8	31.8	31.8	42.5
16	6	25.4	25.1	33.3
16.5	7	26.6	25.3	36.9
17	8	27.9	26.7	39.0
17.5	8	21.2	18.9	42.2
18	8	30.6	29.3	47.8
18.5	8	22.9	20.1	35.3

19	8	26.7	25.8	34.6
19.5	5	33.1	38.8	45.2
20	5	32.8	33.2	54.9
Grouped data				
15 - 20	78	27.7	26	43.4

*Significantly higher than other groups - data not included in calculation of overall range.

B. Neural Tube Defect Pregnancies (Southampton)

A total of 21 sera were analysed, consisting of 4 from cases of anencephaly and 17 from cases of open spina bifida.

Using an upper limit of 2 MOM (multiples of the median) (52) the Enzyme Capture ELISA clearly identified 20 of the 21 neural tube pregnancies, only one case of anencephaly having a level below this cut off (Table 11). When the same criterion was applied to the alfafetoprotein (AFP) results from the same samples, AFP results picked up 19 of the 21 cases, 2 spina bifida cases being just below 2 MOM. Figure 4 shows the spread of sAChE and AFP levels in terms of their respective MOM's. It is noteworthy that the MOM range of sAChE results in neural tube defect pregnancies is almost as wide as that for AFP, and in addition, the two spina bifida cases missed by AFP screening were correctly identified with the Enzyme Capture ELISA for sAChE.

Using the second criterion of whether results were greater than mean + 2SD (97.5 centile) again the Enzyme Capture ELISA for sAChE classified 20 of the 21 neural defect pregnancies as abnormal. (Figure 5)

3) Other abnormal pregnancy samples (Southampton)

i) Samples with raised AFP but normal outcome

Of 13 such samples analysed, 9 had sAChE levels greater than 2 MOM and 12 had levels greater than the 97.5 centile.

ii) Other abnormal pregnancies

Samples from cases of oligohydramnios, renal agenesis, and gastroschisis had raised sAChE levels, as did 3 or 4 samples from twin pregnancies and 3 of 6 cases of miscarriage/missed abortion or intrauterine death (Table 12).

TABLE 11

Neural Tube Defects - Southampton Samples

	Gestation	AFP(KU/L)	AFP(MOM)	*sAChE (Units)	sAchE(MOM)
Anencephaly					
1	17.5	274	6.29	69.4	2.67
2	16	97	2.79	40.6	1.56
3	17.5	287	7.08	142	5.46
4	16.5	120	3.2	172	6.62
Open Spina Bifida					
1	16.5	156	4.17	92.5	3.56
2	16.5	175	4.68	107	4.12
3	17	195	4.80	108	4.15
4	15.5	116	3.59	20.5	7.88
5	17	131	3.23	164.5	6.33
6	17.5	139	3.19	205	7.88
7	13	107	4.12	152.5	5.86
8	14	249	8.89	178	6.85
9	15.5	102	3.15	99.6	3.83
10	17.5	83	1.9	93.1	3.58
11	16	262	7.52	102	3.92
12	17	402	9.92	128	4.92
13	18.5	362	7.16	111	4.27
14	19.5	129	2.2	155	5.96
15	13	50	1.92	108	4.15
16	16.5	120	3.2	172	6.62
17	15.5	114	3.5	147	5.65

*Calculated using the median for the appropriate weeks gestation.

TABLE 12

OTHER ABNORMAL PREGNANCY SAMPLES (SOUTHAMPTON)

i) High AFP but Normal Outcome

No	Gest Age	AFP(KU/L)	sAChE (UNITS)
1	24	342	205
2	17	86	104
3	22	147	194
4	19	159	205
5	17.5	129	67.9
6	19	162	53.7
7	17	189	97.1
8	16	187	72.1
9	17.5	220	61.5
10	17.5	107	48.5
11	19	109	49.4
12	18.5	107	47.1
13	17	266	40.3

ii) Abnormal Pregnancies

Oligohydramnios			
1	19.5	199	100
2	16	168	49.9
Renal Agenesis			
3	19.5	224	119.7
Cong. Abnormalities			
4 (dwarf)	19	135	97.8
5 (gastroschisis)	16.5	192	67
Twins			
6	16	159	62.5
7	16.5	114	36.3
8	16	108	65
9	18.5	218	51
Abortion/intrauterine death			
10	16	560	201
11	18.5	191	30.2
12	15.5	95	67.8
13	16	113	45.9
14	16.5	102	56
15	16	156	42

D OVERALL CORRELATION BETWEEN sAChE AND AFP LEVELS

Although sAChE and AFP levels were both raised in neural tube defect cases, when correlations were assessed for sAChE with AFP either in absolute terms or in terms of MOM results, no correlation between the two tests was found.

For NTD samples (Southampton)

1) sAChE (units) vs AFP (KU/L) - correlation coefficient (r) = -0.075 (not significant)

2) sAChE (MOM) vs AFP (MOM) - correlation coefficient (r) = 0.028 (not significant).

EXAMPLE 7

1. Materials

Microtiter plates were "Immunoplate II" from Gibco (UK).

Poly-L-lysine hydrobromide cat. no. P-1399 molecular weight 289,000 was from Sigma Chemical Co. Ltd (UK).

Monoclonal anti-rabbit immunoglobulins alkaline phosphatase conjugate (clone RG-16) was from Sigma Chemical Co Ltd (UK).

Rabbit (Rb35) anti-sAChE was as desribed in Reference Example 2.

All other chemicals were of analytical grade and were purchased from Sigma Chemical Co Ltd (UK), F.S.A. (UK) or B.D.H. (UK).

Samples

Human serum samples were obtained from the Research Institute for the Care of the Elderly, St Martins Hospital, Bath, BA2 5RP (RICE). Putative Alzheimer's disease had been diagnosed in patients using DSM3R criteria and NINCDS ADRDA criteria (McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., and Stadlan, E.M., Neurology, 34, 939-944, July 1984.

Normal sera were from donors with no suspected neurodegenerative disease.

2. Methods

Enzyme Capture ELISA

Where Tween 20 (polyoxyethylenesorbitan monolaurate) was used it was at a concentration of 0.05%. In all incubation steps 100μl aliquots of solutions per well were used. Wash steps used 150 μl aliquiots per well. Wells were quickly shaken dry inbetween all wash and incubation stages. Microtitre wells were coated overnight at 4°C with poly-L-lysine solution (50μg/ml poly-L-lysine in PBS). Wells were next washed with PBS/Tween, a total of five times, the final wash being left at 37°C for 1 hour. Human serum samples were used at a dilution of 1/10 and assays were performed in duplicate on every sample. A dilution curve of CSF was included on the plate, each dilution being assayed in duplicate.

All serum samples at a 1/10 dilution and CSF were diluted in PBS/Tween/0.35M sodium chloride. Wells were incubated with the appropriate sample for 1½ hours at 37°C. Wells were washed five times with PBS/Tween.

Rabbit anti-sAChE serum at a dilution of 1/1000 in PBS/Tween/1% casein was incubated in wells for 1½ hours at 37°C. Wells were washed five times with PBS/Tween.

Monoclonal anti-rabbit immunoglobulin-alkaline phosphatase conjugate at a dilution of 1/1000 in PBS/Tween/1% casein was incubated in wells for 1½ hours at 37°C. Wells were washed five times with PBS/Tween. Substrate solution (p-nitrophenyl phosphate 2mg/ml in 0.05M bicarbonate/carbonate buffer pH 9.6) was incubated for 1 hour at room temperature. The substrate reaction was stopped by the addition of 50μl of 1M sodium hydroxide, and optical density readings were measured at 405nm using a Titertek Multiscan MCC plate reader.

Expression of results

As per Example 6. The CSF had been frozen and thawed a further three times and had been stored for a further 9 months following its use in Example 6. Therefore the activity had dropped and the defined units from this Example cannot be compared directly with those of the standard curve from Example 6. The arbitrary unit definition used was however the same as for Example 6.

Results

Seventeen control sera (age matched human sera from donors with no known neurodegenerative disease) gave a mean value of 37.8 sAChE units.

Nine patients with diagnosed putative Alzheimer's disease who were not on medication for the disease gave a mean value of 30.88 sAChE units. This is a significant difference in population.

The efficiency of the criteria used in the diagnosis was estimated to be 80% (personal communication by Dr R Jones RICE).

One putative Alzheimer's disease sample gave a value of 70.5 sAChE units, a value which was vastly higher than for the other patients. This value alters the mean for patients from 25.9 sAChE units to 30.88 sAChE units. This patient is the only one who was on antidepressant drugs at the time of taking of sera.

Conclusions

This was only a preliminary experiment but has shown the diagnostic capability of the measurement of sAChE levels to diagnose the likelihood of the patient having Alzheimer's disease. Further optimisation of this preliminary experiment will be designed to further separate out the two populations, controls versus Alzheimer's disease.

CLAIMS

1. A method of determining human secretory acetylcholinesterase (sAChE) in a test sample, which method comprises contacting a ligand which is capable of binding sAChE and which is not an antibody with the sample and determining whether human sAChE has bound to the ligand.

2. A method according to claim 1, in which the ligand is immobilised on a solid or matrix support.

3. A method according to claim 1 or 2, in which an antibody capable of binding to human sAChE is used in determining whether human sAChE has bound to the ligand.

4. A method according to claim 3, in which the antibody is labelled with an enzyme.

5. A method according to any one of claims 1 to 3, comprising:

(i) bringing into contact the sample and a said ligand which is immobilised on the surface of a solid support;

(ii) washing the surface of the solid support;

(iii) contacting the washed surface with a first unlabelled antibody which is capable of binding to human sAChE;

(iv) further washing the surface of the solid support;

(v) contacting the further washed surface with a second antibody which is labelled and which is capable of binding to the first antibody;

(vi) yet further washing the surface of the solid support; and

(vii) detecting the presence or absence of the label of the second antibody on the yet further washed surface.

6. A method according to any one of the preceding claims, in which the test sample is a sample of biological fluid which has been mixed with a 0.1 to 0.5M sodium chloride solution.

7. A method according to any one of the preceding claims, in which the ligand comprises an amino acid capable of being positively charged.

8. A method according to claim 7, wherein the amino acid is arginine, histidine, lysine or ornithine.

9. A method according to claim 8, in which the ligand is selected from poly-L-arginine, poly-L-histidine, poly-D-lysine, poly-L-lysine and poly-L-ornithine or a mixture of two or more thereof.

10. A method according to any one of the preceding claims for diagnosing Neural Tube Defects in a fetus.

11. A method according to any one of claims 1 to 9 for diagnosing Alzheimer's disease.

12. A method of determining an enzyme in a test sample, which method comprises:

i) bringing into contact the sample and a ligand which is immobilised on the surface of a solid or matrix support, which ligand is:

a) a substrate or substrate analogue of the enzyme;

b) an effector or effector analogue of the enzyme;

c) a co-factor or co-factor analogue of the enzyme;

d) a co-enzyme or a co-enzyme analogue of the enzyme; or

e) a prosthetic group or prosthetic group analogue of the enzyme; and

ii) determining whether the enzyme has bound to the ligand.

13. A test kit suitable for use in determining human sAChE in a test sample, which comprises a ligand which is capable of binding sAChE and which is not an antibody and means for detecting human sAChE when bound to the ligand.

14. A kit according to claim 13, in which the ligand is immobilised on a solid or matrix support.

15. A kit according to claim 13 or 14, wherein the said means for detecting human sAChE bound to the ligand comprises an enzyme-labelled antibody which is capable of binding to human sAChE and a substrate for the enzyme.

16. A kit according to claim 13 or 14, wherein the said means for detecting human sAChE bound to the ligand comprises a first unlabelled antibody which is capable of

binding to human sAChE, a second enzyme-labelled antibody which is capable of binding to the first antibody and a substrate for the enzyme label.

17. A kit according to any one of claims 13 to 16, in which the ligand comprises an amino acid capable of being positively charged.

18. A kit according to claim 17, wherein the amino acid is arginine, histidine, lysine or ornithine.

19. A kit according to claim 18, in which the ligand is selected from poly-L-arginine, poly-L-histidine, poly-D-lysine, poly-L-lysine and poly-L-ornithine or a mixture of two or more thereof.

20. A test kit suitable for use in determining an enzyme in a test sample, which comprises a ligand as defined in claim 12 which is immobilized on the surface of a solid or matrix support and which is capable of binding the enzyme and means for detecting the enzyme when bound to the ligand.

21. A method for determining antibodies to sAChE in a test sample, which method comprises:

a) contacting the sample with sAChE which is prebound to a ligand; and

b) measuring bound antibody.

Fig.1.

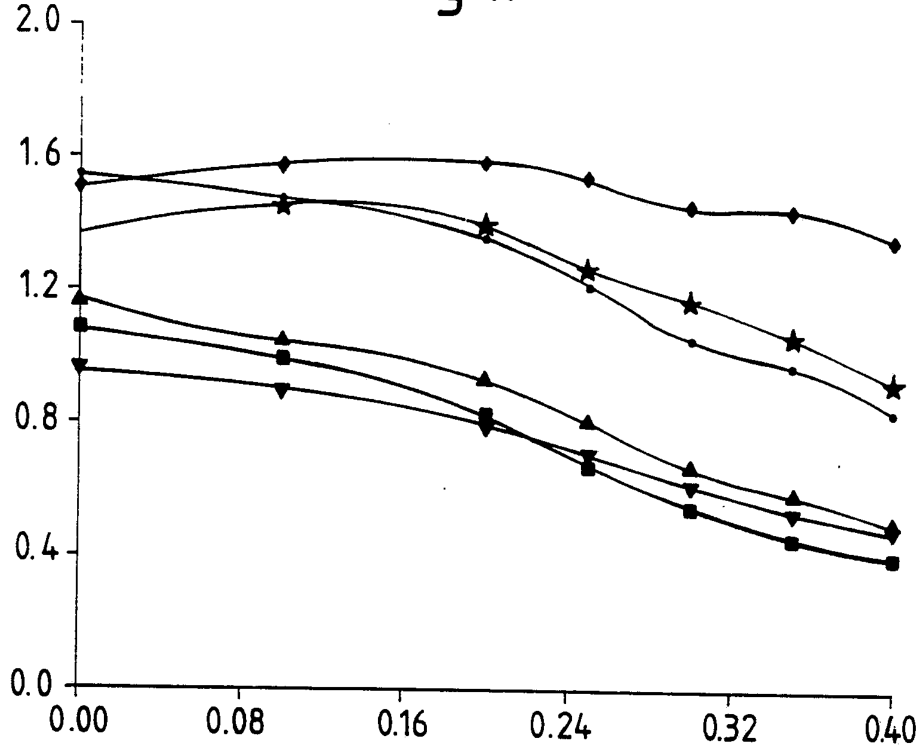

2.0
1.6
1.2
0.8
0.4
0.0
0.00
0.08
0.16
0.24
0.32
0.40

Fig.2.

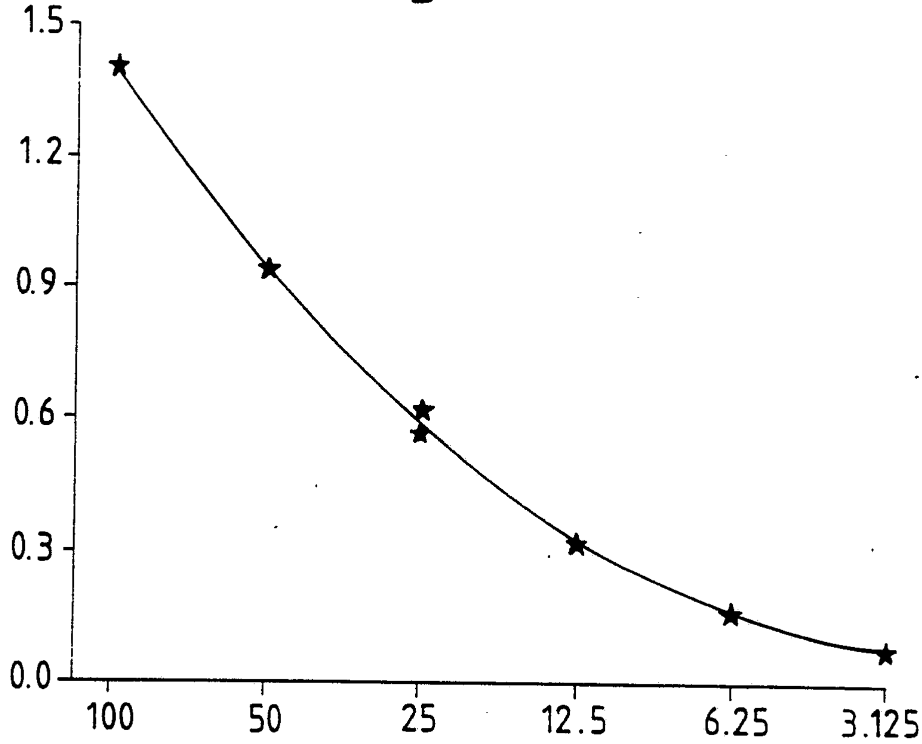
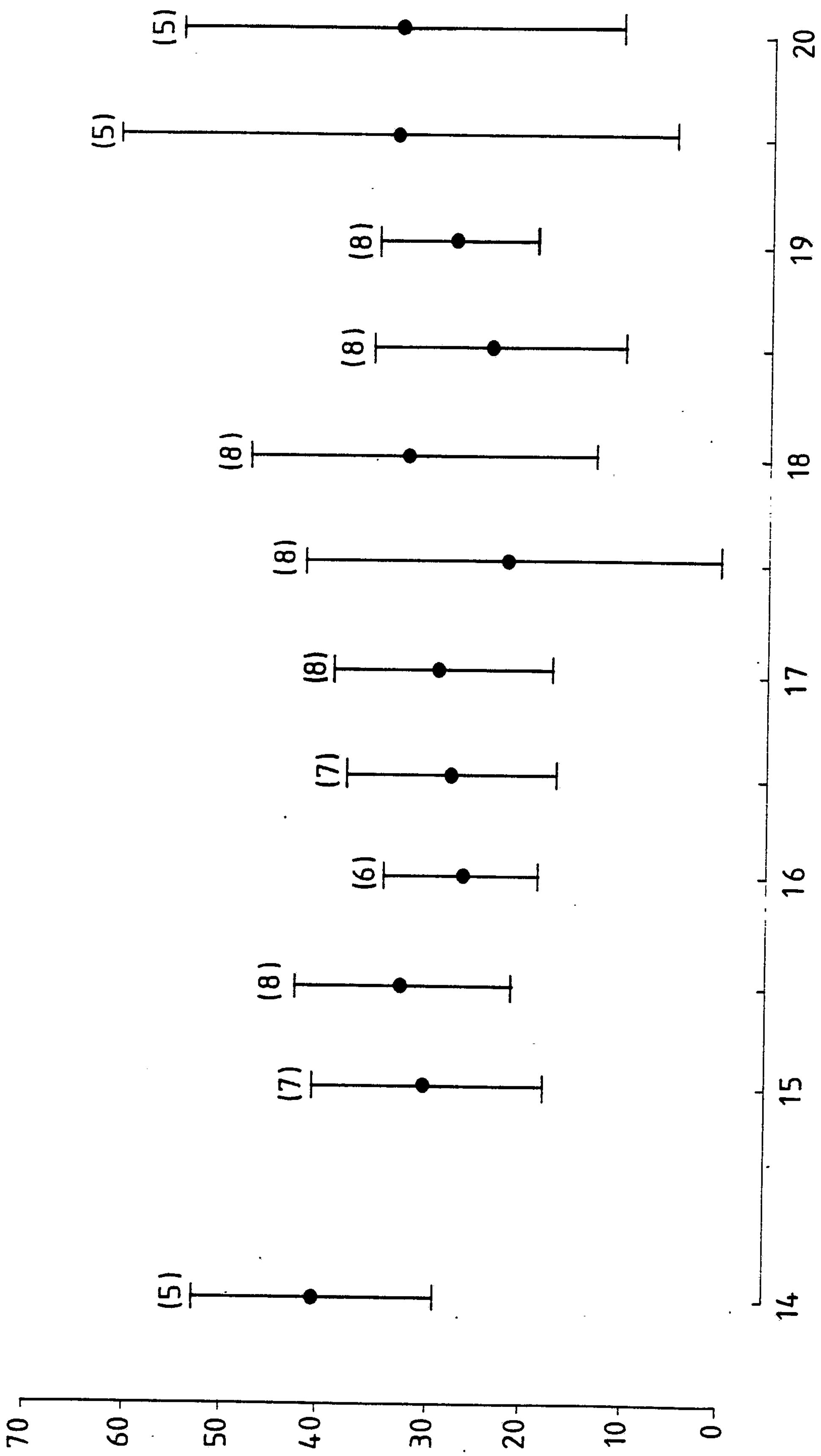

1.5
1.2
0.9
0.6
0.3
0.0
100
50
25
12.5
6.25
3.125

Fig. 3.



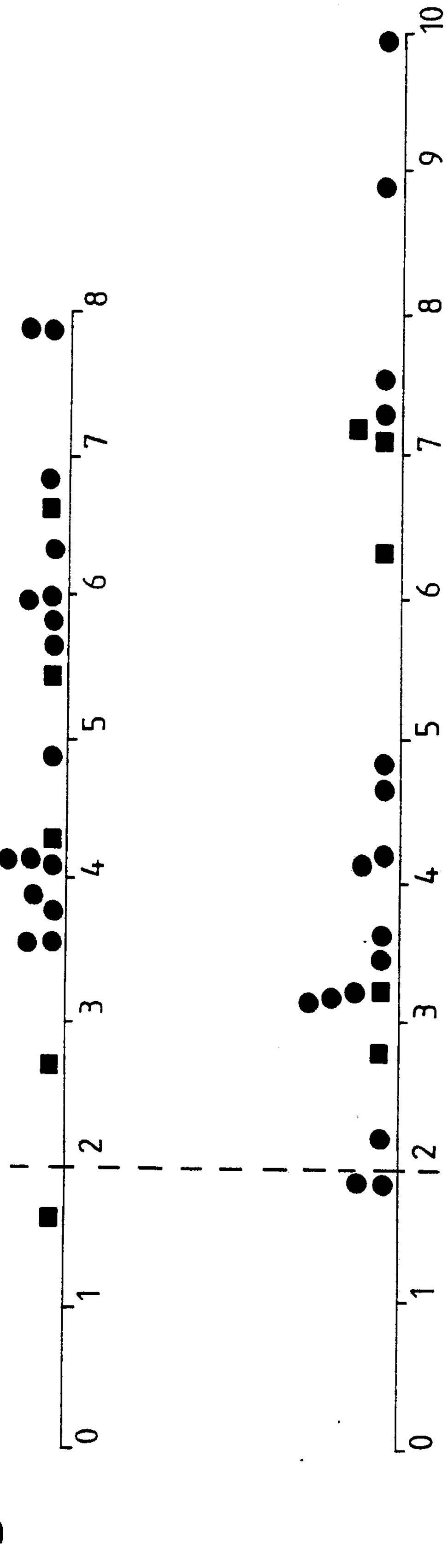


Fig.4.

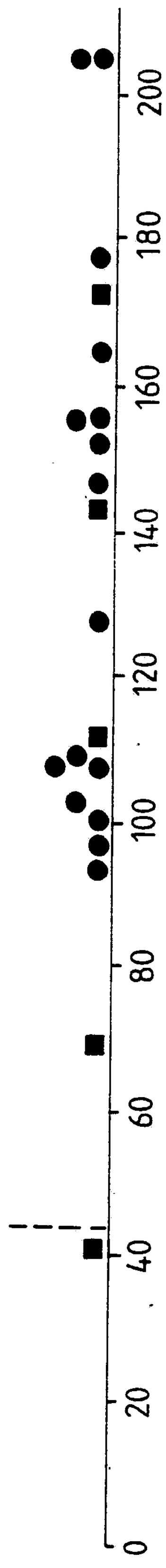


Fig. 5.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01650

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/573; G01N33/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	WO,A,9 108 302 (AVALON BIOSCIENCES LIMITED) 13 June 1991 cited in the application see the whole document ---	1-6, 10-16,21
Y	BIOCHEMISTRY vol. 30, no. 44, 1991, EASTON, PA US pages 10795 - 10799 D. H. SMALL ET AL. 'A protease activity associated with acetylcholinesterase releases the membrane-bound form of the amyloid protein precursor of alzheimer's disease' cited in the application see the whole document ---	1-6,10, 11, 13-16,21

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

1

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 NOVEMBER 1992	10. 12. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	GRIFFITH G.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201650
SA 64229

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 19/11/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9108302	13-06-91	AU-A-	6885591	26-06-91
		EP-A-	0504173	23-09-92
EP-A-0206200	30-12-86	CA-A-	1301095	19-05-92
		JP-A-	62111683	22-05-87
EP-A-0124103	07-11-84	AU-B-	594640	15-03-90
		AU-A-	2747084	01-11-84
		CA-A-	1229564	24-11-87
		DE-A-	3485795	06-08-92
		JP-A-	59208462	26-11-84
WO-A-9003577	05-04-90	EP-A-	0436654	17-07-91
		JP-T-	4501460	12-03-92

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82