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Studies on the stabilization and interconversion of multiple forms of brain acetylcholinesterase (AChE, E.C. 3.1.1.7)

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STUDIES ON THE STABILIZATION AND INTERCONVERSION OF MULTIPLE FORMS OF BRAIN ACETYLCHOLINESTERASE (AChE, E.C. 3.1.1.7)

by

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[redacted]

To Osório, Milena and Melissa

and

To the memory of my father, Amaro P. de Barros, M.D.,

and my uncle, Paulino P. de Barros, M.D.

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ABSTRACT

Acetylcholinesterase (AChE, E.C. 3.1.1.7) was obtained from bovine caudate nucleus tissue, using two methods of solubilization involving a chelating agent EDTA or EDTA with tetracaine. The enzyme was found to exist in two major forms of small (S) and large (L) molecular weights. With time, an aggregation process occurred which converted small to large molecular weight forms via an intermediate form (I).

The molecular weights of brain AChE forms as determined by gel filtration techniques were: 110,000; 210,000 and 340,000 for the S, I and L forms, respectively. A fourth component (void volume peak) with a molecular weight of over 4,000,000 was also detected. It was only separated from the other AChE forms on Sepharose 6B column.

The treatment of the AChE preparation with DEAE Sephadex A-25 resulted in the release of the enzyme in a non-aggregated state. A similar result was also obtained by increasing the ionic strength of the medium with KCl or NaCl.

An interesting finding was the fact that the intermediate molecular weight form (I) of AChE was reconverted to the small form (S), after their separation on gel filtration using a low ionic strength buffer as the eluting solvent. This result was obtained not only with a fresh preparation where S was the predominant form but also with an old enzyme preparation where I was the predominant form, instead.

Neuraminidase and Concanavalin A (Con A) treatments were used as tools for detecting carbohydrate moieties on the enzyme molecule. The results suggested, indeed, the presence of these substances in the enzyme preparations. Carbohydrates, then, might be involved with the AChE

aggregation process and perhaps with the binding of the enzyme to membrane components.

A hypothesis is suggested to explain the mechanisms of the AChE aggregation-deaggregation process, based on the presence of glycoproteins and neuraminidases and/or other specific glycosidases in the brain tissue.

INTRODUCTION

The Cholinergic System

The cholinergic neurons and their synapses, defined as those which release acetylcholine on stimulation, are widely distributed throughout the nervous system in both vertebrates and invertebrates (52). It is generally agreed that while neurotransmitter substances, and the enzymes that synthesize them, are found in highest concentration at nerve terminals, they are also distributed in smaller amounts throughout the entire neuron (145).

Studies carried out on the neuroanatomical distribution of the main components of the cholinergic system--acetylcholine (ACh), choline acetyltransferase (choline acetylase, ChAc), and acetylcholinesterase (AChE)--have demonstrated that only a fraction of the neurons of the brain and spinal cord are cholinergic (51). Regions containing particularly high contents of these components are: the cerebral cortex, hippocampus, caudate nucleus, thalamus, spinal cord and cerebellum. Histochemical studies on rats demonstrated that reticular and tegmental nuclei of the brainstem have AChE-containing fibers which project into cortical and subcortical structures. The function of these fibers may be to facilitate or, in some instances, to depress synaptic transmission (133).

Microelectrophoretic studies showed that some central neurons are excited and others are depressed by ACh (37). Such experiments, using various cholinergic ligands and blocking agents, have shown that both muscarinic receptors (similar to those found on effector cells innervated by postganglionic cholinergic fibers) and nicotinic receptors (defined as those present in autonomic ganglia and at the neuromuscular junction) occur in the central neurons. In addition the transmitter substance ACh

has been found to be released from central synapses, especially after inactivation of AChE (42).

Cholinergic neurons are characterized by their high content of ACh and the enzyme responsible for its synthesis, ChAc. By contrast, studies on the distribution of AChE are complicated by the fact that the enzyme is distributed over the length of the cholinergic neuron and, in certain locations (e.g., the cerebellum), appears to be present in non-cholinergic neurons (145). Thus, the presence of AChE is not a clear-cut indication of a cholinergic neuron.

According to Seiller (131), the best criterion for a cholinergic neuron is the presence of high concentrations of ACh in its ending. Feldberg and Vogt (51) and Hebb (70) have shown, in general, a fairly good correlation among enzymes of the ACh metabolism (ChAc and AChE) and ACh content, in different regions of the brain. Table 1 shows the quantitative distribution of ACh in the central nervous system.

AChE Distribution in the Nervous System

Through the direct association with the metabolism of ACh and also through the relationship existent between AChE activity and the electrical activity in excitable membranes, it became evident that AChE plays a fundamental role not only in the control of nerve excitability but, also, in the control of excitability of muscle and other tissues at postsynaptic sites. Other postulated roles for this enzyme are that it might regulate the level of free ACh at presynaptic terminals, and that it could function as an integral component of cholinergic receptors (29,98).

ACh can undergo rapid breakdown in cerebral tissues, which is brought about by cholinesterases. In most parts of the brain, the enzyme present

TABLE 1. ACh CONTENT IN THE CENTRAL NERVOUS SYSTEM

Tissue	ACh Content	
	µg/g fresh tissue	species
Spinal cord, ventral horn of grey matter	1.5	dog
Cerebellar cortex	0.1 - 0.3	man, cat
Retina	5.0 - 6.0	ox, sheep
Optic nerve	0.0 - 0.3	ox, cat
Superior colliculus	1.7	cat
Basal ganglia	7.0	cat
Occipital lobe cortex	2.2	cat
Frontal lobe cortex	4.5	cat
Olfactory bulb	1.3	cat

Values are from MacIntosh (1941) and Feldberg (1945). Table adapted from: C. O. Hebb, "Formation, Storage, and Liberation of Acetylcholine," in Handbuch der Experimentellen Pharmakologie, Suppl. XV. Cholinesterases and Anticholinesterase Agents, ed. by G. B. Koelle (Berlin: Springer-Verlag, 1963), p. 65.

is the "true" cholinesterase, i.e., AChE which degrades ACh more rapidly than other closely related substrates. Much of the pseudocholinesterase of the brain appears, by histochemical methods, to be located mainly at the walls of blood vessels and in glial cells.

The activity of cholinesterase appears to differ greatly in different parts of the brain. In the hypothalamus, AChE is at very high level in cells of specific hypothalamic nuclei. Rodent cerebellar regions show marked variation in AChE activity, but activities usually parallel those of ChAc (107). This supports a role for the enzyme as limiting the topographic spread of ACh action. Average rates determined for AChE of the whole brain, in different mammalian species, vary between 200 and 500 μmoles of ACh hydrolysed per gram per hour. The rates attained by cerebral AChE show it to be among the most rapid cerebral processes. The mean rate is a quarter to a tenth that of several phosphokinases (107). Table 2 shows the rate of synthesis and hydrolysis of ACh in the dog brain.

The highest activity of ChAc is found in the caudate nucleus, retina, corneal epithelium and central spinal roots. In the cerebellum, ChAc and AChE are distributed differently. There is a high concentration of both enzymes in the cerebellar nuclei, whereas in the cortex, except in the archicortex, only low levels of ChAc occur (59,85). Abnormally high ratios of AChE to ChAc activities are found in substantia nigra and globulus pallidus together with the cerebellar regions (17). The caudate nucleus has the highest content of ACh, ChAc and AChE in the brain (52).

The cytological distribution of AChE has been clarified in some cases, in a quantitative manner, by the use of microgasometric analysis by the Cartesian-diver technic and microscopic histochemistry. Neurons that give rise to the three categories of peripheral cholinergic fibers

TABLE 2. ACh SYNTHESIS AND HYDROLYSIS IN THE DOG BRAIN

Tissue	Rate of ACh Metabolism (μmoles/g fresh tissue/h)	
	Synthesis	Hydrolysis by AChE
Cerebral cortex (different areas)	1.3 - 3.7	60 - 100
Cerebellar cortex	0.09	460
Corpus callosum	--	10 - 15
Caudate nucleus	13.0	1900
Thalamus	3.1	220 - 310
Hypothalamus	2.0	190

Values are from Hebb and Silver, 1956; Hebb and Morris, 1969; Burgen and Chipman, 1951; and Kasa and Silver, 1969. Table adapted from: H. McIllwain and H. S. Bachelard, Biochemistry and the Central Nervous System (4th ed.; Edinburgh:Churchill Livingstone, 1971), p. 454.

(postganglionic parasynpathetic, preganglionic autonomic, and somatic motor) contain relatively high concentrations of AChE throughout their entire length (dendrites, perikarya, axons). The concentrations present in noncholinergic neurons (adrenergic, primary afferent) are considerably lower (87). The presence of AChE in presumably noncholinergic neurons has led to some speculation concerning its function at such sites, including a putative role in amine release.

In neurons of some regions, such as the caudate nucleus and the putamen, the concentration of AChE is high; neurons of other areas can be classified as having intermediate, low, little, or no activity, according to their specific staining reactions. At the motor end-plates of skeletal muscle, most of the AChE is localized at the surface and infoldings of the postjunctional membrane or subneural apparatus (87).

In autonomic ganglia, histochemical and pharmacological findings suggest that the neuronal AChE consists of two fractions: a functional portion, with its active sites directed externally to the surface of the cell membrane and directly concerned with the hydrolysis of ACh; and an internal or reserve portion, representing enzyme more recently synthesized within the endoplasmic reticulum and serving as a source of replacement for the former in the course of the cell cycle of protein turnover (86,88).

Very recently (142), the rate of degradation of AChE was studied in mature rat brain. The half-life of the enzyme was calculated to be 2.8 days. These results demonstrated that AChE is turning over more rapidly, compared to the average of brain proteins.

In sympathetic ganglia of the cat, the external or junctional AChE is almost entirely presynaptic, whereas in parasympathetic ganglia it is both pre- and postsynaptic. In skeletal muscle several additional sites of

AChE can be found, such as the musculotendinous junction where no established functional role is known (87). The same is true of the enzyme in erythrocytes, thrombocytes and placental tissue.

Subcellular Distribution of AChE

The localization of AChE has been reviewed from two rather different points of view. At the molecular level, it is recognized that AChE is a membrane-associated enzyme; at the cellular level, it has been shown in increasingly finer detail that AChE is localized in the synaptic apparatus (77). The enzyme is present in the plasma membrane underneath the myelinic sheath of nerves and, in high concentrations, on the external membrane of the cholinergic nerve terminal. The coexistence of cytoplasmic ACh and AChE in synaptosomes seems less paradoxical if it is assumed that the functional groups of the enzyme face outward and are, therefore, not accessible to endogenous ACh (144).

Studies on the intracellular localization of the enzymes of the ACh metabolism have produced contradictory results. Hebb and Whittaker (69) found ChAc-containing particles distinct from mitochondria. A small amount of the enzyme activity was found in microsomes, but 20 to 28% was in a soluble state. The AChE activity was shown to be up to 70% in the fractions containing the large cytoplasmic particles and the mitochondria of the caudate nucleus. The soluble tissue fraction had only 8% of the total enzyme activity (112).

Toschi (137) and Aldridge and Johnson (2) showed that the highest specific activity of AChE was in the microsomal and mitochondrial fractions. De Robertis *et al.* (40,41) found parallelism in the activity of the three components of the ACh system in mitochondrial subfractions

of the brain. In the primary fractions (nuclear, mitochondrial, microsomal and soluble) there is a difference in the distribution: in contrast to ACh and ChAc, the microsomal fraction contains high AChE activity. All three components were found in the fraction containing nerve endings, and also in an additional subfraction with membranes and fragmented nerve endings. The particles of the second fraction contained nerve endings which did not have any of the enzymes of the ACh system. After treatment of the primary mitochondrial fraction with distilled water and density gradient centrifugation, the AChE was shown to be bound tightly to membranes which were considered to be cholinergic synaptic membranes. The incorporation of C^{14}-tubocurarine, by the isolated subfractions, parallels the AChE activity. This observation strengthens the view that the subcellular distribution of the cholinergic receptor (nicotinic) is similar to that of AChE.

Pharmacological and enzymological investigations have suggested that AChE is located both inside and outside cell membranes. Such suggestions have been grounded on comparisons of the effects of tertiary and quaternary ammonium bases with anticholinesterase activity. Results show that the quaternary compounds penetrate cell membranes poorly, due to their lipid insolubility (76).

Comparison of the distributions of AChE in autonomic ganglia indicated that practically all of the external AChE of the sympathetic ganglia is associated with the presynaptic axonal terminations: most of the cytoplasmic AChE of the cholinergic neuronal perikarya is internal to a relatively impermeable membrane. It has been proposed that an important function of ganglionic AChE may be the protection of the preganglionic terminations from depolarization and antidromic firing, in response to ACh

liberated by these and adjacent terminations. If this represents only a small portion of the total AChE, its inactivation and reactivation might not be detectable by the usual techniques (76).

Cholinergic Receptors (AChR)

Over the last ten or fifteen years a number of authors have claimed to have isolated certain cholinergic receptors (22,48). Some of the earliest efforts, based upon the binding of cholinergic ligands, led to the isolation of a variety of macromolecules that were identified respectively as protein, acid mucopolisaccharide, nucleoprotein, phospholipoprotein or lipoprotein. Most of these studies failed to present enough proof concerning the specificity of ligand binding expected for identification of a cholinergic receptor (113).

The adequate identification of a tissue component as a pharmacological receptor requires satisfaction of a number of criteria (128):

1. The component should demonstrate a high specificity for interaction with many molecules established to react with the receptor of the tissue *in vitro* and *in situ*;
2. The specificity of such interactions should be commensurate with known physiological observations;
3. Putative receptor components should show little affinity for non-specific drugs;
4. The isolated component should be shown to originate from the cellular sites where it is considered to exist *in situ*;
5. The quantity of material isolated should be consistent with estimates of the amount of receptor present in the original tissue.

Until the present time, none of the investigations on isolation and characterization of cholinergic receptors seem to fulfill all of the requirements necessary for its precise identification. The discovery (27) that some snake neurotoxins act as very specific ligands for the nicotinic cholinergic receptor has supplied this field with an excellent approach for assaying and thus for isolating, purifying and characterizing these receptors from different sources. α-Bungarotoxin is a particularly useful label for the AChR because of its physiological specificity. It has been made radioactive with a variety of labels at high specific activities. Its irreversibility of binding permits easy separation of the unreacted toxin from that which has reacted with the receptor.

The cholinergic receptor of the electroplax of the electric eel has been, so far, considered a prototype for a more direct approach to molecular receptor pharmacology. Karlin (84) has pointed out the difficulties involved with the study of receptors in the postsynaptic cell membrane. These problems are mainly concerned with the isolation of the system from its membrane-bound form, and with the fact that it is no longer clearly identifiable after membrane disruption.

Various procedures have been used in attempts to solubilize the cholinergic ligand binding macromolecules from membrane fractions, including salt extractions, use of chelating agents, enzymatic digestions, ultrasonication, and detergent treatment, among others (128). The molecular nature of the putative cholinergic receptor materials, so far isolated, is somewhat controversial; however, most of the investigators seem to agree that a protein component is present and that it is apparently distinguishable from AChE.

In electric tissue, the separated materials considered to be AChR appear to have their origin in innervated membranes. Similarly, the cholinergic binding macromolecules of brain tissue appear to be located in synaptosomal membrane fractions. Identification of AChR *in vitro* has greatly contributed to the progress in the isolation of AChR. Because of high concentrations of AChR found in electric organs most of the advance in its isolation has been in this tissue.

Nicotinic and Muscarinic AChR in Mammalian Brain

Electrophysiological studies on mammalian brain have demonstrated the presence of nicotinic and muscarinic AChR as well as, possibly, an intermediate type, each one predominating in certain areas of the brain. Muscarinic AChR predominates in the cerebral cortex and caudate nucleus (32); nicotinic AChR in cerebellar cortex (33); and the intermediate type in the brain stem (15) and in the thalamus (106).

More recently, Schleifer and Eldefrawi (129), in studies done on the binding of radioactive compounds to mitochondrial and synaptosomal preparations from mouse brain, by means of equilibrium dialysis, suggested that the ACh binding sites are on the AChR, which is found at concentrations similar to AChE. Those AChR were divided almost equally between muscarinic and nicotinic types.

Hiley and Burger (73) measured the concentration of muscarinic receptors in twenty-two areas of the dog nervous system. They showed that, when propylbenzilylcholine mustard (PrBCM), an alkylating agent analogue of atropine, reacts with brain tissue, a considerable fraction of the uptake by cortex or caudate nucleus homogenate can be inhibited by atropine. The binding of PrBCM could also be inhibited by ACh, pilocarpine and other

muscarinic agonists, but was unaffected by nicotinic agonists or antagonists or still other compounds without known pharmacological reactivity on putative ACh receptors. These tests, together with the quantitative aspects of the inhibitory effects and the close similarity of the results obtained by these methods when applied to the longitudinal smooth muscle of the guinea pig ileum, led those investigators to suggest that they were dealing with muscarinic receptors. They also observed a considerable range in concentrations: from the highest level of muscarinic receptor concentration, found in the caudate nucleus (470 pmol/g protein), to values in some areas that by their technique are not detectably different from zero (e.g., pes pedunculi, medullary pyramids, retina, posterior half of the lumbar spinal cord, posterior spinal roots, and optic nerve).

The uneven distribution of receptor concentration in the nervous system is in broad agreement with the known uneven distribution of ACh and its synthetic and degradative enzymes. The presence of the highest concentration of the receptor in the caudate nucleus agrees with this being the site of highest ACh synthesis (68) and of AChE (18).

In studies performed by Yamamura and Snyder (150), it was observed that low concentrations of 3-quinuclidinylbenzilate (QNB), a potent muscarinic cholinergic antagonist, bound specifically to muscarinic cholinergic receptors in brain homogenates. These investigators also measured the specific binding of [^{3}H] QNB in 30 regions of monkey brain. They observed marked regional variations in QNB binding with a 33-fold difference between the putamen, which contained the highest density of binding sites (1126 pmoles/g protein), and the optic chiasm, which had the lowest one (34.4 pmoles/g protein). The head and body of the caudate nucleus contained about the same amount of binding (976 and 1061 pmoles/g

protein, respectively) as the putamen. The presence of substantial QNB binding in most brain regions and its tendency to correlate with choline uptake and choline acetyltransferase activity suggested to them that a major portion of AChR in the brain was muscarinic.

Relationship between AChE and AChR

Several attempts have been made to correlate the molecular behavior of AChE with events at the AChR level, and suggestions have been presented as to the possibility of the anionic binding site of AChE being identical to a portion of the AChR macromolecule (9). The effects of ACh at the synaptic membrane have been explained in terms of an induced conformational change in the receptor. It is well known that the ionic quaternary part of ACh is sufficient to cause this change, supporting the view that the receptor molecule need not possess any of the esterolytic properties of AChE. It would appear, then, that only that part of the enzyme which carries the anionic sites needs to be retained and adapted to the specific requirements of synaptic membranes in order to function as a cholinergic receptor (9).

Belleau and DiTullio suggested that the anionic site of AChE may serve as a model in studies of the mechanisms of quaternary ion interactions with the AChR of excitable membranes (9). Assuming that AChE is a primitive enzyme, the possibility exists that, during the course of evolution, gene duplications and mutations may have adapted the enzyme, or part of it, to the role of quaternary ion receptor in a variety of synaptic membranes. For this purpose, only the anionic site-rich part of the enzyme may have been retained and adapted.

Changeux (28) has suggested that AChE is closely related to the cholinergic receptor in the postsynaptic membrane. He further proposes that the receptor-esterase unit (protomer) is an equilibrium between two conformationally different states: $P \rightleftharpoons D$. P represents the state of the protomer when the postsynaptic membrane is polarized, and D represents the depolarized state. Membrane potential (and thus permeability) is determined by the fraction of protomers which undergo D state transition. The activities of agonists and antagonists are described in terms of differential stabilization of either D or P conformational forms. This theory of allosteric transitions is modeled after that described for regulatory enzyme by Monod, Changeux and Jacob (110).

However, several pieces of evidence demonstrate differences between the catalytic site of AChE and the ACh binding site of the AChR: (a) ACh agonists and antagonists (e.g., muscarine, nicotine, decamethonium, curare and atropine) have much higher affinities for AChR than for AChE; (b) ACh, acetylthiocholine and acetylselenocholine are hydrolysed by AChE at similar rates, but have widely different depolarizing potencies; (c) several organophosphates phosphorylate AChE irreversibly, at low concentrations, without affecting depolarization of the innervated membrane of eel electroplax; only at much higher concentrations do they cause its reversible repolarization and block the binding of cholinergic ligands *in vitro*; (d) analogs of benzoquinonium and ambenonium derivatives have effects on depolarization of eel electroplax that differ markedly from their effects on the inhibitory action of AChE *in vitro*; (e) AChE is degraded by papain and not by phospholipase C, whereas the opposite is true for AChR, measured by binding of muscarone to *Torpedo* extracts; (f) after toluene extraction of the receptor fraction from *Torpedo* electroplax

tissue there is no binding of muscarone, but AChE is still active; (g) they have different sensitivities to pH and temperature. The exposure of AChE to 48°C for 20 minutes destroys it, whereas about 30% of the suspected AChR, as judged by the specific decamethonium binding, survives (113).

Differences found between the active sites of AChE and of AChR do not exclude the possibility that a single molecule carries both sites, especially since the ratio of catalytic sites of AChE to binding sites of AChR is usually near unity.

In studies done with E. electricus (30), the macromolecule which carries the cholinergic receptor site was thermolabile and digestable by proteolytic enzymes, appearing to be a protein or at least to possess a protein moiety. The receptor protein was selectively adsorbed by Naja nigricollis α-toxin, which had been covalently bound to Sepharose, and then it was separated from AChE. Changeux et al. (30), therefore, concluded that the catalytic site of AChE and the AChR site are located on different polypeptide chains. Nothing more is known about the topographical relationships of these two protein units once integrated in excitable membranes.

Physical separation of a macromolecule, that binds α-BGT but does not hydrolyse ACh, has also been achieved by gel filtration or density gradient fractionation in the presence of sodium dodecyl sulphate (SDS). Using a Lubrol XW-solubilized preparation from Torpedo electroplax, O'Brien et al. (unpublished, see 113) claimed to have achieved a partial separation on Sepharose 6B of AChE from AChR, judging the latter by appropriate ACh binding. Also, when most of AChR, in the deoxycholate extract of eel electroplax, bound irreversibly to α-BGT coupled to Sepharose 4B, it sedimented in a low speed pellet, leaving most of AChE in solution. This

partial separation of AChR activity from AChE supports the suggestions that they are separate macromolecules. Furthermore, whereas muscarone and ACh at micromolar concentrations bound to the macromolecules thought to be AChR in different tissue preparations, those compounds did not bind to purified AChE from eel electroplax or erythrocytes.

More recently (118), results of a preparative scale-affinity chromatography showed that the α-BGT binding activity was cleanly separated from the bulk of the protein as well as from the AChE activity. This investigation was mainly concerned with the large-scale isolation and characterization of AChR from the electric organ of Torpedo californica.

From the studies performed on AChR, to date, a general agreement seems to exist between the results obtained with Torpedo and with Electrophorus on the following points: (a) decamethonium alone is a relatively non-specific reagent for receptor binding; (b) snake venom α-toxins are highly specific reagents for the cholinergic receptor site; (c) the receptor contains a protein moiety, as judged by its degradability by proteinases; (d) various detergents can solubilize receptor activity; (e) AChE and AChR activities are clearly separable (30).

AChE: Purification, Properties and Isoenzymes

A useful requirement for the purification of a macromolecule is a large supply of the crude material. This is the major reason why AChE has been, so far, purified mainly from the electric tissues of Torpedo marmorata and Electrophorus electricus. These rich sources of most of the components of the ACh-AChE system were first recognized by Nachmansohn and Lederer in 1939 (111). The value of these sources became apparent when it was verified that 1 kg of electric tissue, with a protein content of

30 g, metabolizes 2 to 4 kg of ACh per hour. Since then, the enzyme has been purified by means of several chromatographic procedures. The specific activity of such preparations ranged from 440 to 750 nmoles of ACh hydrolysed per miligram of protein per hour (89,96,111).

Leuzinger and Baker (96) reported the crystallization of this enzyme in a 35% ammonium sulfate solution. When a solution of these crystals was tested with disc electrophoresis, a single band was obtained. Their starting material was 10 kg of toluene-treated preparation, which gave about 60 mg of homogeneous enzyme protein. The highest specific activity obtained was 750 nmoles ACh hydrolysed per miligram of protein per hour.

Berman and Young (11) derivatized agarose with low molecular weight compounds related to potent AChE inhibitors. Their preparation firmly bound 94 to 100% of the enzyme applied. It was eluted with another strong enzyme inhibitor, edrophonium, at a concentration of 10^{-2} M. This technique has the advantage of simplicity as well as effectiveness: crude material is applied once, and a highly purified preparation is obtained with a specific activity over 900 nmoles of ACh hydrolysed per miligram of protein per hour.

Sedimentation studies done by Kremzner and Wilson (89) showed a sedimentation coefficient of 10.8 S, for a preparation of AChE with a specific activity of 660 nmoles of ACh hydrolysed per miligram of protein per hour. This preparation showed the presence of a single symmetrical peak during sedimentation velocity measurements. These investigators also reported that, when the preparation lost activity on storage, the $S_{20,w}$ value increased to 11.4 S. The molecular weight calculated from this study was 230,000. Using a similar technique, Leuzinger et al. (97) reported a molecular weight of 260,000 for their enzyme preparation.

Data on the subunit structure of AChE from electric organs of E. electricus indicated that the enzyme dissociates into four subunits of 64,000 daltons each. Examination of C-terminal residues, by two independent methods, revealed the existence of two types of polypeptide chains on AChE molecule. This fact suggested that the enzyme has a dimeric hybrid structure with two α and two β chains. Kremzner and Wilson (89), utilizing a different technique, estimated an "equivalent weight" (weight per active site) of 54,000. It appears from their data that the enzyme molecule also consisted of four subunits. From gel electrophoresis experiments, Dudai and Silman (45) reported two major polypeptide chains of molecular weights 88,000 and 64,000, respectively. Powell et al. (117), using the same technique, also found two subunits with molecular weights of 62,000 and 91,000. According to them, these subunits appear as common components of all AChE forms whether native or obtained by proteolytic digestion.

Recently (109), an enzymatically active species of AChE from E. electricus has been isolated, with a molecular weight of 134,000. It apparently had its origin in the Triton X-100 mediated cleavage of higher polymers of AChE. The subunit molecular weight of this species was 65,000, as determined by SDS gel electrophoresis, indicating the enzyme to be a dimer.

Results of previous investigations on the number of active sites are still controversial. The enzyme is assumed to contain either four (53) or two (98) active sites. More recent studies revealed the presence of 3.3 active sites per enzyme molecule, by active site titration measurements (120).

Purification of the brain enzyme has had as a major problem its solubilization from other tissue components. Several approaches have been used which include the utilization of surface-active agents, organic solvents, proteolytic enzymes and chelating agents, among others (23,34, 74,75,81,82,94,115,127).

The nonionic detergents offer some usefulness preferably to cationic and anionic detergents which cause enzyme inactivation, even at relatively low concentrations (94,115). Recently, Yamamura et al. (149) claimed to have achieved an 80% solubilization of AChE from guinea pig brain homogenates, after treatment with Triton X-100. Also, Bellanger et al. (8), studying the optimum conditions of solubilization of brain AChE from baboon monkeys, showed yields of 75 to 80% of the original activity, when 2 to 3 mg of Triton X-100 was added per mg of protein in the homogenate. The maximum recovery, using sodium deoxycholate, was only of 50 to 60% due to some inhibitory effect of this detergent on the enzymatic activity. No change of the catalytic properties of AChE was observed in the presence of Triton X-100, in the range of concentration used.

The purpose of the organic solvent extraction procedures is to remove lipids, which are present in appreciable concentration in brain membrane preparations. The delipidation is followed by the release of AChE into an aqueous medium in a soluble form. Lawler (94) obtained a 33% solubilization of AChE from calf caudate nuclei, by means of homogenization of a dry acetone powder in aqueous medium at pH 8.8. The percentage of solubilization was increased to 80% with the concurrent use of pancreatic lipase. Another more successful procedure (81) used repeated dry n-butanol extraction of a freeze-dried residue from calf caudate nuclei. The extracts were washed with anhydrous ether, dried and powdered, and AChE

was extracted into glycine buffer at pH 9.0. An overall purification of 30- to 35-fold was obtained with a 4 to 6% yield of purified brain AChE. The most active AChE preparation hydrolysed 1.11 mmoles of ACh per hour per milligram of protein.

Several investigators have used enzymes to solubilize AChE from brain tissue (74,82,94,115). Ho and Ellman (74) incubated rat brain microsomes for 20 hours with bacterial protease, which resulted in a 72% solubilization, with a 40-fold purification after DEAE Sephadex and Sephadex G-200 chromatography. Later, Kaplay and Jagannathan (82) obtained a 13% solubilization of AChE activity present in the original homogenate from ox caudate nuclei, by means of pancreatic elastase. The final specific activity of their preparation was 100 mmoles of ACh per milligram of protein per hour.

The use of chelating agents such as EDTA has been very successful (23,24), especially if one considers the fact that, with such a mild extraction process, any possible physicochemical changes on the enzyme macromolecule which could alter its characteristics would be minimized.

The introduction of affinity chromatography (25,149), for the purification of the brain AChE, has made it possible to obtain this enzyme with a specific activity comparable to that of the eel enzyme. However, studies dealing with properties and characteristics of the brain enzyme are not abundant in the literature as yet.

In recent years much evidence has been presented on the multiple nature of enzymes. Such enzyme systems as dehydrogenases, esterases and phosphatases, among others, have been shown to exist in more than one molecular species (isoenzymes) by kinetic, electrophoretic and column separation techniques (12). The term "isoenzyme" was employed for the first time by Markert and Møller (101), to denote different molecular

forms in which proteins may exist with the same type of enzymatic activity. The existence of multiple molecular forms of enzyme within a single tissue may be due to a variety of reasons, including genetic variations manifest at the level of the polypeptide synthetic machinery itself, multiple cell types in one tissue, each producing its own form of enzyme, enzymatic alteration, non-enzymatic processes like folding, aggregation, dissociation, chemical modification, catabolic changes and artifacts of isolation (57).

Over a hundred isoenzyme systems have been described in animals, plants and bacteria. They do not represent any specific class of proteins. Some have their basis in subtle differences in protein conformation, such as a form of creatine kinase found in the brain of sparrow (39). Others, such as serum BuChE forms in the human, are believed to be a series of polymers of the same protein (91). Many isoenzymes are products of the activity of multiple alleles or separate genes, e.g., lactic dehydrogenase (67). Molecular weight variants of placental alkaline phosphatase appear to be due to aggregation and dissociation (57).

Several isoenzyme systems have been shown to change their distribution with development. These include lactic dehydrogenase (21), malic dehydrogenase (72), aldolase (125), phosphorylase (38), creatine kinase (39), non-specific esterase (13,47), and AChE (105,146). Many of these have been studied in developing muscle. Several isoenzymes of AChE and BuChE are supressed during muscle development (146). But, these isoenzymes are not suppressed in muscles from chickens with inherited muscle distrophy, and it is possible that ChE's may play a special role in this abnormality (147). A knowledge of the multiple molecular forms of enzymes has opened new perspectives in molecular pathology. It is now well known that these molecular forms may vary according to tissue, species and age, and also in some pathological conditions (126).

AChE isoenzymes have been found in Torpedo and Electrophorus electric organs (46,61,102), rat diaphragm muscle (66), insects (65,78), erythrocytes (148), chicken embryo (80) and crustacean nervous system (104). The presence of more than one active form of mammalian brain AChE, from different sources, has also been reported (5,8,13,25,74,75,81,108,140).

An interesting aspect of investigations performed on human serum ChE's has been related to the observation that these isoenzymes are interconvertible (93). It has been observed that native isoenzymes may differ from one another in their state of molecular aggregation. LaMotta et al. (93) proposed that interconversion among ChE isoenzymes occurs by a process of molecular aggregation-deaggregation. Little is known about the factors involved in the interconversion process occurring with the brain AChE isoenzymes.

Data on the multiple nature of AChE in nervous tissue might lead to specific information on the relation of the enzyme to the nerve conduction process, or might shed light on the general problem of the physiological meaning of the occurrence of multiple molecular enzyme species.

Scope of the Research Problem

Because of the importance of brain AChE for a better understanding of membrane excitability and cholinergic synaptic transmission, this area of study was chosen for the present work. A better knowledge of AChE isoenzymes would permit the development of more efficient enzyme reactivators used to reverse the inhibition of cholinesterases by anticholinesterases. The pharmacological aspects of the use of anticholinesterase agents as "war gases" and as insecticides are based on an understanding of the changes which occur in the organism, following the inactivation of

cholinesterases. Each organ innervated by cholinergic nerves responds to the anticholinesterase agent and the response can be attributed to the accumulation of ACh.

In addition, Tripathi et al. (138) have shown that the reactivity of eel and housefly isoenzymes toward substrates and inhibitors differs over a range of between 1.5 and 4.2-fold. These investigators observed that no single isoenzyme predominates in either tissue.

AChE isoenzymes from beef brain are also known to differ not only in molecular sizes, but also in pH and substrate optima, in Km and in their inhibition by physostigmine and fluoride ion, as well. Chan et al. (26) showed difference in sensitivities of AChE brain isoenzymes to some of the inhibitor agents tested.

Consequently, the possibility exists that the AChE isoenzymes may differ in their physiological role. The effects of anticholinesterases are, then, perhaps not accurately measured by studies performed on the total AChE.

In the present investigation two methods for enzyme extraction, using EDTA (24) and EDTA plus tetracaine (75) as solubilizing agents, were employed. The major interest was to study AChE forms on mammalian brain. It was expected that mild extraction procedures would avoid or decrease the possibility of artifactual changes on the molecular species of the enzyme.

AChE isoenzymes from several sources undergo, under certain conditions, an aggregation process. This process is not well understood, but most investigators agree that the larger forms appear to be multiple molecular weight aggregates of the smaller ones.

Early investigations, reported on AChE from E. electricus (94,122), indicated molecular weights for the enzyme ranging from two to thirty million. Apparently the molecular size is salt dependent, being very high in medium of low salt concentrations. High salt concentrations, on the other hand, produce enzyme species with low molecular weights.

Grafius et al. (63) suggested that high molecular weight species are aggregates, and that reversible aggregation of AChE may be of biologic importance. The significance of the phenomenon of aggregation is still unknown. It has been suggested that a reversible aggregating AChE system, as part of the macromolecule structure of electrogenic membranes, may regulate ionic permeability during nerve impulse transmission (62). Such aggregation phenomenon could be of physiological importance, considering the supposedly regulatory nature of the enzyme (28).

In the past few years evidence has been accumulating in the literature concerning the presence of carbohydrates in AChE isoenzymes from several sources (31,117,119). Some investigations have even suggested the involvement of the carbohydrates with the AChE aggregation process (46,134). A glycoprotein is a protein that has attached to it one or more carbohydrate groups. Such carbohydrate groups may be quite simple (di, tri or oligosaccharides) or more complex heteropolysaccharide chains containing N-acetylneuraminic acid (NANA), mannose, galactose, N-acetylglucosamine and/or fucose. Glycoproteins are found in brain tissues (16) and some of these compounds are known to be good substrates for neuraminidases. In the plasma membranes they are believed to account for part of the carbohydrate-containing material on the outer surface of the cell. Glycoproteins are also present in neuronal membranes (60).

The analysis of different subcellular fractions, obtained from synaptosomes isolated from adult rat brain, showed that synaptosomal plasma membranes (SPM) have the highest specific activity of protein-bound sialic acid of the synaptosomal subfractions (60). Neuraminidase also is known to be present in brain tissues; higher activity has been found in grey matter, e.g., caudate nucleus (58,114,124). There is also evidence that particulate neuraminidase is linked to nerve tissue membranes (136). The enzyme has an optimum pH close to 5, presenting little or no activity below pH 4 or above pH 8 (19).

Investigations (136) have shown that when rabbit brain homogenate was divided into the conventional nuclear, crude mitochondrial and microsomal fractions, the activity of particulate neuraminidase was almost completely recovered in these two later fractions and equally distributed between them. After subfractionation of the crude mitochondrial fraction on a sucrose density gradient, 80% of the activity was recovered in the bands rich in nerve endings.

As a result of the present investigation a hypothesis is proposed which is mainly based on the presence of neuraminidase and complex carbohydrates in the brain enzyme preparation. Glycoproteins by causing the association among AChE molecules may be a factor involved with the aggregation process. Neuraminidase by splitting NANA (N-acetylneuraminic acid) residues from glycoproteins may cause the dissociation of AChE macromolecules and, thus, might contribute to AChE deaggregation or stabilization.

However, only a general hypothesis is offered here, and much more complex interactions might exist, and other types of linkages besides glycosidic ones could also be involved with the aggregation process and attachment of the enzyme molecule to the membrane surface.

Objectives of the Study

In the present work, multiple forms of AChE from bovine brain (caudate nucleus tissue) were studied, utilizing two different extraction procedures. The main objectives were:

1. To study methods of solubilization of the membrane-bound enzyme with the use of a chelating agent such as EDTA (1^{st} method) and EDTA plus tetracaine (2^{nd} method);
2. To compare enzyme activity and protein content in the several sub-cellular fractions from fresh and frozen tissues;
3. To study interconversion between AChE enzyme forms, as judged by changes in molecular weights, using gel filtration chromatography of the soluble and solubilized membrane-bound enzymes;
4. To determine the effect of ionic strength (I = 0.09-1.0) *per se* and ions (KCl and NaCl) on the AChE forms;
5. To study the effect of different pH's on the AChE isoenzymes;
6. To gain further support for the possibility of obtaining the enzyme in a non-aggregating state, using DEAE Sephadex A-25 and high salt concentration (e.g., 1 M KCl);
7. To attempt to determine the possible factor(s) responsible for or involved with the aggregation process, by means of procedures (reconstitution experiments) whereby combined fractions were examined for aggregation, and by affinity chromatography;
8. To detect ways of reversing the aggregation of AChE forms after gel filtration;
9. To detect the presence of carbohydrate residues on AChE macromolecules;
10. To determine the molecular weights of AChE forms by means of Sephadex G-150, Sephadex G-200, and Sepharose 6B.

Most of the studies on AChE in the literature deal with the eel enzyme. On the other hand, characterization of the mammalian enzyme is important for the understanding of the molecular events associated with the cholinergic synaptic transmission at the central nervous system, and one cannot simply extrapolate the results from one system to another. Finally, it is hoped that this work may shed some light on this fascinating but sometimes controversial field.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade. Acetylthiocholine iodide (ATC), alkaline phosphatase (from E. coli, type III), p-nitrophenyl-phosphate (ditris salt), thyroglobulin (bovine, type I), concanavalin A (from jack beans, grade III), neuraminidase (from C. perfringens, type VI), α-methyl-D-mannoside, 5,5'dithio-bis-(2-nitrobenzoic acid) (DTNB), crystalline tetracaine, and Blue Dextran B-2000 were obtained from Sigma Chemical Co. (St. Louis, Mo.); hydrogen peroxide (30%) came from Fisher Scientific Co. (Pittsburgh, Pa.); catalase (from bovine liver), apo-ferritin (from horse), albumin (bovine), and γ-globulin (human, fraction II) were purchased from Schwartz/Mann Bio-research Inc. (Orangeburg, N.Y.); DEAE Sephadex A-25, Sephadex G-150, Sephadex G-200, and Sepharose 6B were purchased from Pharmacia (Uppsala, Sweden); affinose-202 was obtained from Bio Rad Labs. (Richmond, Ca.); and edrophonium chloride came from Hoffman La Roche (Nutley, N.J.).

Solutions and Gels

0.32 M Sucrose Solution.

Sucrose-EDTA-Tris Solution, pH 8.0: This was a 0.32 M sucrose solution, containing final concentrations of 1 mM EDTA and 6 mM Tris. The pH was adjusted by slowly adding Tris (Trizma base) to the solution stirring at room temperature.

Sucrose-EDTA-Tetracaine-Tris Solution, pH 7.0: This was a 0.32 M sucrose solution, containing final concentrations of 0.1 mM EDTA, 0.01 mM tetracaine and 30 mM Tris. The pH was adjusted with 4 N HCl.

0.3 M Tris Buffer Stock-Solution, pH 8.0: The pH was adjusted with 4 N HCl. The solution was diluted to 30 mM before use.

0.3 M Phosphate Buffer Stock-Solution, pH 8.0: The pH was also adjusted with 4 N HCl. The solution was diluted to a final concentration of 30 mM before use.

DEAE Sephadex A-25 Preparation: DEAE Sephadex A-25 was slowly added to the 0.32 M sucrose, to the 0.32 M sucrose-EDTA-Tris, pH 8.0, or to the 0.32 M sucrose-EDTA-Tetracaine-Tris, pH 8.0, solutions. The mixtures were stirred slowly at room temperature, for 30 min. The gel was then left to swell for at least 3 hours. The slurry was kept at 4°C. This preparation was used to obtain non-aggregating forms of the enzyme.

KCl-Tris Stock-Solution, pH 8.0: This was a 1 M KCl solution, containing 0.1 M Tris (final concentrations), at pH 8.0 adjusted with 4 N HCl. The solution was used for elution of the enzyme from DEAE Sephadex A-25 preparation.

500 mM Sodium Acetate Buffer Stock-Solution, pH 5.5: The pH was adjusted with 4 N HCl.

Neuraminidase Stock-Solution: Neuraminidase was added to 500 mM sodium acetate buffer, pH 5.5, to obtain a final concentration of 1 unit per ml.

Concanavalin A (Con A) Stock-Solution: Con A was added to a solution of 500 mM sodium acetate buffer, pH 5.5, to obtain a final concentration of 40 μM.

10 mM $MnCl_2$, and 10 mM $CaCl_2$ Stock-Solutions: These were prepared in distilled water.

100 mM α-Methyl-D-Mannoside Stock-Solution: Prepared in distilled water.

Edrophonium Chloride Stock-Solution: Edrophonium in 30 mM phosphate buffer, pH 8.0, was added to 100 mM NaCl, to a final concentration of 10 mM.

All solutions were prepared in distilled and deionized H_2O.

Assays

AChE: The method of Ellman et al. (49) was used. One unit of enzyme activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 μmole of acetylthiocholine per hour, at 25°C, unless otherwise specified. The specific activity is expressed as units per milligram of protein.

Alkaline phosphatase: The Garen and Levinthal method (56) was used; the rate of release of p-nitrophenol from p-nitrophenylphosphate was determined by following absorbancy changes at 410 nm. One enzyme unit is that activity liberating 1 μmole of p-nitrophenol per minute, under the defined conditions at 25°C.

Catalase: The Beers and Sizer technique (7) was used. The disappearance of peroxide was followed spectrophotometrically at 240 nm. One enzyme unit is equal to 1 μmole of hydrogen peroxide decomposed per minute, under the specified conditions at 25°C.

Blue dextran: It was estimated by extinction at 625 nm.

Protein: It was determined by following the absorbance at 220 nm or 280 nm, or by the method of Lowry et al. (100) using Bovine Serum Albumin (BSA) as the standard.

All spectrophotometric assays were carried out with a Norelco-Unicam model SP 500 spectrophotometer from Unicam Instruments Ltd. (Cambridge, England).

Solubilization of Brain AChE

Solubilization of brain AChE was carried out by two different methods (Figures 1 and 2). The first procedure was that of Chan et al. (24), and the second one was that of Hollunger and Niklasson (75). Both were modified.

1. First method (Chan et al.): Bovine brains were obtained from a local slaughterhouse, 1 to 2 hours after death, and transported to the laboratory on ice. Caudate nucleus tissue was excised, chopped finely with scissors, and a 15% homogenate was prepared in 0.32 M sucrose, by means of 8 to 10 passes (at 2000 rev./min) in a motor-driven Potter-Elvehjem Teflon tissue grinder (clearance between pestle and tube: 0.004"-0.006"). Centrifugations were carried out at 4°C in a Lourdes model A-2 Betafuge, using the 9RA rotor, and in a Spinco model L centrifuge, using the rotor FA 30. In some experiments, the mitochondrial-microsomal fraction was resuspended and left stirring at 4°C overnight.
2. Second method (Hollunger and Niklasson): Bovine brains were obtained from the same source and in the same conditions as described above. Caudate nucleus tissue was excised and a 15% homogenate (w/v) was prepared in a 0.32 M sucrose-EDTA-tetracaine-Tris solution, at pH 7.0. Centrifugations were carried out at 4°C in a Spinco model L centrifuge, using an FA 30 rotor.

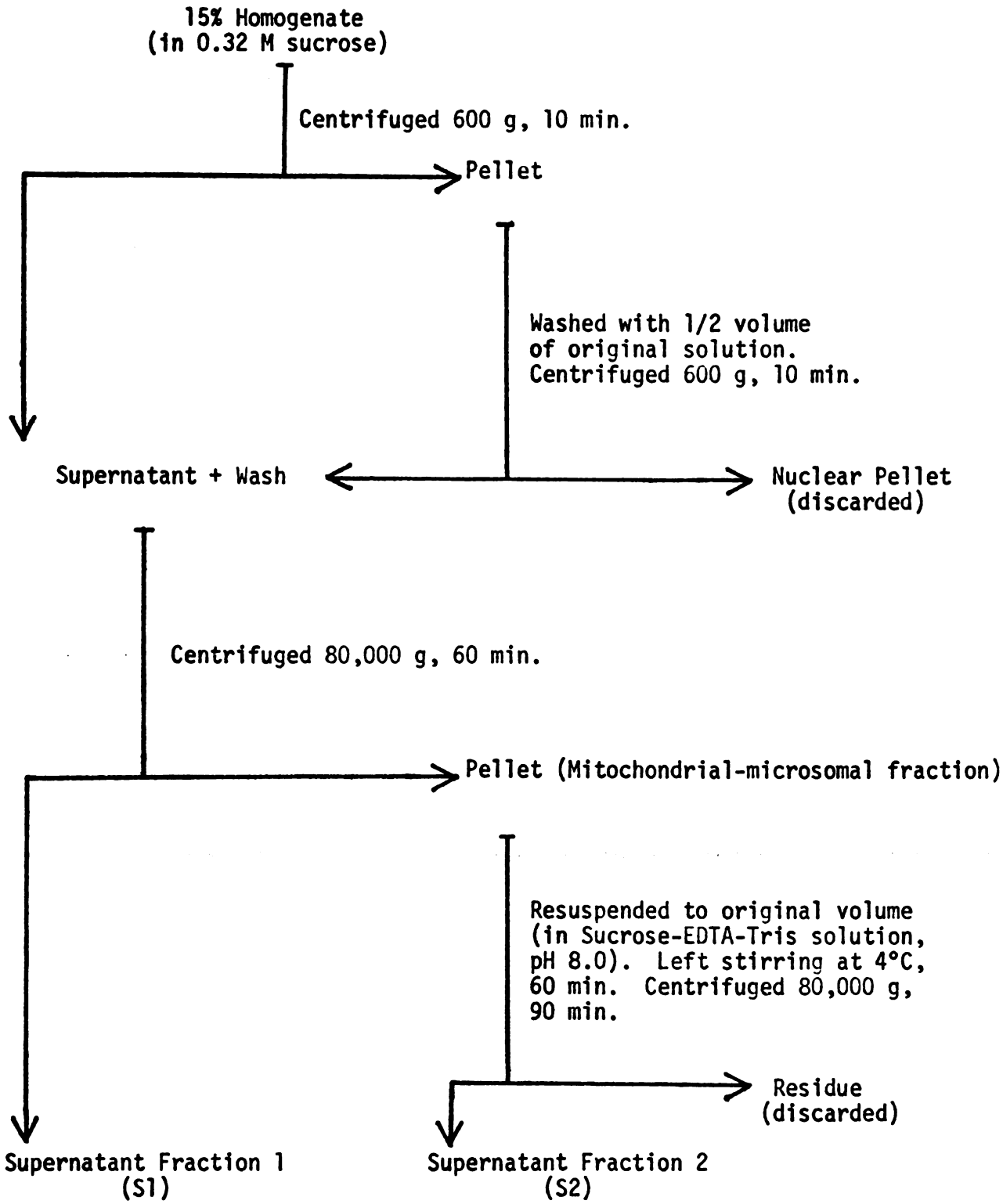

Figure 1. Fractionation Scheme for AChE Solubilization by the 1st Method: Chan et al. (24), Modified.

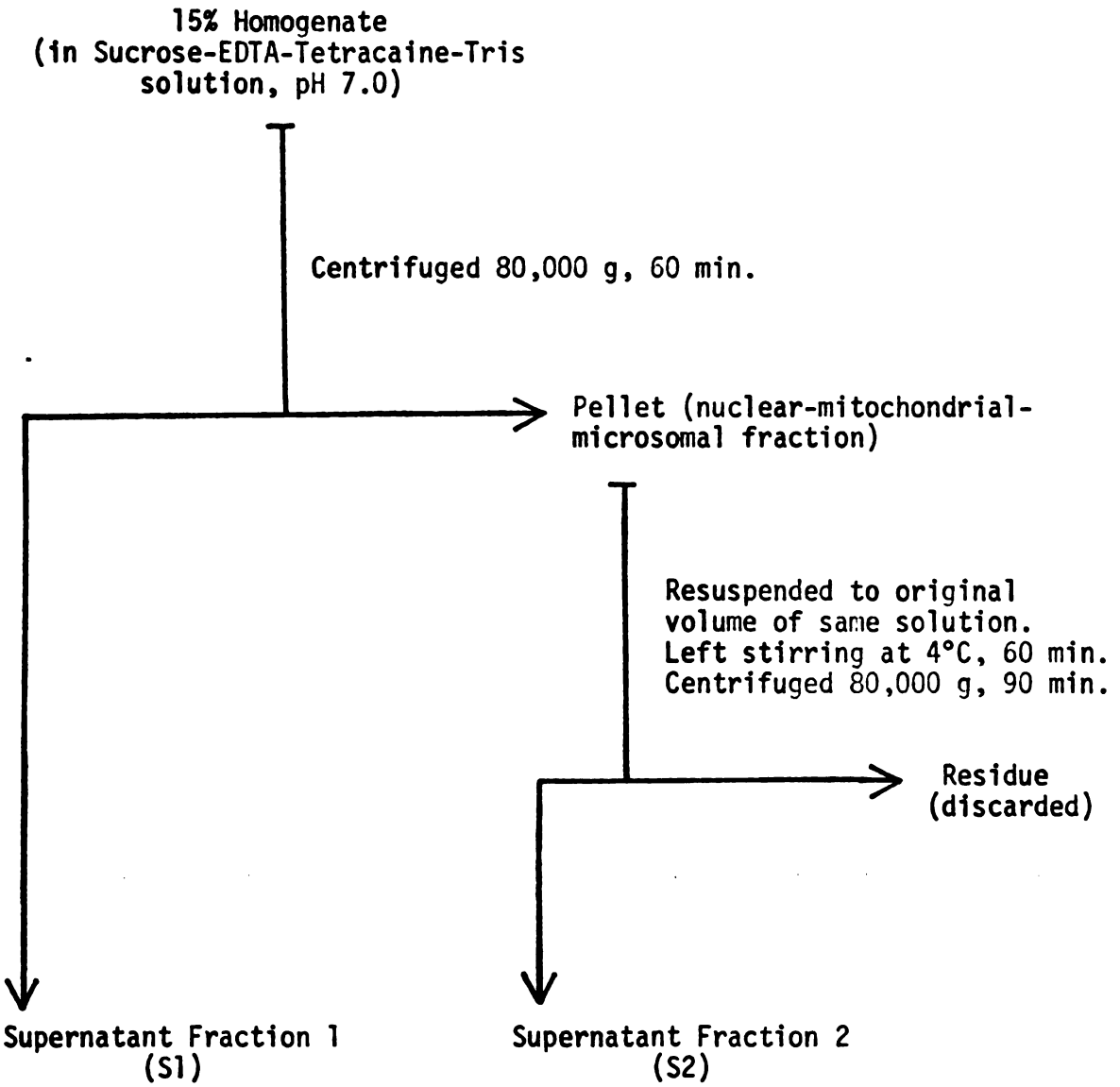

Figure 2. Fractionation Scheme for AChE Solubilization by the 2nd Method: Hollunger and Niklasson (75), Modified.

In both procedures, the supernatant fraction 1 (S1) was saved for studies on the soluble AChE, and the supernatant fraction 2 (S2) was the source of membrane-bound enzyme.

Gel Filtration Experiments

All chromatography was done at approximately 4°C. The enzyme solution to be chromatographed (AChE: 120-800 units; protein: 6-36 mg, usually) was applied to the column after concentration with no loss of enzyme activity. The concentration was carried out in a collodion bag (Round-bottom suction apparatus from Schleicher and Schuell Inc., Keene, N.H.), without previous dialysis against buffer, unless otherwise specified. The concentrated material was applied to the column, alone or together with an enzymatic protein used as internal marker. The fractions were separated with a fraction collector model 1200 Pup from Instruments Specialties Co. (ISCO), Lincoln, Neb. The percentage of enzyme recovery was 72.0 ± 3.3 (S.E.M.) for S2, and 90.3 ± 1.3 (S.E.M.) for S1.

1. Sephadex G-150 and Sephadex G-200: AChE preparations (supernatant fractions 1 or 2) were applied to a Sephadex column (1.5 x 90 cm), previously equilibrated with 30 mM phosphate buffer at pH 8.0, or with 30 mM Tris buffer at pH 8.0. Proteins were eluted with the same buffer, at a flow rate of 8 to 9 ml/h. Fractions of usually 2.3 ml were collected and assayed for AChE activity and protein content.

   The columns (Sephadex G-150 or Sephadex G-200) were standardized with the following proteins of known molecular weight: apo-ferritin, 480,000; γ-globulin, 205,000; catalase, 195,000; alkaline phosphatase, 78,000; bovine serum albumin, 71,000.

Blue dextran 2000 of molecular weight 2 x 10^6 daltons was used to determine the void volume (Vo); it is totally excluded from all Sephadex G-type columns. The exclusion limits of molecular weight for peptides and globular proteins are 300,000 on Sephadex G-150, and 600,000 on Sephadex G-200 column.

2. Sepharose 6B: The chromatographic procedure here was essentially the same as the one above. However, the flow rate was 6 to 7 ml/h. The column was standardized with the same protein markers used above (with exclusion of BSA), and thyroglobulin with a molecular weight of 670,000 was included.

   Due to the presence of very high molecular weight components, Blue dextran 2000 was also used here for void volume determination. The exclusion limit of molecular weight for both peptides and globular proteins is 4,000,000 on Sepharose 6B column.

The calibration method was that due to Andrews (3), based on the observed linear relationship between the logarithm of the molecular weight of a protein and the ratio of its elution volume (V) to the void volume (Vo), on gel filtration.

Preparation of Stable (Non-Aggregating) Forms of AChE

1. DEAE Sephadex A-25 treatment: To prepare non-aggregating forms of AChE, the desired supernatant fraction, at day 0 (day of enzyme preparation), was mixed with 0.25 volume of DEAE Sephadex A-25, suspended in the same supernatant fraction medium. The mixture was stirred, at room temperature for about 15 minutes, and then centrifugated at 12,800 g, for 15 minutes, in a Lourdes model A-2 Betafuge.

After removal of the supernatant, the enzyme was eluted from the gel, by adding 0.5 M KCl, containing 0.05 M Tris, pH 8.0, to obtain a final concentration of 0.25 M and 0.025 M, respectively. The mixture was again left stirring, at room temperature for 15 minutes, and then centrifugated at 28,700 g for 15 minutes. The resulting supernatant was assayed for enzyme and protein contents and kept at 4°C as the source of Seph E.

2. KCl or NaCl treatments: Non-aggregating enzyme was also prepared by the addition of KCl or NaCl to the supernatant fraction, at the desired final concentration (up to 1 M). When pH effects were studied the pH of the supernatant fraction was adjusted, at day 0, with 1 N HCl or 1 N NaOH, before the addition of the salt.
3. Neuraminidase experiments: 0.02 unit of neuraminidase per mg of protein was added to the supernatant fraction 2, adjusted to pH 6.0, at day 0. The mixture was incubated overnight, at room temperature, and the pH was, afterwards, maintained as such or readjusted to pH 8.0. This material was kept at 4°C, for subsequent chromatographic studies. The reaction medium contained: enzyme (AChE: 1761 units; protein: 30 mg), 0.32 M sucrose-EDTA-Tris solution at pH 6.0, and 0.5 unit of neuraminidase. The control mixture had everything specified in the reaction medium, except neuraminidase.

Affinity Column Chromatography

The synthesis of meta-trimethylammonium aniline HCl was carried out as described by Chan *et al.* (25). The side arm of affinose-202 (10 ml) was extended to twice its length, and the m-trimethylammonium aniline was bonded to this side chain, as described by Cuatrecasas (36). A

volume of 5 to 10 ml of affinity gel was packed in a (1.0 x 15 cm) column and washed overnight with 30 mM phosphate buffer, pH 8.0, before application of the brain AChE preparation. The bound material was then washed with 50 bed volumes of 100 mM NaCl, pH 8.0. The elution of AChE was accomplished by 10 bed volumes of 10 mM edrophonium chloride in 100 mM NaCl, pH 8.0. The eluted fractions (each equal to a bed volume) were dialysed against 30 mM phosphate buffer, pH 8.0, for two days at 4°C, to reduce edrophonium chloride to less than 10^{-9} M. AChE activity and protein content of each fraction were determined subsequently. Active fractions (1-3) were pooled and kept as a source of affinity enzyme (Aff E). The enzyme recovery averaged 78%.

Chromatography on DEAE Sephadex A-25 Column

A DEAE Sephadex A-25 column (1.5 x 30 cm) was equilibrated at 4°C with 30 mM Tris buffer, pH 8.0. At day 0, the fresh supernatant fraction 2, 1^{st} method, (AChE: 875-2600 units; protein: 15-45 mg) after concentration in a collodion bag was applied to the column and washed with 10 ml of 30 mM Tris buffer at pH 8.0. Elution was carried out with a linear gradient of KCl (0.0-2.0 M or 0.25-1.0 M) in a total volume of 260 ml, at an average rate of 16 ml/h. Fractions of 2.8 ml were collected and analyzed for AChE activity and protein content. Fractions containing enzyme activity were pooled, concentrated in a collodion bag under reduced pressure, and used for further chromatography on Sephadex G-200, and for other experiments. The enzyme recovery was approximately 70%.

Ammonium Sulphate Precipitation

This experiment was carried out according to Chan et al. (25). The first precipitation was done by slowly adding 12.5% $(NH_4)_2SO_4$ to S2, 1st method, (AChE: 1525 units; protein: 15 mg). The mixture was stirred for 30 minutes at room temperature and centrifugated, at 14,000 g for 40 minutes, in a Lourdes Beta-Fuge model A-2. The supernatant was removed for further precipitation by adding 26.5% $(NH_4)_2SO_4$, and the above procedure was repeated. The second protein precipitate was redissolved in a small volume (2.5 ml) of 30 mM phosphate buffer, pH 8.0, and kept at 4°C for further chromatographic studies. This enzyme preparation had a specific activity of 100 μmoles of ATC hydrolysed per milligram of protein per hour.

Reconstitution Experiments

This set of experiments was done with the enzyme isolated from DEAE Sephadex A-25, Seph E, and with a purified enzyme preparation obtained after affinity chromatography, Aff E, in an attempt to learn more about AChE form conversion. For more details the reader is referred to sections on the preparation of stable (non-aggregating) forms of AChE, and on affinity column chromatography, presented earlier.

1. With Seph E: At day 0, supernatant fraction 2 was treated with DEAE Sephadex A-25. The resulting supernatant, which represented non-adsorbable material, was designated Seph Sup 1. After enzyme release, the gel was submitted to a second extraction with 1 M KCl, containing 0.1 M Tris at pH 8.0, which was anticipated to liberate from the gel most of the components not eluted with the enzyme. This was designated Seph Sup 2. Seph E, Seph Sup 1 and Seph Sup 2 were then

assayed for enzyme activity and protein content. Two volumes of Seph Sup 1 or Seph Sup 2 were added to one volume of Seph E (2:1 ratio). For control experiments the KCl elutions were performed on the gel without previous adsorbed enzyme, under the same experimental conditions. The mixtures (Seph E + Seph Sup 1 or Seph E + Seph Sup 2) were kept at 4°C for further Sephadex G-200 chromatography.

2. With affinity enzyme (Aff E): At day 0, supernatant fraction 2 (AChE: 2500-4500 units; protein: 45-75 mg) was dialysed for three hours against 30 mM phosphate buffer, pH 8.0. The material was, then, passed through and retained in the affinity column. It was eluted from the column with edrophonium chloride (10^{-2} M) and dialysed against 30 mM phosphate buffer, pH 8.0, until the concentration of edrophonium chloride was less than 10^{-9} M. This preparation was used as the source of affinity enzyme. Whatever was not retained in the column was called Aff Sup. This was assumed to represent most of the components of the supernatant fraction, except AChE and other materials that could interact with the affinity ligand. One volume of the Aff E or of Seph E was, then, added to two volumes of the Aff Sup (1:2 ratio). The mixtures were kept at 4°C and, after concentration in a collodion bag, used for chromatographic studies of AChE forms.

Concanavalin A (Con A) Experiments

The experiments were done with AChE forms, separated by gel filtration techniques, as a means of detecting carbohydrate moieties in the enzyme preparation. The starting material used was supernatant fraction 2, 1^{st} method. The reaction medium contained in final concentrations: enzyme (corresponding on average to a $\Delta A_{412\ nm}$/min = 0.5/ml), Con A

(0.08-2.0 μM), 1 mM $MnCl_2$, 1 mM $CaCl_2$, 50 mM sodium acetate buffer, pH 5.5, in a volume of 1 ml. Both Mn^{2+} and Ca^{2+} are necessary for saccharide binding (6). The mixture was incubated for 15 minutes at 4°C, centrifuged at 28,700 g for 30 minutes in a Lourdes Beta-Fuge model A-2. AChE activity was determined in the supernatants. The controls contained everything specified in the reaction medium, except Con A.

Reversibility of Con A Effects: The reaction medium as described above was left incubating at 4°C for 15 minutes, and 10 mM of α-methyl-D-mannoside, a sugar which serves as a haptenic determinant of Con A (134), was added to it. The mixture was further incubated at 4°C for an additional 15 minutes and centrifuged at 28,700 g for 30 minutes.

The effect of α-methyl-D-mannoside per se on enzyme activity was also determined. In this case, the reaction medium contained in final concentrations: enzyme (corresponding to a $\Delta A_{412\ nm}$/min = 0.5/ml), 1 mM $MnCl_2$, 1 mM $CaCl_2$, 10 mM α-methyl-D-mannoside, 50 mM sodium acetate buffer, at pH 5.5, in a volume of 1 ml. Controls contained everything specified above, except α-methyl-D-mannoside.

In all Con A experiments the resulting supernatants were assayed for AChE activity.

Reversion of AChE Form Aggregation

Supernatant fractions when run on Sephadex G-200 column gave two (fresh preparation, at day 0) or three (old preparation, after 24 h) active peaks of AChE. The peaks correspond to a small (S), an intermediate (I), and a large (L) molecular weight form of AChE. The method below describes the experiments done with semi-purified enzymatic forms, separated by gel filtration, in an attempt to reverse AChE aggregation.

1. With AChE forms from gel filtration on Sepharose 6B: At day 0, 35 ml of S2, 1st method, (AChE: 3000 units; protein: 52 mg) were concentrated and applied to a Sepharose 6B column. On day 1, the fractions were assayed for enzyme activity and pooled as follows:

   Fraction A (tubes No. 20 to 30 = 24.5 ml): contained mainly the void volume (Vo) peak, which was a very large molecular weight form, over 4,000,000, and constituted about 9% of the total recovered enzyme activity;

   Fraction B (tubes No. 31 to 41 = 24.0 ml): contained mainly the large molecular weight form (L), which represented 12% of the total enzyme activity;

   Fraction C (tubes No. 42 to 50 = 19.5 ml): contained mainly the small molecular weight form (S) and the intermediate molecular weight form (I), which represented 58% and 13% of the total enzyme activity, respectively;

   Fraction D (tubes No. 51 to 61 = 24.2 ml): contained 8% of the total enzyme activity;

   Fraction E (tubes No. 62 to 70 = 20.5 ml): contained mainly proteins which come out from the column after S.

   Fraction C was added to fraction A, B, D or E in a ratio of 1 volume of C to 2 volumes of the desired fraction. The mixtures were kept at 4°C and used after concentration for chromatographic studies.

2. With AChE forms from Sephadex G-200: On day 0, 30 ml of S2, 1st method, (AChE: 2600 units; protein: 45 mg) were concentrated to 3 ml, and applied to the column. The amount of enzyme activity recovered from the column was 70%. On the next day, after enzyme activity determinations, the fractions were pooled as follows:

Fraction A (tubes No. 19 to 29 = 23.5 ml): was a combination of L and I forms (representing 42% of the total recovered enzyme activity), plus the void volume peak, not separable here;

Fraction B (tubes No. 30 to 38 = 18.5 ml): was mainly S form (about 55% of the total enzyme activity) with I form as contaminant;

Fraction C (tubes No. 39 to 60 = 46.0 ml): contained, besides small molecular weight compounds, 3% of the total enzyme activity.

As the volume of each fraction varied, the mixtures were carried out in such a way that the protein ratio in each mixture ranged from 1:2 to 1:1. One volume of B was then added to 1.5 volumes of A or to 3 volumes of C. The mixture of B + (A+C) was done by adding 1 volume of B to 4 volumes of A plus C (previously mixtured in a 1:2 ratio). The mixtures were kept at 4°C and used, after concentration, for chromatographic studies.

Calculations and Statistics

The relative amounts of enzyme activity of each AChE form were calculated from the ratio between the sum of changes in absorbance, at 412 nm per minute, under the peak and of those constituting the total amount of enzyme recovered from the column:

$$\% \text{ of AChE Activity} = \frac{\Sigma \text{ Peak } \Delta A_{412\ nm}/min}{\Sigma \text{ Total } \Delta A_{412\ nm}/min}$$

Student's t-test: The small sample method was used for the test of hypothesis concerning the difference between the means of two populations:

$$t = \frac{\bar{x}_1 - \bar{x}_2 - (\mu_1 - \mu_2)}{\sqrt{(n_1-1)s_1^2 + (n_2-1)s_2^2}} \cdot \sqrt{\frac{n_1 n_2 (n_1+n_2-2)}{n_1 + n_2}}$$

$$d.f. = n_1 + n_2 - 2$$

Analysis of Variance: Two-way layout analysis of variance and the Scheffé-test for contrasts among groups were applied. The differences in AChE specific activity and percentage of recovery were, then, tested for the supernatant fractions and fresh or frozen tissues. Experimental data obtained with the 1st: Chan et al. (24), and the 2nd: Hollunger and Niklasson (75) methods, modified, were used.

RESULTS

Solubilization of Brain AChE

The yield and specific activities of AChE solubilized from bovine caudate nucleus tissue with the modified procedure of Chan et al. (24), 1st method, using fresh and frozen tissues are summarized in Table 3. Similar data for the modified procedure of Hollunger and Niklasson (75), 2nd method, using fresh tissue are shown in Table 4.

Supernatant fractions 1 (S1, soluble enzyme) and 2 (S2, membrane-bound enzyme) were the sources of enzyme used in both methods. With fresh tissue, the percentage of enzyme recovery and the specific activity in S1 were similar in both methods (Tables 3 and 4). On the other hand, under the same experimental conditions the percentage of AChE solubilized and the specific activity were significantly greater for S2, 2nd method, as compared to S2, 1st method.

Tables 5 and 7 show the results of the two-way layout analysis of variance for the AChE specific activity and the percentage of recovery performed with the different supernatant fractions and fresh and frozen tissues. Tables 6 and 8 present the Scheffé tests to detect contrasts among the four supernatant fractions using the data of the analyses of variance, above.

When the same method of solubilization was applied, there was a statistically significant difference between the AChE specific activity in S1 and S2, in both methods, at a 95% confidence level (Table 6). However, the percentage of enzyme recovery was significantly greater in S2 than in S1 only when the 2nd method was used (Table 8). No statistical difference

TABLE 3. SOLUBILIZATION OF BRAIN AChE IN FRESH AND FROZEN TISSUES
1st Method: Chan et al. (24), modified

Fraction	AChE Activity		Protein	
	Specific (μmoles/mg protein/h)	% Recovery	mg	%
Homogenate				
Fresh (9)	27.5 ± 1.7	100.0	1607.0 ± 101.9	100.0
Frozen (6)	25.8 ± 1.8	100.0	1608.0 ± 72.9	100.0
Mitochondrial-microsomal				
Fresh (8)	27.8 ± 1.7	83.1 ± 6.2	1292.0 ± 146.1	81.2 ± 3.8
Frozen (5)	27.6 ± 3.3	90.7 ± 7.8	1352.0 ± 137.6	82.9 ± 6.6
Supernatant 1				
Fresh (9)	20.5 ± 2.9	9.7 ± 1.9	186.0 ± 12.7	12.7 ± 1.1
Frozen (6)	23.5 ± 3.5	14.1 ± 2.6	195.0 ± 4.1	15.3 ± 3.6
Supernatant 2				
Fresh (8)	62.3 ± 5.3	14.8 ± 2.1	135.0 ± 3.5	6.5 ± 0.8
Frozen (6)	55.1 ± 3.1	17.6 ± 2.9	168.0 ± 10.6	7.8 ± 0.7

AChE activity was determined according to the procedure of Ellman et al. (49). Protein was estimated by the method of Lowry et al. (100). Number of experiments, for fresh and frozen tissues, are indicated in parentheses. Values are means ± S.E.M.

TABLE 4. SOLUBILIZATION OF BRAIN AChE IN FRESH TISSUE
2nd Method: Hollunger and Niklasson (75), modified

Fraction	AChE Activity		Protein	
	Specific (μmoles/mg protein/h)	% Recovery	mg	%
Homogenate (4)	24.7 ± 3.4	100.0	2035.0 ± 292.9	100.0
Nuclear-mitochondrial-microsomal (3)	22.2 ± 3.2	91.4 ± 3.4	2017.0 ± 276.9	90.1 ± 2.1
Supernatant 1 (4)	18.5 ± 3.6	10.1 ± 1.7	303.0 ± 26.1	13.4 ± 1.9
Supernatant 2 (3)	105.7 ± 14.6	24.8 ± 4.9	168.0 ± 8.2	7.0 ± 1.2

AChE activity was determined according to the procedure of Ellman et al. (49). Protein was determined by the method of Lowry et al. (100). Number of experiments is indicated in parentheses. Values are means ± S.E.M.

TABLE 5. ANALYSIS OF VARIANCE FOR AChE SPECIFIC ACTIVITY WITH DIFFERENT SUPERNATANT FRACTIONS AND FRESH AND FROZEN TISSUES

Source	Sum of squares	d.f.	Mean squares	F	$F_{.95}$
Between supernatants	24,720.054	3	8240.018	60.362	2.917
Between fresh and frozen tissues	238.190	1	238.190	1.745	4.166
Error	4,231.789	31	136.509	--	--
TOTAL	29,190.033	35	--	--	--

Data were from experiments using supernatant fractions 1 and 2 from both methods [1st: Chan et al. (24), and 2nd: Hollunger and Niklasson (75); modified]. Values from frozen tissue were obtained with the 1st method, only.

TABLE 6. SCHEFFÉ'S TEST* (α = 0.05) FOR AChE SPECIFIC ACTIVITY CONTRASTS AMONG SUPERNATANT FRACTIONS

Contrast	Confidence limits	Result
S1, 1^{st} - S2, 1^{st}	37.6 ± 12.8	Significant
S1, 2^{nd} - S2, 2^{nd}	87.2 ± 26.4	Significant
S1, 1^{st} - S1, 2^{nd}	3.2 ± 19.4	Not significant
S2, 1^{st} - S2, 2^{nd}	46.5 ± 22.0	Significant

*Test performed with the data of Table 5. 1^{st} = 1^{st} method, Chan et al. (24), modified; 2^{nd} = 2^{nd} method, Hollunger and Niklassen (75), modified.

TABLE 7. ANALYSIS OF VARIANCE FOR PERCENTAGE OF AChE RECOVERY WITH DIFFERENT SUPERNATANT FRACTIONS AND FRESH AND FROZEN TISSUES

Source	Sum of squares	d.f.	Mean squares	F	$F_{.95}$
Between supernatants	852.390	3	284.130	7.332	2.917
Between fresh and frozen tissues	32.760	1	32.760	0.845	4.166
Error	1201.292	31	38.751	--	--
TOTAL	2086.442	35	--	--	--

Data were from experiments using supernatant fractions 1 and 2 from both methods [1st: Chan et al. (24) and 2nd: Hollunger and Niklasson (75); modified]. Values from frozen tissue were obtained with the 1st method, only.

TABLE 8. SCHEFFÉ'S TEST* (α = 0.05) FOR PERCENTAGE OF AChE RECOVERY CONTRASTS AMONG SUPERNATANT FRACTIONS

Contrast	Confidence limits	Result
S1, 1^{st} - S2, 1^{st}	4.5 ± 6.8	Not significant
S1, 2^{nd} - S2, 2^{nd}	18.7 ± 14.1	Significant
S1, 1^{st} - S1, 2^{nd}	1.4 ± 10.4	Not significant
S2, 1^{st} - S2, 2^{nd}	12.8 ± 11.7	Significant

*Test performed with the data of Table 7. 1^{st} = 1^{st} method, Chan et al. (24), modified. 2^{nd} = 2^{nd} method, Hollunger and Niklasson (75), modified.

was detected between fresh and frozen tissues for the specific activity or for the percentage of enzyme recovery (Tables 5 and 7).

By extracting the enzyme (S2, 1st method) for a longer period, at 4°C, an increase in the percentage of solubilization from 14% (1 hour) to 31% (overnight) was observed, while the AChE specific activity increased from 67 to 121 μmoles of acetylthiocholine (ATC) hydrolysed per milligram of protein per hour. After an overnight extraction the amount of solubilized enzyme from the resuspended mitochondrial-microsomal fraction decreased progressively, as well as the specific activity. The one-hour extraction procedure was adopted due to the need for the chromatography to be done with very fresh enzyme preparations.

AChE in Supernatant Fraction 2 (S2)

1. Conversion of multiple AChE forms with time: Studies of the multiple forms of brain AChE in S2 were carried out on a time basis using Sephadex G-200 chromatography (Figures 3 and 4). Table 9 shows the effect of storage on the percentage of change of AChE forms, with enzyme prepared by both methods. At day 0, with enzyme from fresh tissue, there was a predominance of the S form, usually only a trace of I, and a small L form. With time, S gradually disappeared, I increased at the expense of S, and L underwent a small decrease (between 20 and 12%). The amount of the L form was slightly smaller with the 1st method of solubilization as compared to the 2nd one, within a period of one week. In addition, the percentages of increase of I and decrease of S were greater with the 1st method.

Although with time S almost disappeared completely when the 1st method was used for enzyme preparation, the enzyme obtained with the 2nd

TABLE 9. EFFECT OF STORAGE TIME ON THE PERCENTAGE OF CHANGE OF AChE FORMS FROM SUPERNATANT FRACTIONS 2

AChE Form	1st Method			2nd Method	
	Day 0	Day 2	Days 4-8	Day 0	Days 4-8
L	20.2 ± 2.9 (5)	11.8 ± 2.4 (3)	16.1 ± 4.4 (4)	22.6 ± 2.5 (4)	29.0 ± 5.0 (4)
I	12.8 ± 2.2 (5)	70.1 ± 2.1 (3)	70.8 ± 2.3 (5)	10.8 ± 0.5 (4)	48.0 ± 5.6 (4)
S	67.0 ± 4.0 (5)	18.1 ± 2.1 (3)	12.5 ± 2.9 (4)	66.7 ± 4.4 (4)	23.1 ± 4.3 (4)

The relative amounts of enzyme in each AChE form were calculated from the ratio:

$$\% \text{ of AChE activity} = \frac{\Sigma \text{ Peak } \Delta A_{412\ nm}/min}{\Sigma \text{ Total } \Delta A_{412\ nm}/min}$$

Number of experiments is indicated in parentheses. Values are means ± S.E.M. 1st method: Chan et al. (24), modified; 2nd method: Hollunger and Niklasson (75), modified.

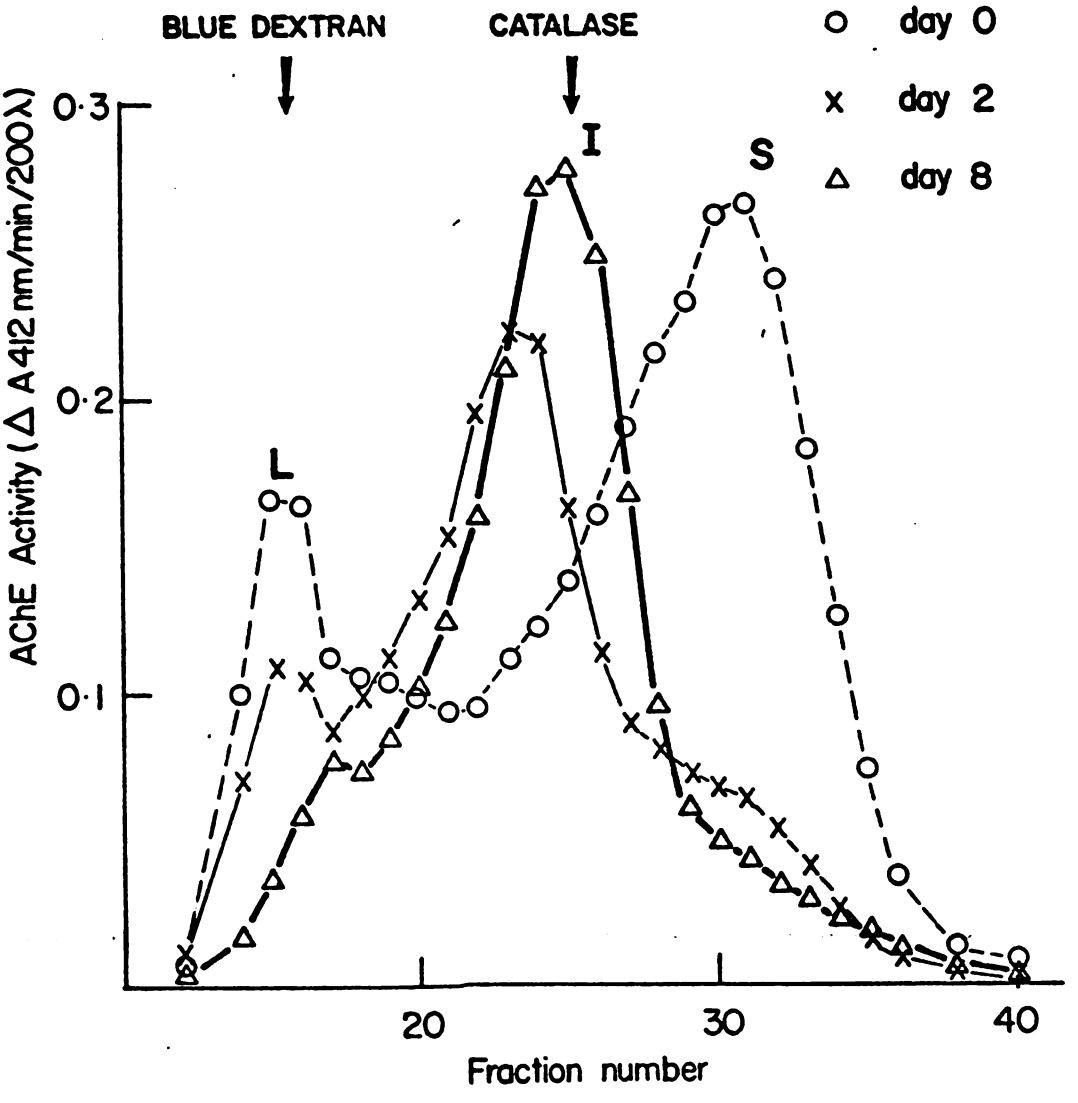

Figure 3. Changes with Time of AChE Forms (L, I, S) Present in S2, 1st Method, Shown by Sephadex G-200 Chromatography.

At time intervals indicated, a concentrated enzyme preparation (AChE: 432 units; protein: 6 mg) was applied to a 1.5 x 90 cm column, previously equilibrated with 30 mM phosphate buffer, pH 8.0. The arrows indicate the position of catalase and blue dextran, used as internal marker and for the void volume (Vo) determination, respectively. Fraction size = 2.3 ml.

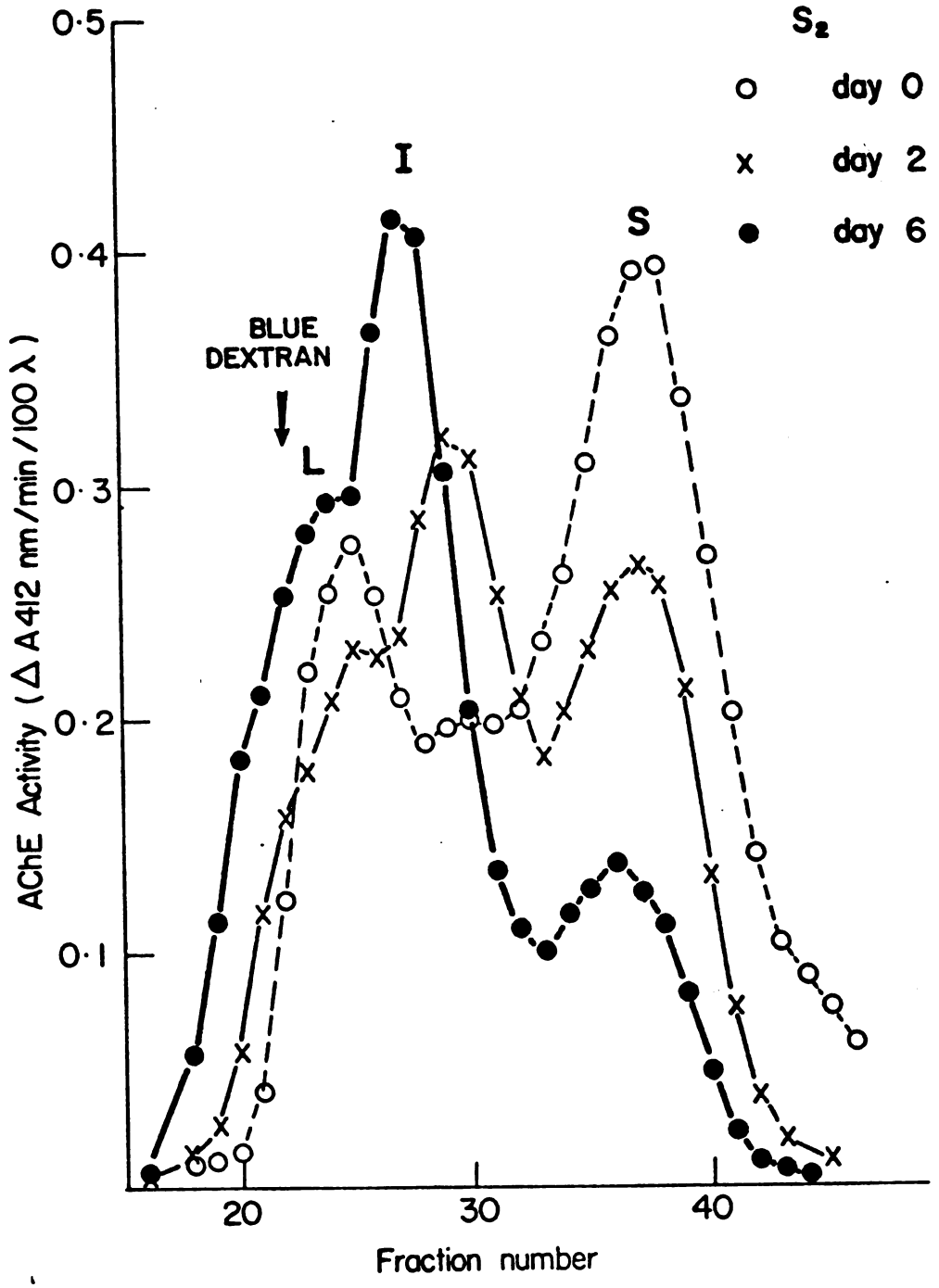

Figure 4. Changes with Time of AChE Forms Present in S2, 2nd Method, Shown by Sephadex G-200 Chromatography.

Aliquots: AChE = 710 units; protein = 7 mg.

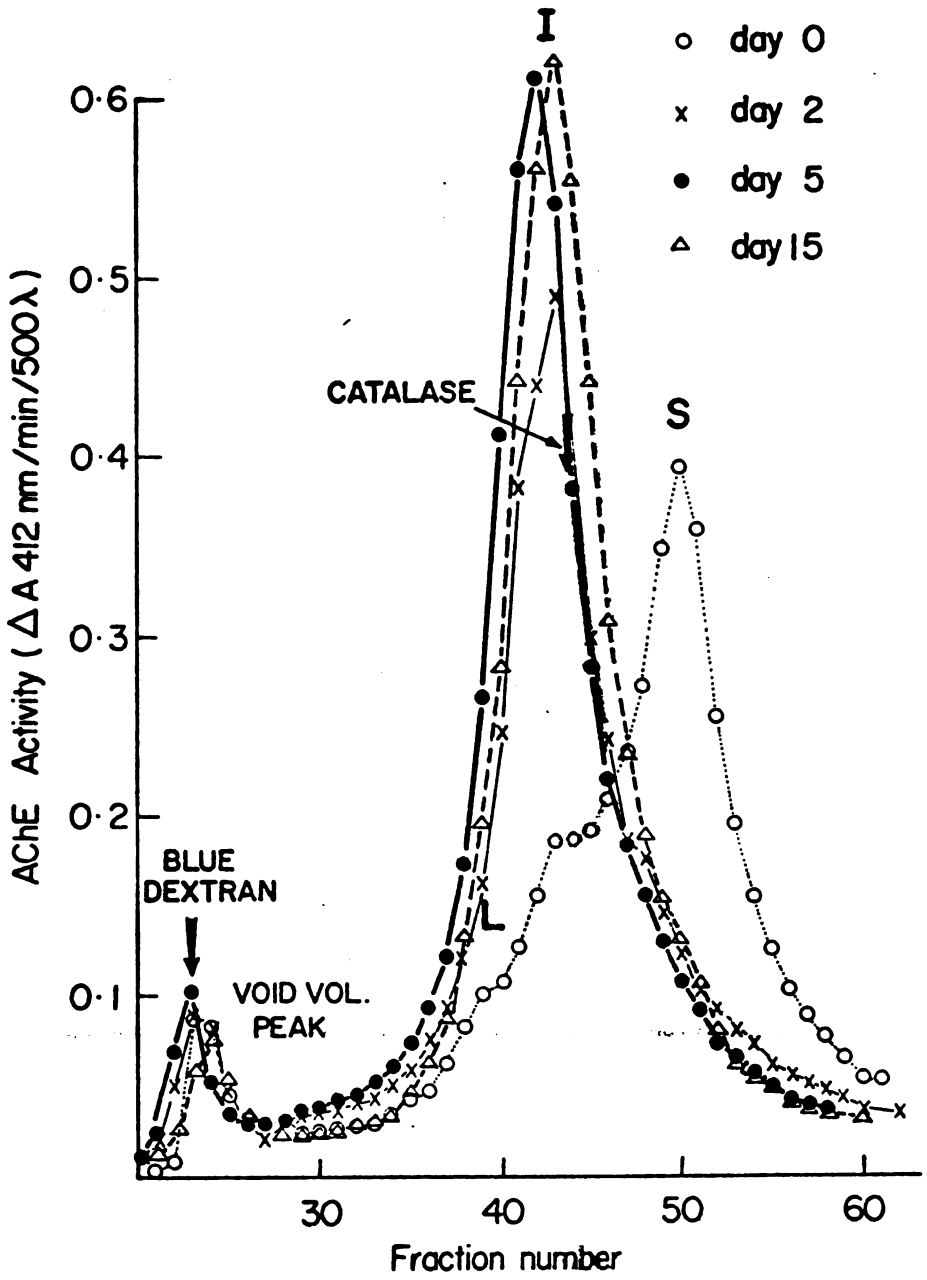

Figure 5. Changes with Time of AChE Forms (Void Vol. Peak, L,I,S) Present in S2, 1st Method, Shown by Sepharose 6B Chromatography.

Aliquots: AChE = 504 units; protein = 8 mg.

method appeared to be more stable, and S could still be detected after an 18-day period. The conversion (S → L) especially with the 1st method took place rapidly and, within 24 hours, one could not only detect the conversion of S to the I form, but also the predominance of the latter. After two days, there was a tendency toward a plateau and no more dramatic changes were observed.

When the source of enzyme was frozen tissue, the initial picture at day 0 was different, with a greater percentage of L form (35%) and a smaller S form (45%), as compared to fresh tissue. The changes with time were, however, similar. Figure 5 shows a time-course of S2 (1st method) on Sepharose 6B column, which is more useful for the separation of very high molecular weight components (its exclusion limit is 4 million daltons). At day 0, besides the three usual forms (L, I and S), a fourth enzyme component in the void volume was detected. The S form here was also predominant. With time, S was converted to the I form, which seemed to be the only component besides the void volume peak. The L form then was not detected, but might be present in the I form as a contaminant.

2. Effect of pH on form conversion: Figure 6 shows the time-course of S2 (1st method), at pH 6.5. Figures 7, 8 and 9 show the effects of storage time and different pH's on the percentage of change of AChE forms. The observed AChE form conversion was dependent on the pH of the medium: it was slowed down at lower pH, and accelerated at higher pH. At low pH (6.0), the enzyme was very stable, little build up of the I form was observed, and the percentage of enzyme activity at day 17 was not very different from that shown on day 1.

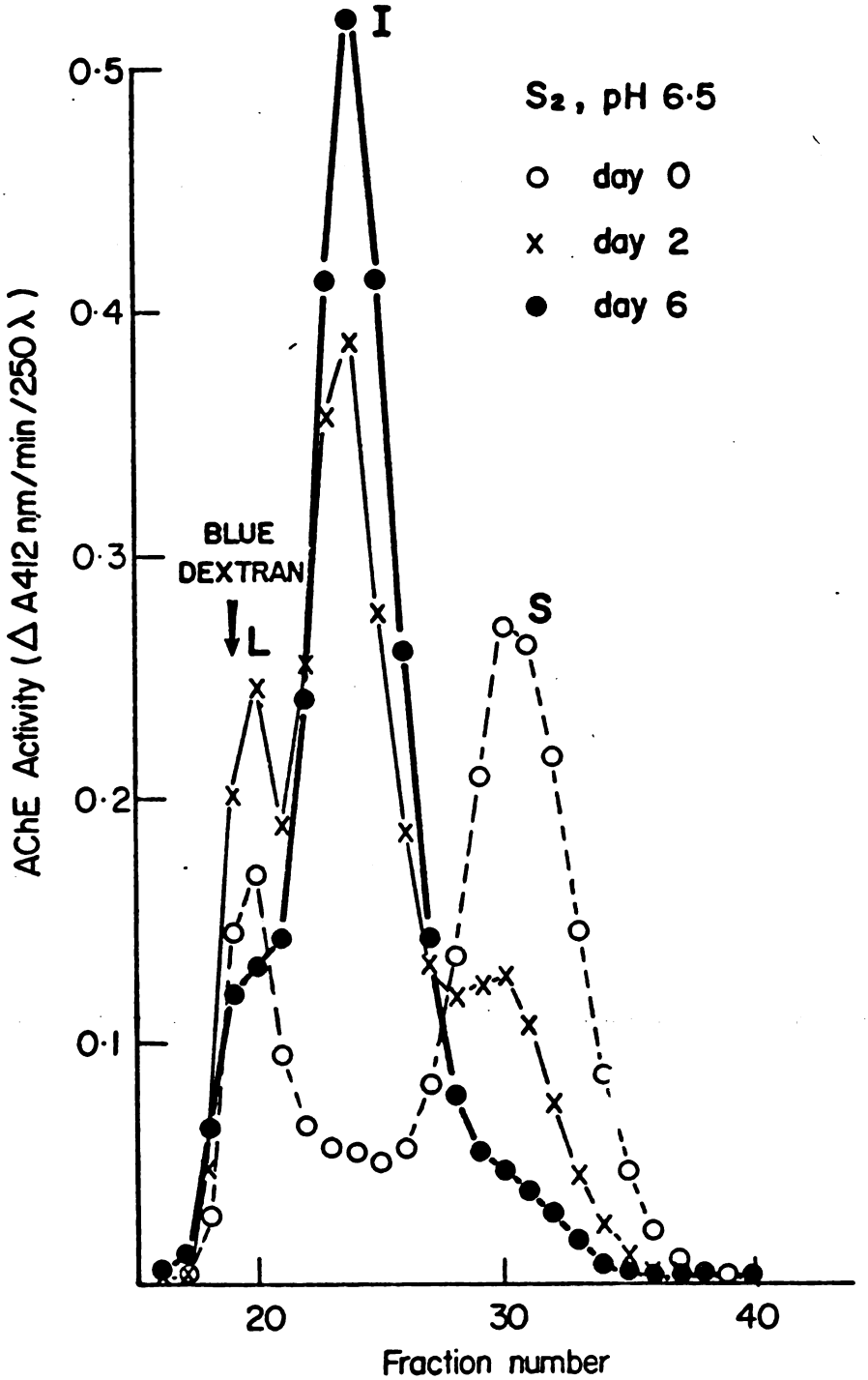

Figure 6. Changes with Time of AChE Forms Present in S2, 1st Method, at pH 6.5, Shown by Sephadex G-200 Chromatography.

Aliquots: AChE = 405 units; protein = 8 mg. The pH was adjusted at day 0.

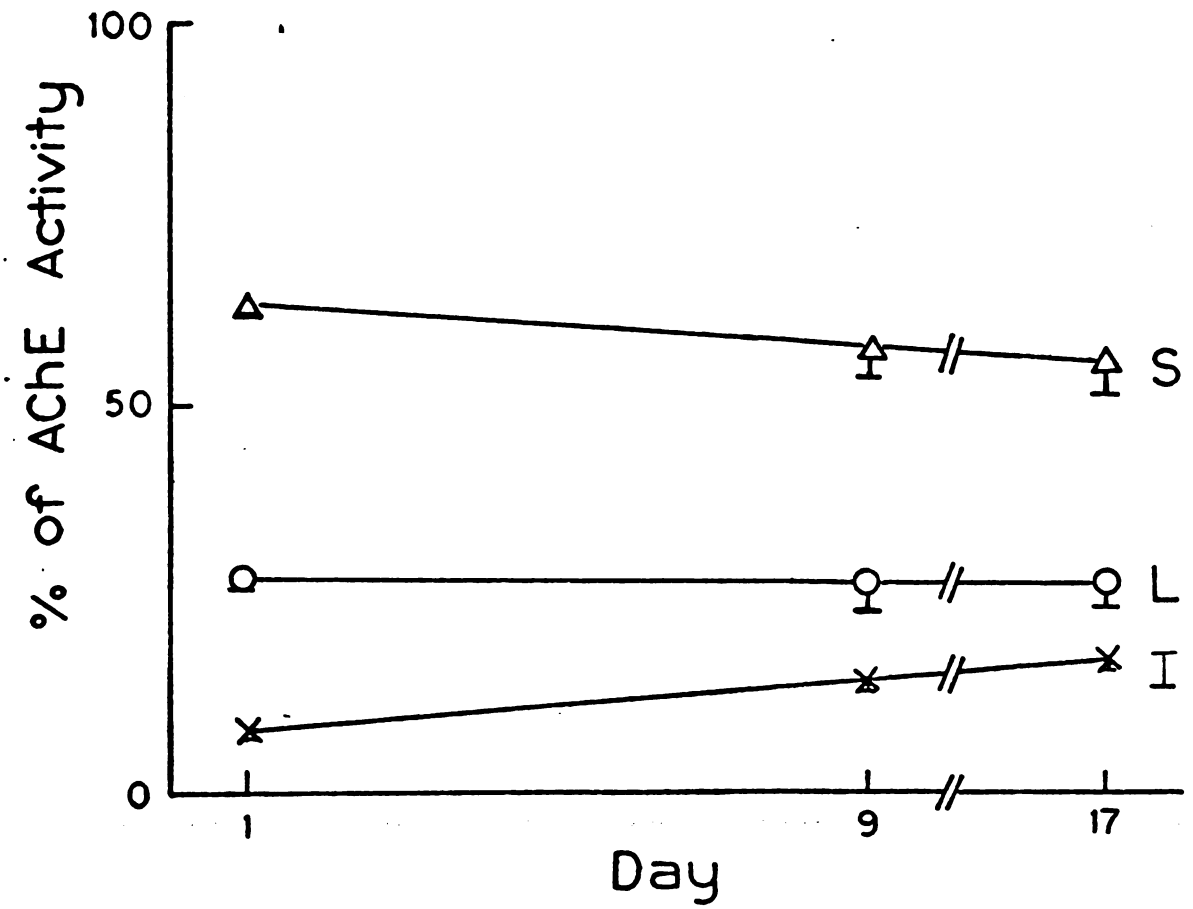

Figure 7. Effect of Storage Time on the Percentage of Change on AChE Forms Present in S2, 1st Method, at pH 6.0.

Points are means ± S.E.M. of 2 experiments.

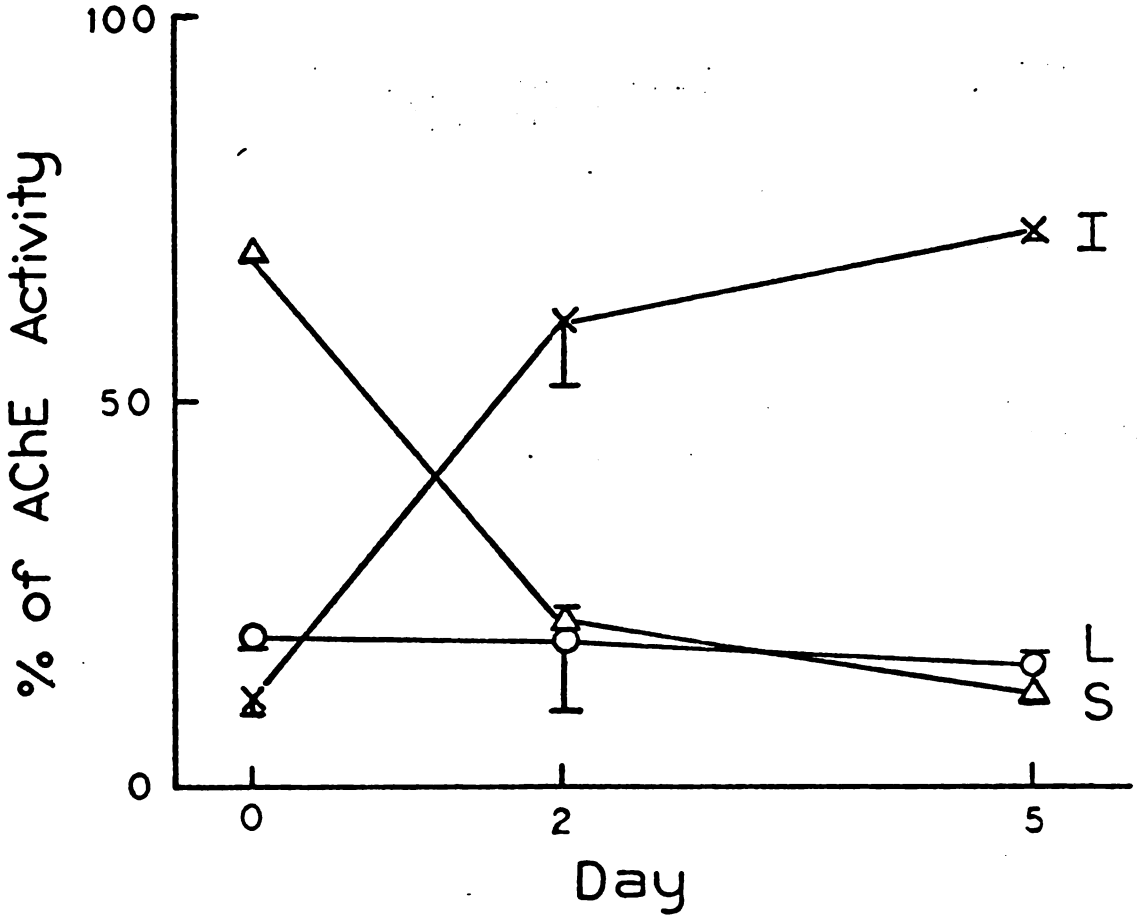

Figure 8. Effect of Storage Time on the Percentage of Change on AChE Forms Present in S2, 1st Method, at pH 7.0.

Points are means ± S.E.M. of 2-3 experiments.

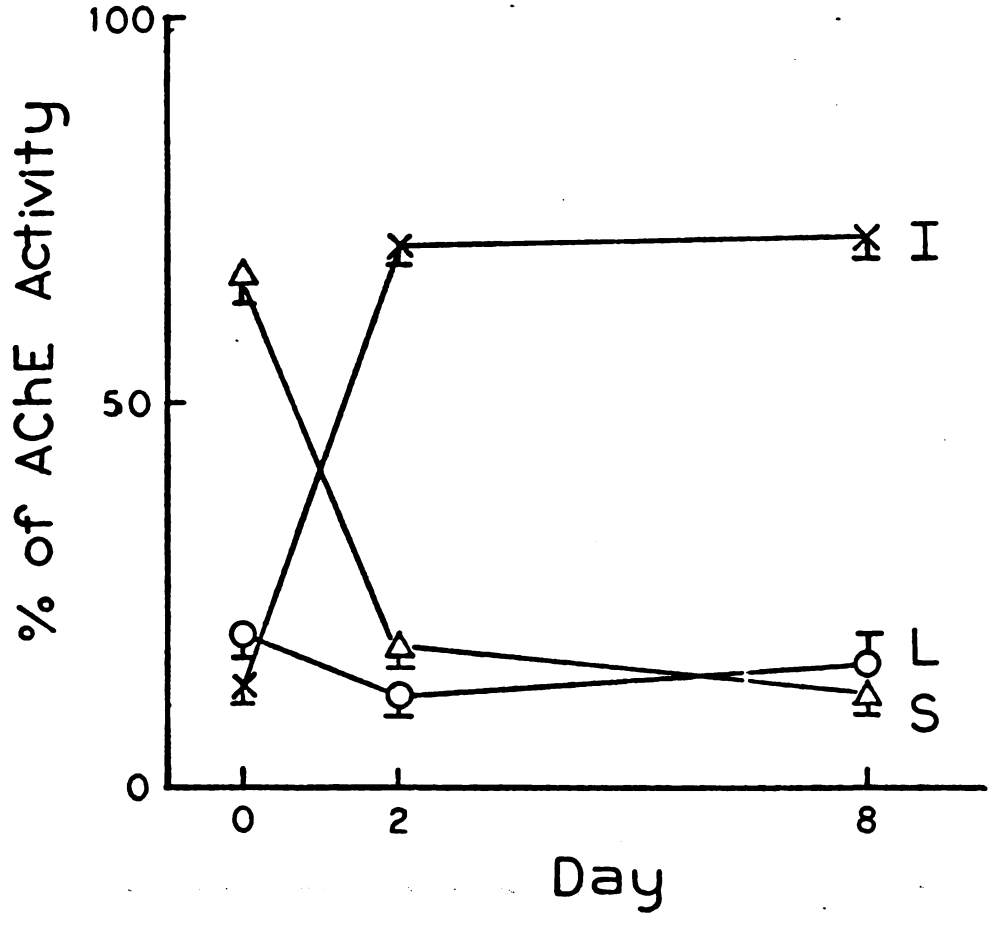

Figure 9. Effect of Storage Time on the Percentage of Change on AChE Forms Present in S2, 1st Method, at pH 8.0.

Points are means ± S.E.M. of 3-5 experiments.

3. Non-aggregating (stable) forms of AChE:

a. With DEAE Sephadex A-25: The addition of an anion exchanger, DEAE Sephadex A-25, to the supernatant fraction 2, at day 0, resulted in adsorption of the solubilized enzyme. The subsequent release of AChE by 0.25 M KCl gave non-aggregating forms of the enzyme. The enzyme was more stable, changes in the forms took place slowly, and the I form did not accumulate (Figure 10). The picture was similar with both methods of enzyme solubilization, but the 2^{nd} method gave a somewhat more stable enzyme (Figure 11).

The specific activity of AChE in S2 was always higher after DEAE Sephadex A-25 treatment (Seph E). This increase was well observed with S2, 2^{nd} method. The percentage of enzyme recovery ranged from 45 to 54 in Seph E from S2, prepared using the 2^{nd} method and the 1^{st} method, respectively (Table 10).

The Seph E preparation, obtained from frozen and fresh brain tissue, showed a predominance of the S (fresh tissue) or the L (frozen tissue) forms, according to the tissue age. Seph E also contained the void volume peak detected after Sepharose 6B chromatography. The treatment of an old aggregated-enzyme preparation with DEAE Sephadex A-25 did not reverse the aggregation process within a 24-hour period (not shown).

b. With KCl or NaCl treatments: Non-aggregating enzymatic forms were also obtained by adding 1 M KCl or NaCl to recently prepared supernatant fractions. As the concentration of salt increased there was a decrease in the I form and an increase in the S form present; while a decrease in salt concentration from 1.0 to 0.06 M KCl caused a progressive decrease in the S form followed by an increase in the L form (Figures 12 and 13). With this type of treatment the I form was less stable and there was a gradual increase of the L form. An increase in pH from 6.5 to 7.0

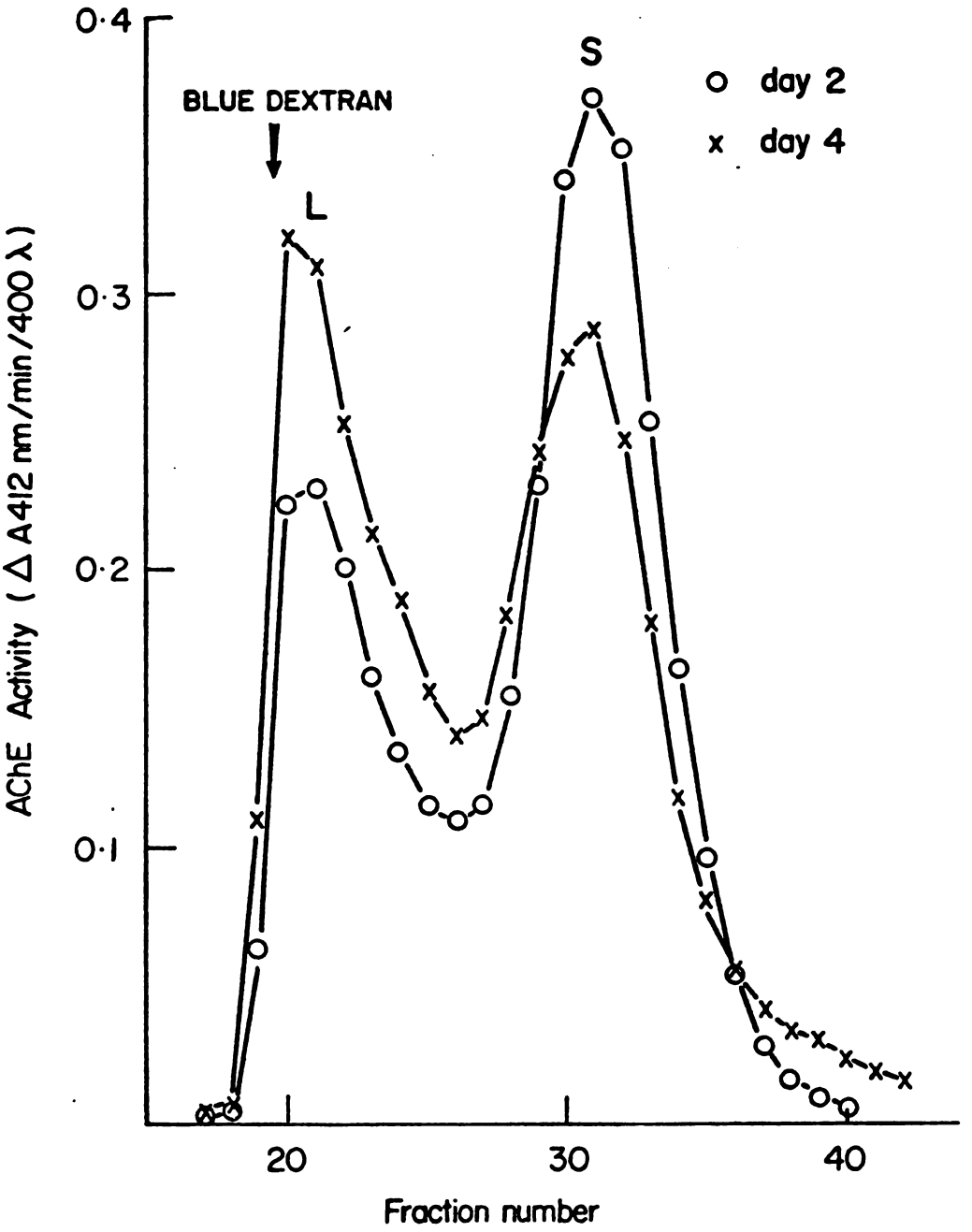

Figure 10. Effect of DEAE Sephadex A-25 Treatment on the Stabilization of AChE Forms Present in Seph E from S2, 1st Method, Shown by Sephadex G-200 Chromatography.

Aliquots: AChE = 376 units; protein = 6 mg.

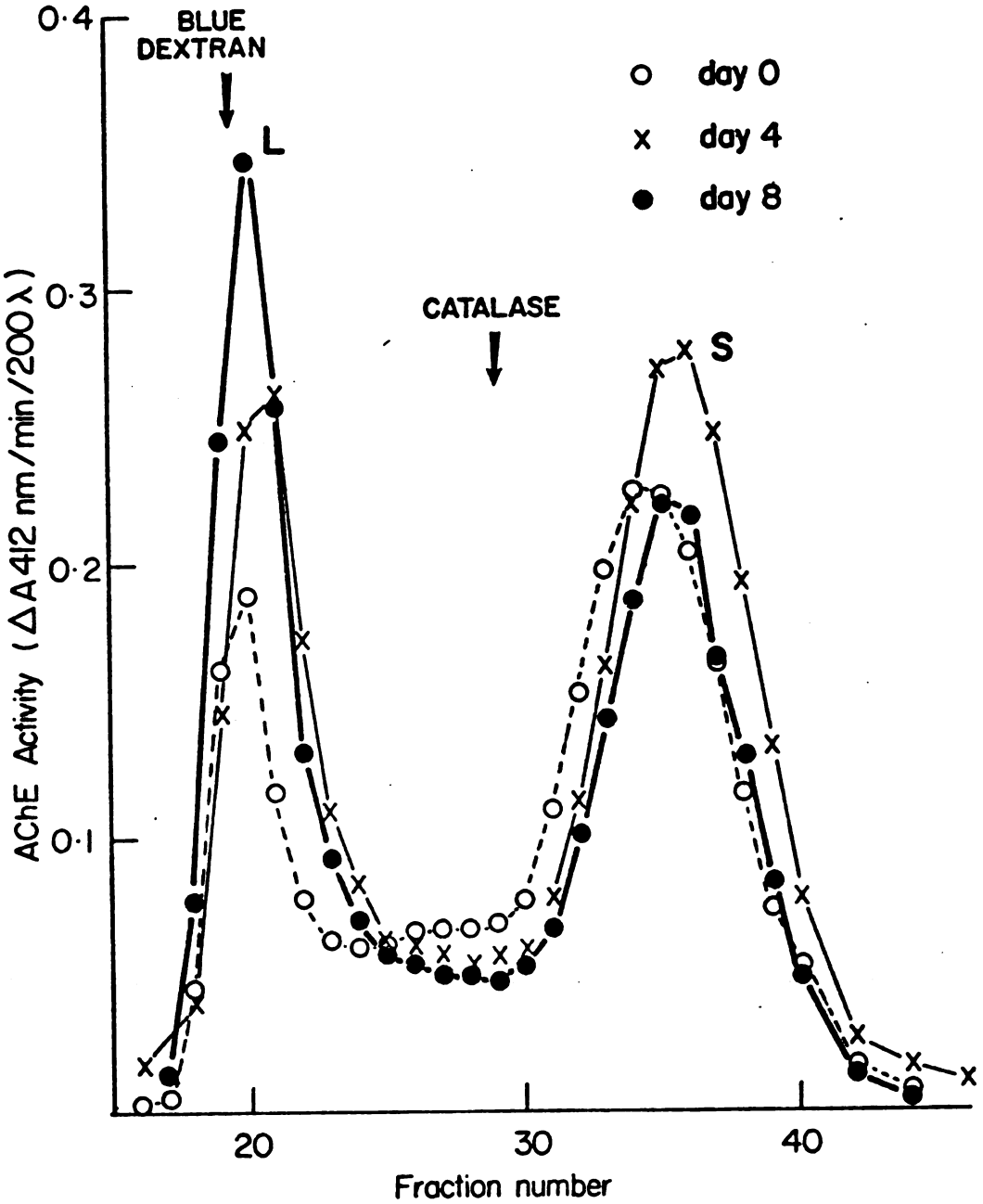

Figure 11. Effect of DEAE Sephadex A-25 Treatment on the Stabilization of AChE Forms Present in Seph E from S2, 2nd Method, Shown by Sephadex G-200 Chromatography.

Aliquots: AChE = 661 units; protein = 9 mg.

TABLE 10. AChE ACTIVITY AND PROTEIN CONTENT IN SEPH E FROM SUPERNATANT FRACTIONS 1 AND 2

Method	Fraction	AChE Activity		Protein	
		Specific (μmoles/mg protein/h)	% Recovery	mg	%
1st	S2 (8)	56.5 ± 3.9	100.0	141.5 ± 13.0	100.0
	Seph E (8)	62.6 ± 4.3	53.7 ± 5.9	195.0 ± 23.5	48.0 ± 4.5
2nd	S2 (3)	82.4 ± 16.7	100.0	173.0 ± 1.0	100.0
	Seph E (6)	95.2 ± 12.4	45.4 ± 4.3	110.0 ± 24.0	37.7 ± 5.5
1st	S1 (3)	13.4 ± 0.6	100.0	216.3 ± 5.5	100.0
	Seph E (3)	17.6 ± 1.6	41.3 ± 5.1	216.0 ± 20.3	31.3 ± 2.8

AChE activity was estimated according to the procedure of Ellman et al. (49). Protein was determined by the method of Lowry et al. (100). Number of experiments is indicated in parentheses. Values are means ± S.E.M. 1st method: Chan et al. (24), modified; 2nd method: Hollunger and Niklasson (75), modified.

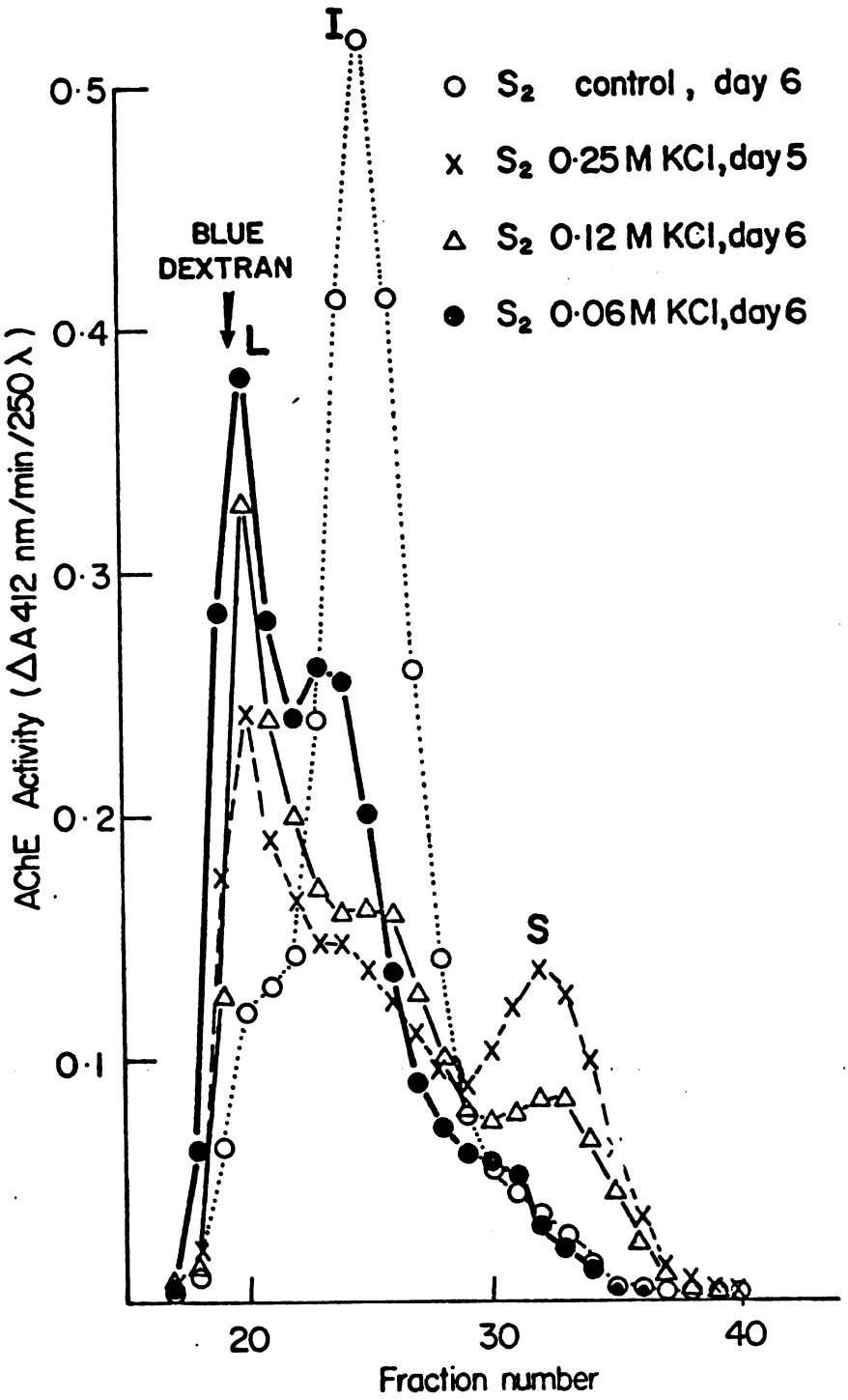

Figure 12. Sephadex G-200 Chromatography of AChE Forms in S2, 1st Method, at pH 6.5, in the Absence (Control) and Presence of Different Concentrations of KCl.

pH adjustment and addition of KCl were carried out at day 0. Aliquots: AChE = 405 units; protein = 8 mg.

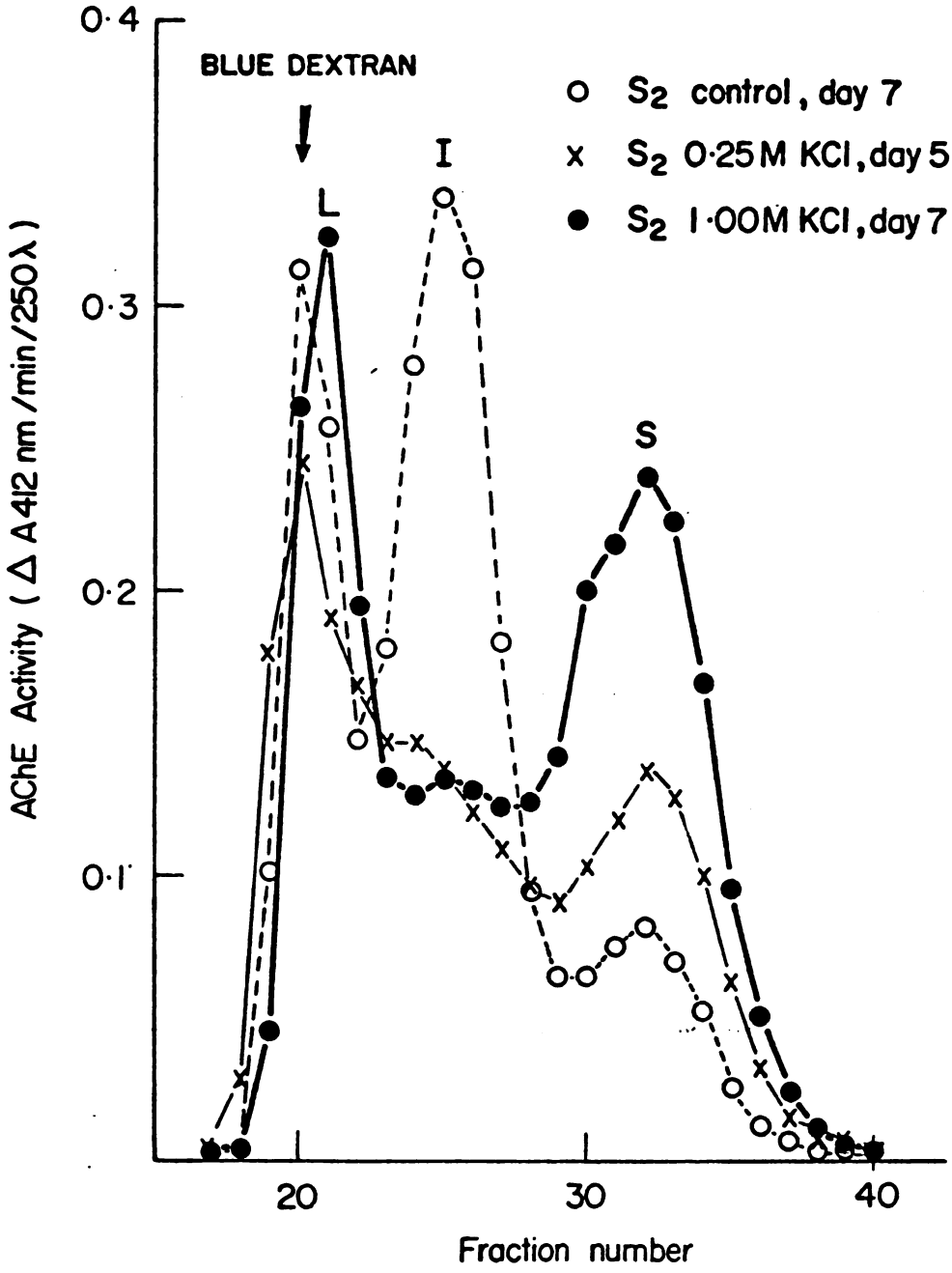

Figure 13. Sephadex G-200 Chromatography of AChE Forms in S2, 1st Method, at pH 6.5, in the Absence (Control) and Presence of 0.25 and 1.00 M KCl.

Aliquots: AChE = 405 units; protein = 8 mg.

or 8.0 tended to decrease the salt effect on the stabilization of the S form and, although there was an increase in L, a blockade of the I form was still detected (Figure 14).

With S2 prepared with the 2nd method, a more dramatic effect was observed. The enzyme was well protected against form conversion in the presence of 1 M KCl or NaCl, and the picture was similar to the one observed with a fresh preparation at day 0, even at day 6. Lower salt concentrations (0.25 M) offered a partial protection (Figure 15).

These data support the suggestion that the presence of KCl or NaCl prevents the formation of intermediate molecular forms (I) of AChE.

4. Effect of dialysis and lower ionic strength on DEAE Sephadex A-25 or KCl treatments: Some conclusion may be drawn from the results presented so far. The first one is that high ionic strength protected the S2 enzyme against aggregation (conversion from smaller to larger forms), and low ionic strength caused its aggregation. The effect was pH and concentration dependent. The second conclusion is that the enzyme treatment with DEAE Sephadex A-25 (Seph E) also slowed down AChE aggregation.

The enzyme was eluted from Sephadex with 0.25 M KCl and, so, a certain ionic strength was present throughout the elution process. Seph E, after dialysis for three hours against KCl, containing Tris, pH 8.0, at concentrations ranging from 0.12 to 1.0 M KCl, remained virtually unchanged in terms of AChE forms present. Aggregation was also absent after dialysis against buffer of lower ionic strength (such as 30 mM phosphate buffer at pH 8.0), for the Seph E as well as for the KCl treated enzyme at pH 6.5 (Figure 16).

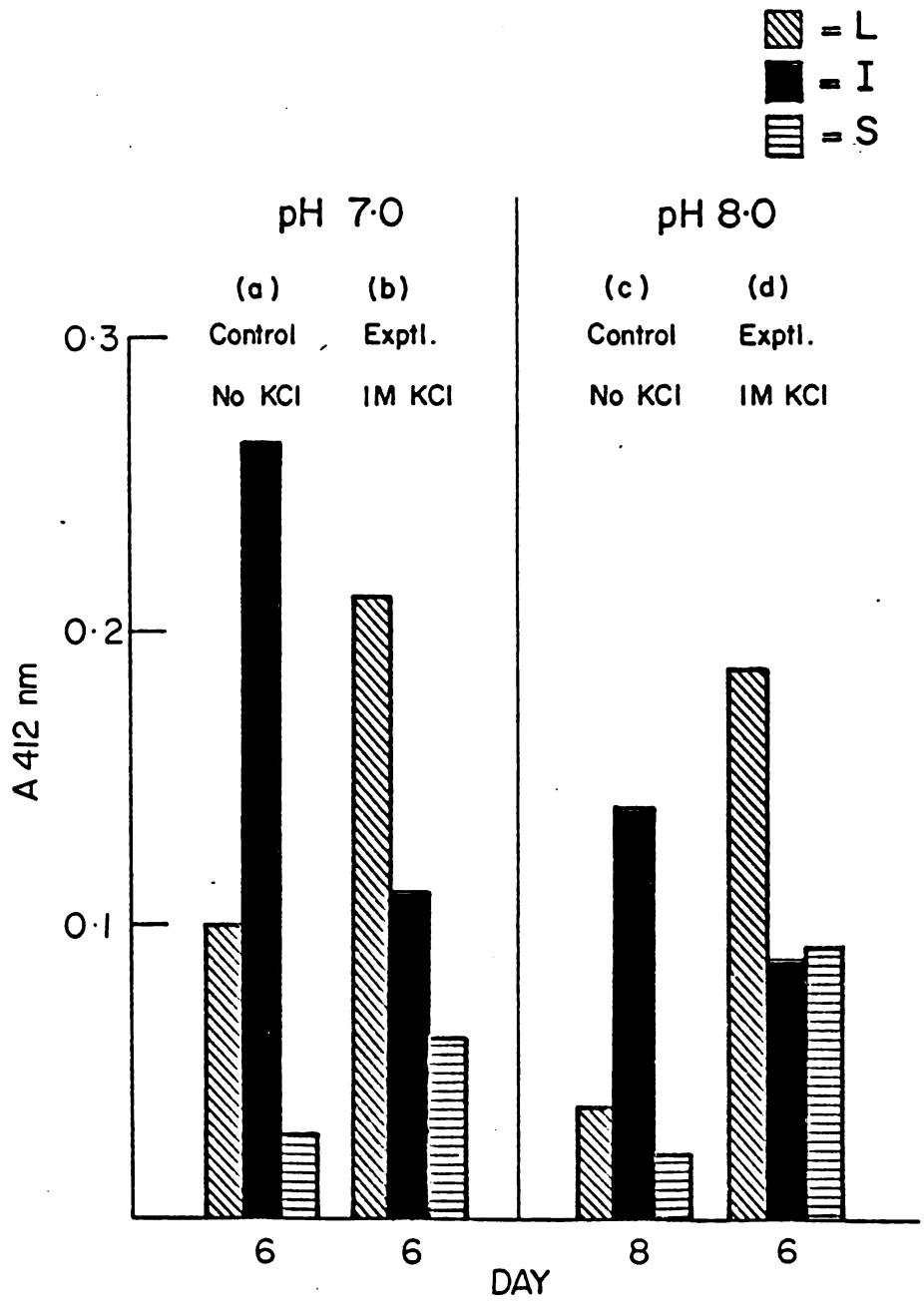

Figure 14. Sephadex G-200 Chromatography Pattern of AChE Forms (Peak Heights) in S2, 1st Method, in the Absence (Controls) and Presence (Experimentals) of 1.0 M KCl, at Different pH's and Time Intervals.

pH's were adjusted at day 0. Aliquots for chromatography: AChE: a,b = 252 units; c,d = 216 units; protein: a,b = 4 mg; c,d = 3 mg. Aliquots for enzyme assay: a,b = 250 λ; c,d = 200 λ.

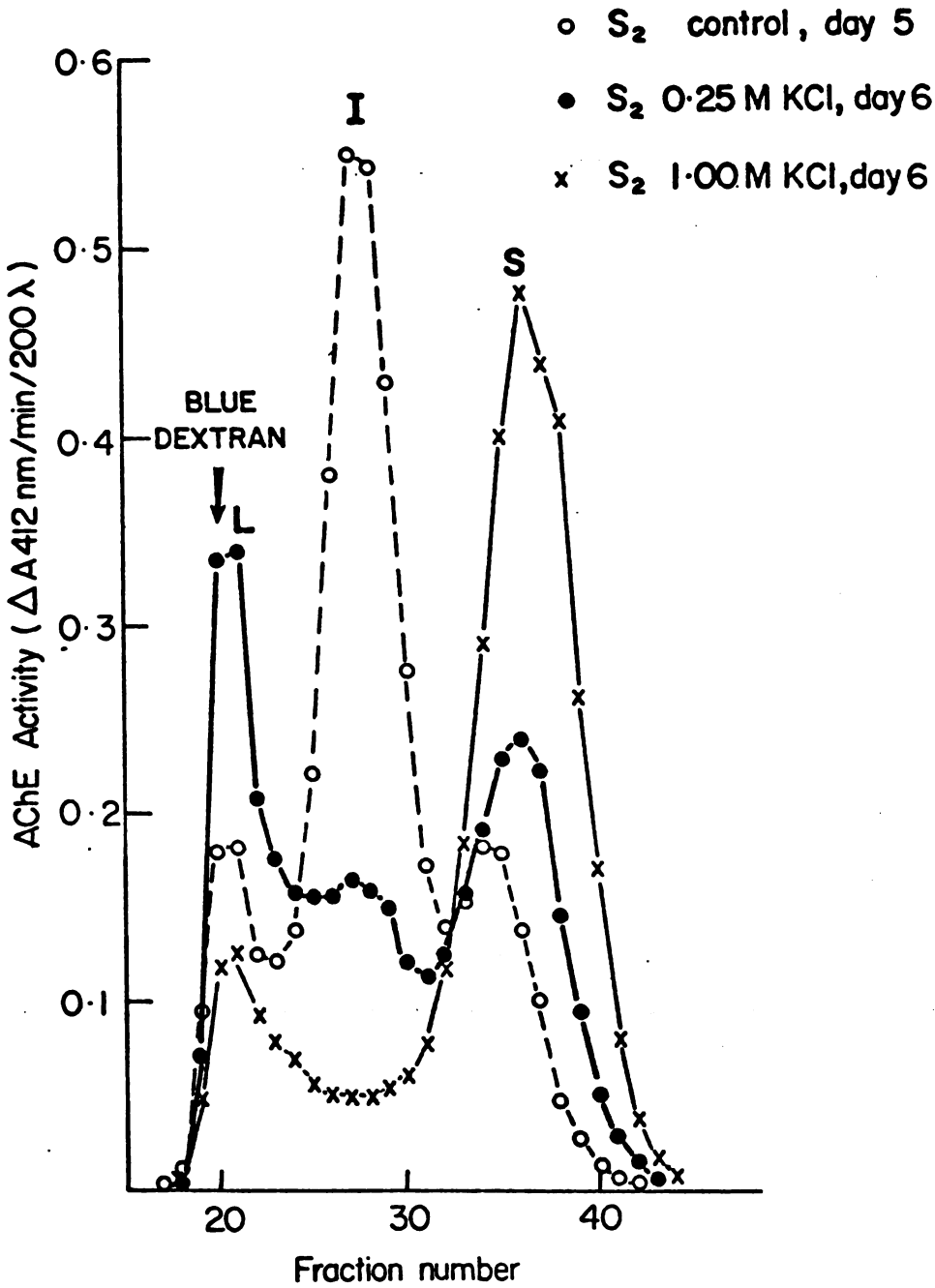

Figure 15. Sephadex G-200 Chromatography of AChE Forms in S2, 2nd Method, in the Absence (Control) and Presence of 0.25 and 1.00 M KCl.

The Addition of KCl was carried out at day 0. Aliquots: AChE = 823 units; protein = 7 mg.

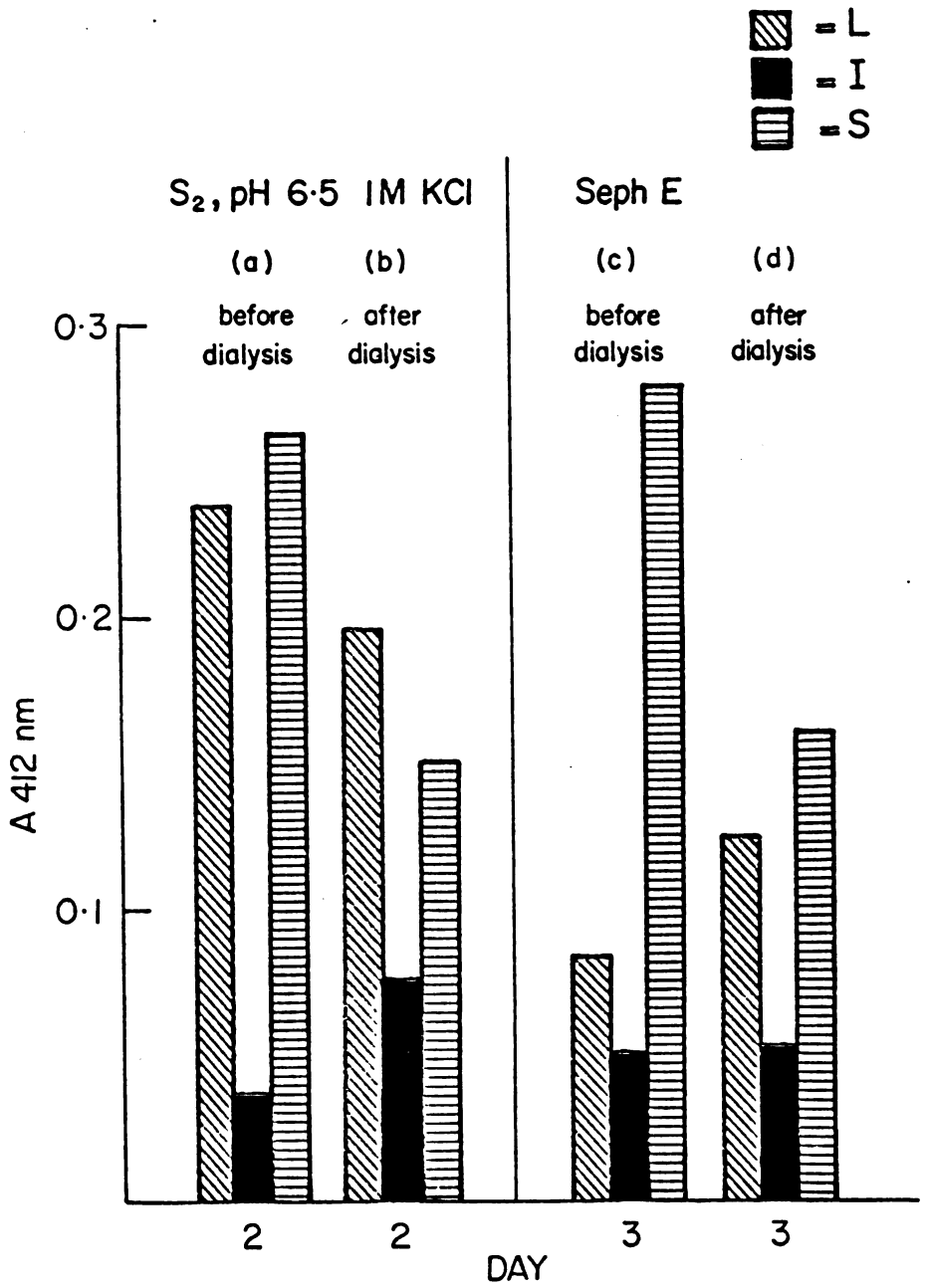

Figure 16. Sephadex G-200 Chromatography Pattern of AChE Forms (Peak Heights) Present in S2, with 1 M KCl at pH 6.5, and in Seph E, 1st Method, Before and After Dialysis.

pH adjustment and addition of KCl were carried out at day 0. At indicated time intervals, respective aliquots (AChE = 446 and 490 units; protein = 8 and 7 mg) were dialysed for 3 h against 30 mM phosphate buffer, pH 8.0. Aliquots for enzyme assay: 125 λ.

The observed ratios in the dialysis experiments were at least 1/20. It may be concluded, then, that a dialysis effect per se, or the increasing or decreasing of the ionic strength of the medium did not reverse the effect of DEAE Sephadex A-25 or KCl treatments on the enzyme preparation.

AChE in Supernatant Fraction 1 (S1)

Much of the data to be introduced now are similar to those studied above. However, a more easily solubilized enzyme of a lower specific activity was used in this instance.

1. Conversion of AChE forms with time: S1, prepared by method 1, gave a somewhat different picture from that obtained with S2 (same method), even at day 0, since the L form rather than the S form predominated. However, conversion to the I form still occurred with time (Figure 17). S1 prepared by the 2nd method was similar to S2 prepared by the same method, with a predominance of the S form, at day 0, and a gradual conversion to the I form, with time, which in this case was as large as the L form (Figure 18). Table 11 shows the effect of storage time on the percentage of change on AChE forms from S1 obtained by the two methods.

The chromatography of S1 (1st method) on Sepharose 6B gave the same changes, with time, as those obtained with S2 under the same experimental conditions. It was noticeable that a peak of AChE activity occurred in the void volumes of both chromatographic patterns.

2. Non-aggregating (stable) forms of AChE:

a. With DEAE Sephadex A-25 treatment: The effect of DEAE Sephadex A-25 on S1, obtained by both methods of solubilization, was even more marked than that on the isoenzymes present in S2 (1st method).

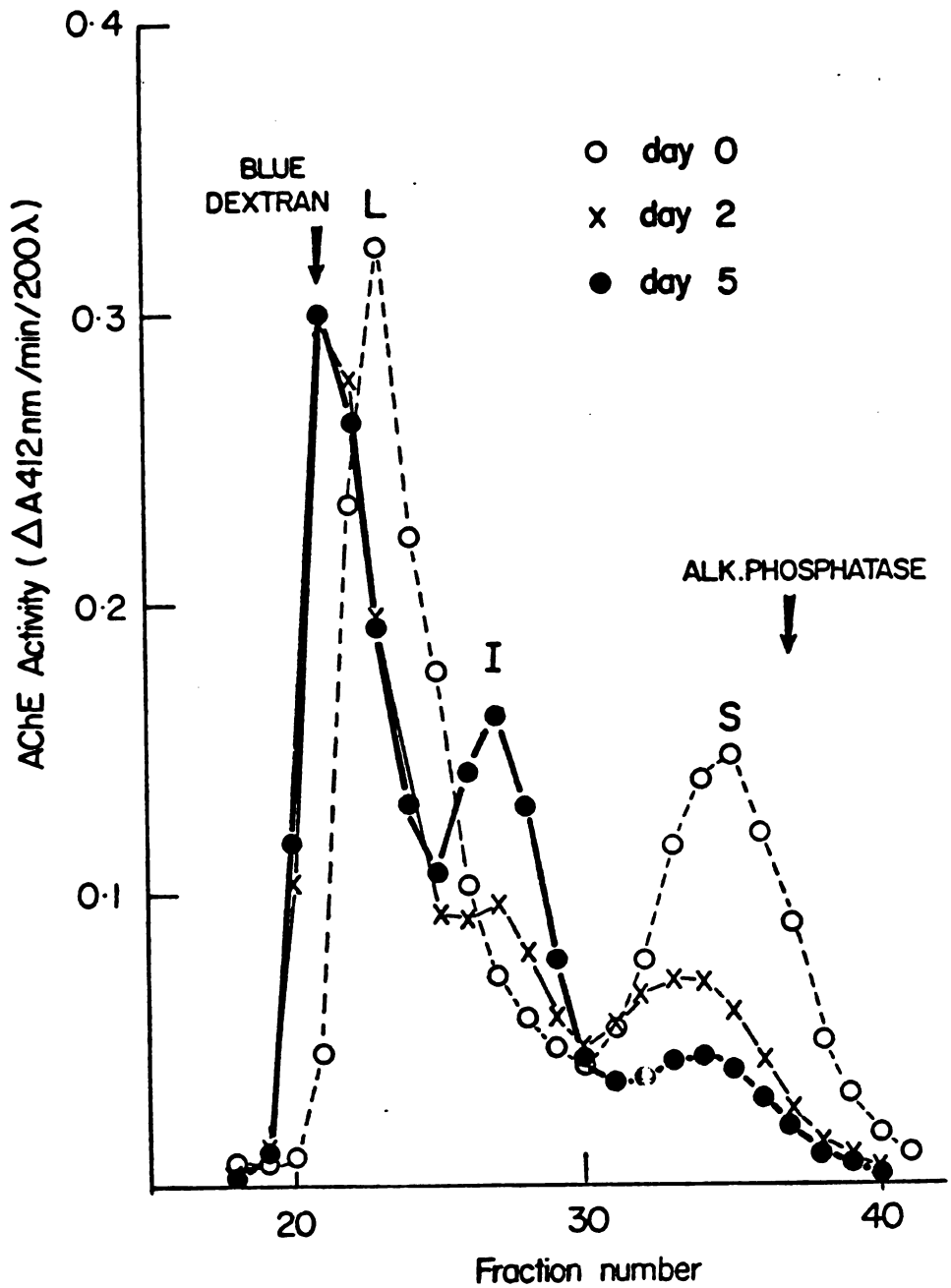

Figure 17. Changes with Time of AChE Forms Present in S1, 1st Method, Shown by Sephadex G-200 Chromatography.

Aliquots: AChE = 305 units; protein = 22 mg.

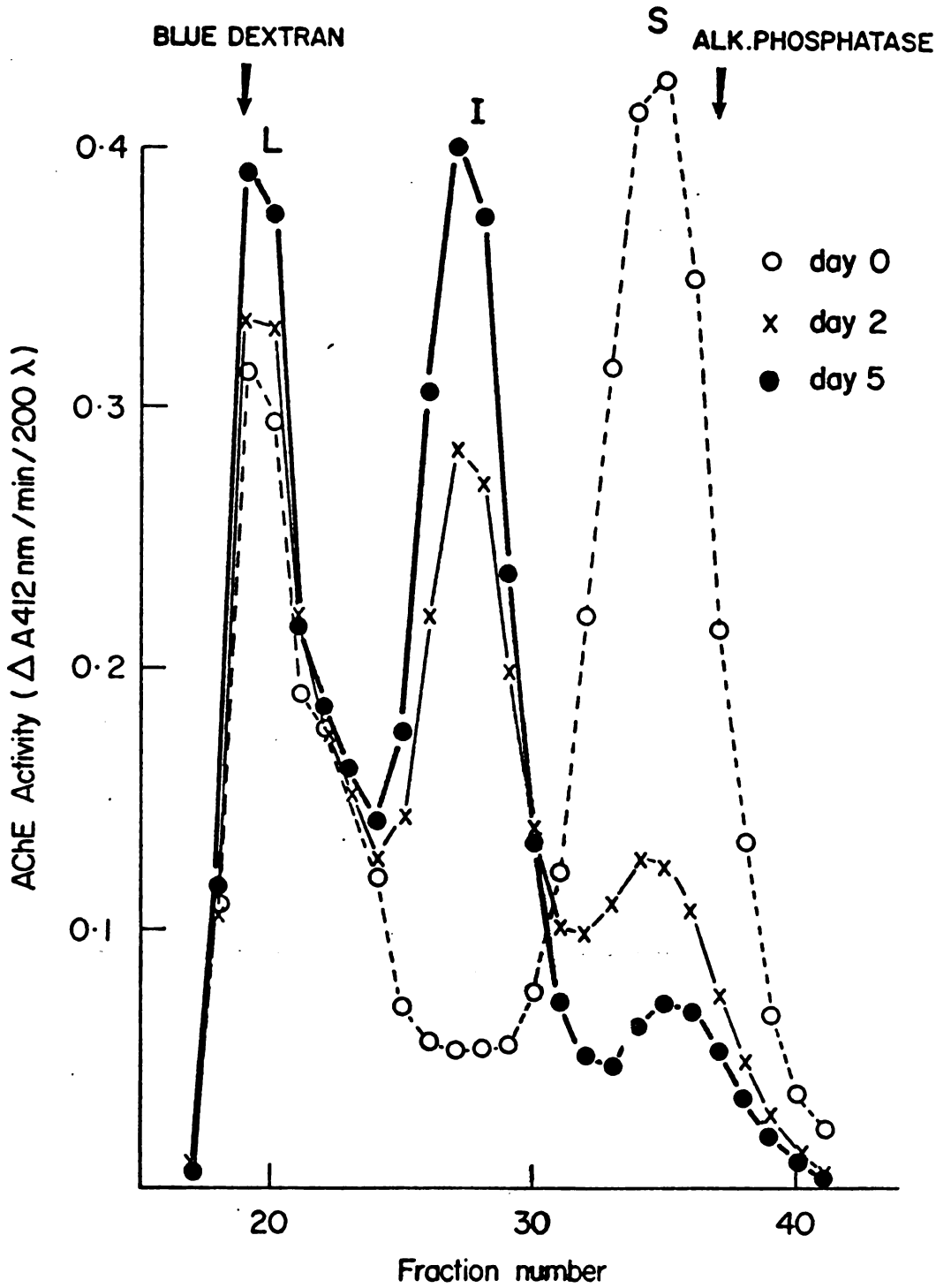

Figure 18. Changes with Time of AChE Forms Present in S1, 2nd Method, Shown by Sephadex G-200 Chromatography.

Aliquots: AChE = 569 units; protein = 36 mg.

TABLE 11. EFFECT OF STORAGE TIME ON THE PERCENTAGE OF CHANGE OF AChE FORMS FROM SUPERNATANT FRACTIONS 1

AChE Form	1st Method			2nd Method	
	Days 0-1	Days 1-2	Days 4-7	Day 0	Days 2-5
L	52.2 ± 5.0 (2)	54.1 ± 4.4 (2)	51.1 ± 3.3 (3)	19.8 ± 1.5 (3)	41.7 ± 1.3 (2)
I	7.2 ± 0.7 (2)	18.2 ± 1.4 (2)	34.1 ± 3.3 (3)	16.2 ± 1.2 (3)	44.7 ± 3.7 (2)
S	40.6 ± 4.2 (2)	27.7 ± 5.8 (2)	14.7 ± 1.8 (3)	64.1 ± 2.7 (3)	13.6 ± 4.7 (2)

The relative amounts of enzyme in each AChE form were calculated from the ratio:

$$\% \text{ of AChE Activity} = \frac{\Sigma \text{ Peak } \Delta A_{412\ nm}/min}{\Sigma \text{ Total } \Delta A_{412\ nm}/min}$$

Number of experiments is indicated in parentheses. Values are means ± S.E.M. 1st method: Chan et al. (24), modified; 2nd method: Hollunger and Niklasson (75), modified.

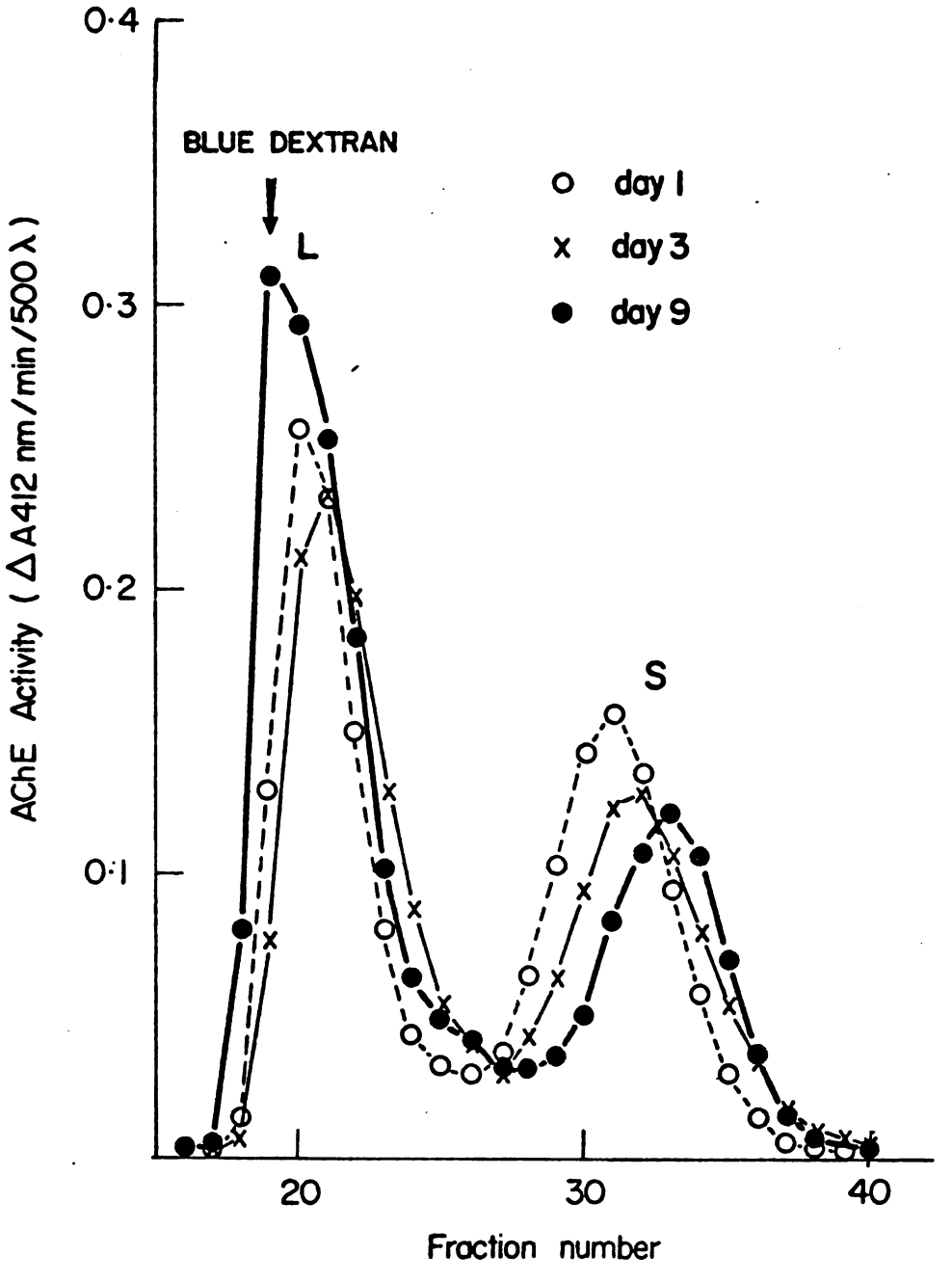

Figure 19. Effect of DEAE Sephadex A-25 Treatment on the Stabilization of AChE Forms Present in Seph E from S1, 1st Method, Shown by Sephadex G-200 Chromatography.

Aliquots: AChE = 162 units; protein = 9 mg.

Figure 19 shows the stabilization of AChE forms present in Seph E from S1, 1st method. This effect on the forms was not reversed by dialysis against 30 mM phosphate buffer, pH 8.0, and again S1 (1st method) was more resistant to dialysis than its counterpart. The specific activity of AChE in S1 (1st method) was also higher after DEAE Sephadex A-25 treatment (Seph E). The amount of enzyme recovered from the gel was 41% (Table 10).

b. With KCl treatment: This treatment protected the enzyme preparation against form conversion by decreasing formation of the I form. Figure 20 shows the effect of KCl at concentrations ranging from 0.06 to 0.25 M. The effect was concentration dependent with increasing protection of the S form and less formation of the I form when salt concentration increased, a total protection being observed with 1 M KCl. At higher pH (8.0), the effect of KCl was less intense in terms of the S form, but a blockade of I formation could still be detected (Figure 21).

Enzyme stabilization was well demonstrated in S1 (2nd method) after treatment with DEAE Sephadex A-25, 1 M KCl or NaCl (Figure 22). The results were basically the same under the three experimental conditions. This salt effect was not reversed by dialysis against buffer of lower ionic strength (Figure 23). However, the treatment of a two-day old enzyme preparation with 1 M KCl reversed the usual aggregation process (not shown).

3. Effect of KCl on the extraction of AChE forms in S1: There is evidence in the literature (75,95) suggesting a decrease in the percentage of AChE solubilization when the enzyme is extracted in the presence of KCl

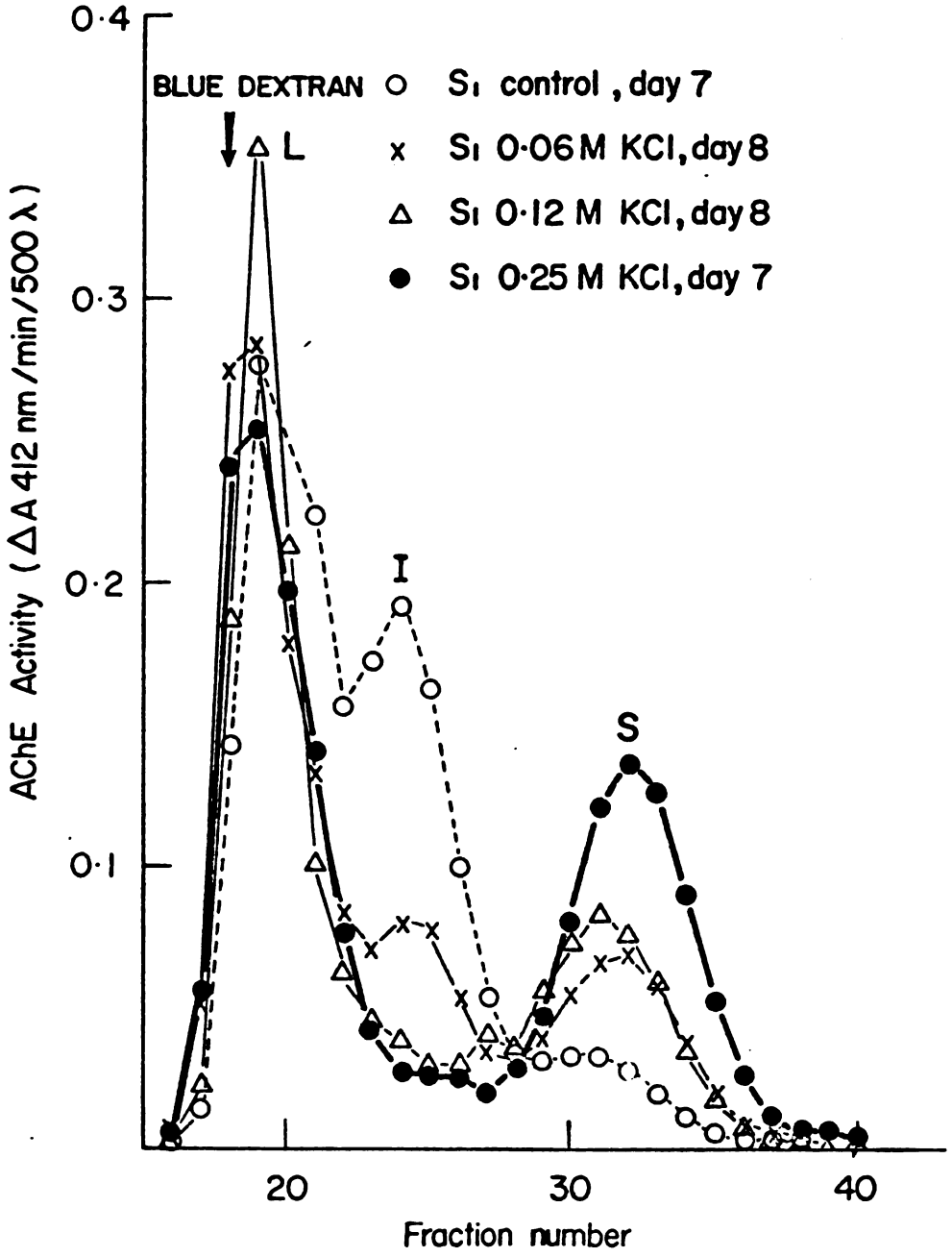

Figure 20. Sephadex G-200 Chromatography of AChE Forms in S1, 1st Method, in the Absence (Control) and Presence of Different Concentrations of KCl.

The addition of KCl was performed at day 0. Aliquots: AChE = 126 units; protein = 10 mg.

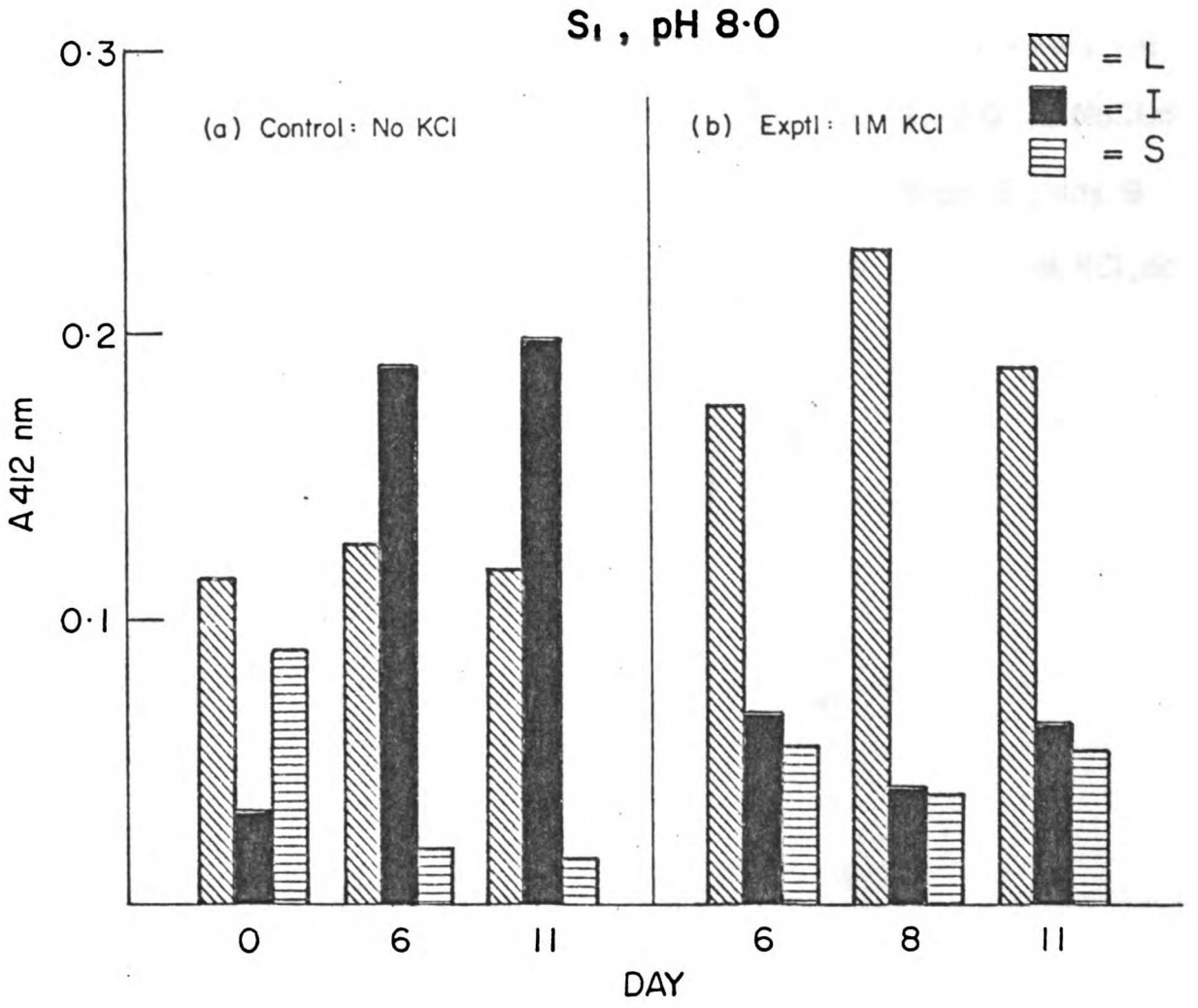

Figure 21. Sephadex G-200 Chromatography Pattern (Peak Heights) of AChE Forms in S1, 1st Method, in the Absence (Control) and Presence (Experimental) of 1 M KCl, at pH 8.0.

pH adjustment and addition of KCl were performed at day 0. Aliquots for chromatography: AChE = 175 units; protein = 9 mg. Aliquots for enzyme assay: 250 λ.

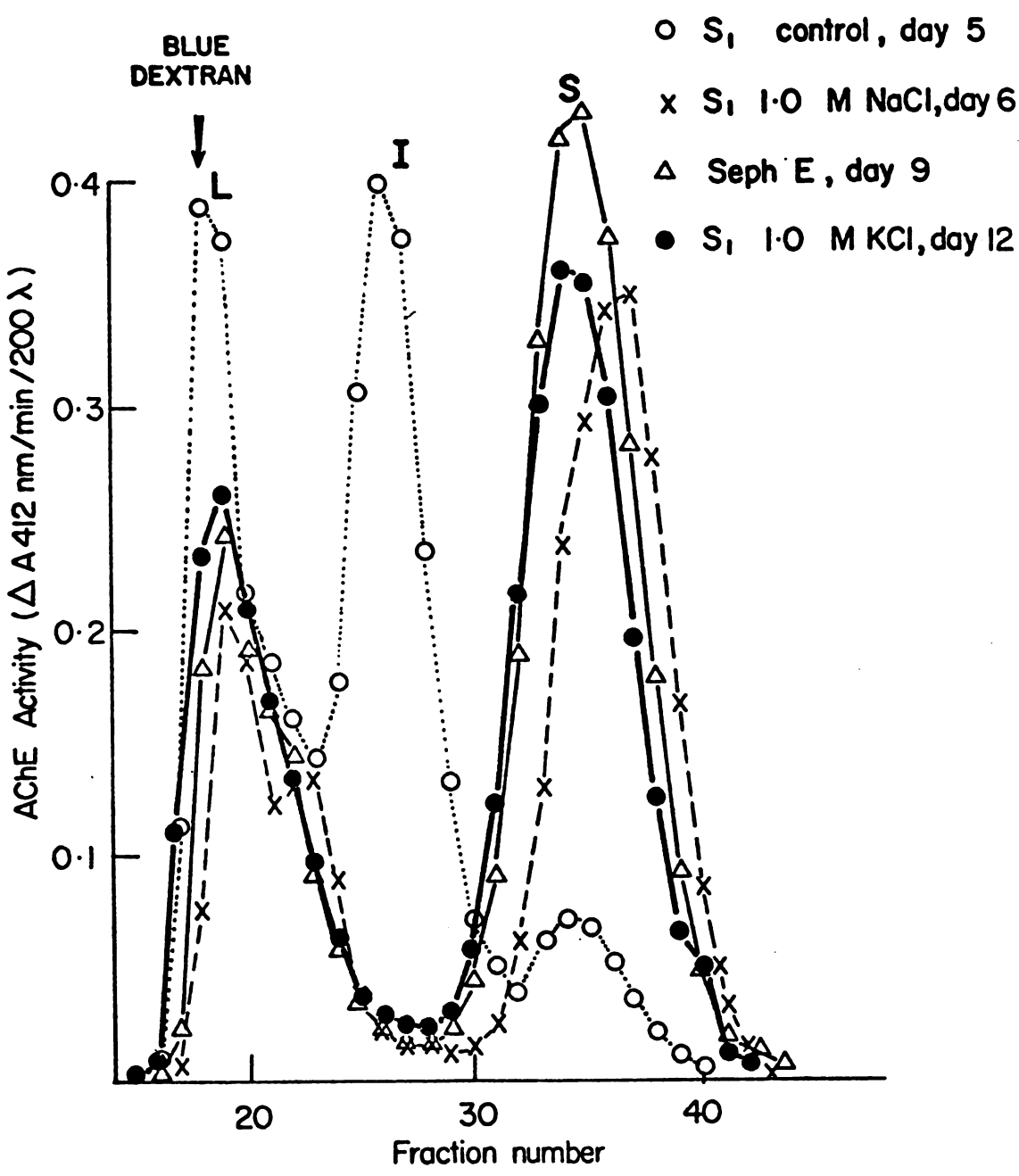

Figure 22. Effect of DEAE Sephadex A-25, KCl and NaCl Treatments on the Stabilization of AChE Forms Present in Sl and Seph E, 2nd Method.

Treatments were performed at day 0, and the Sephadex G-200 chromatography was carried out at indicated times. Respective aliquots for Seph E and for the other cases: AChE = 501 and 569 units; protein = 21 and 36 mg.

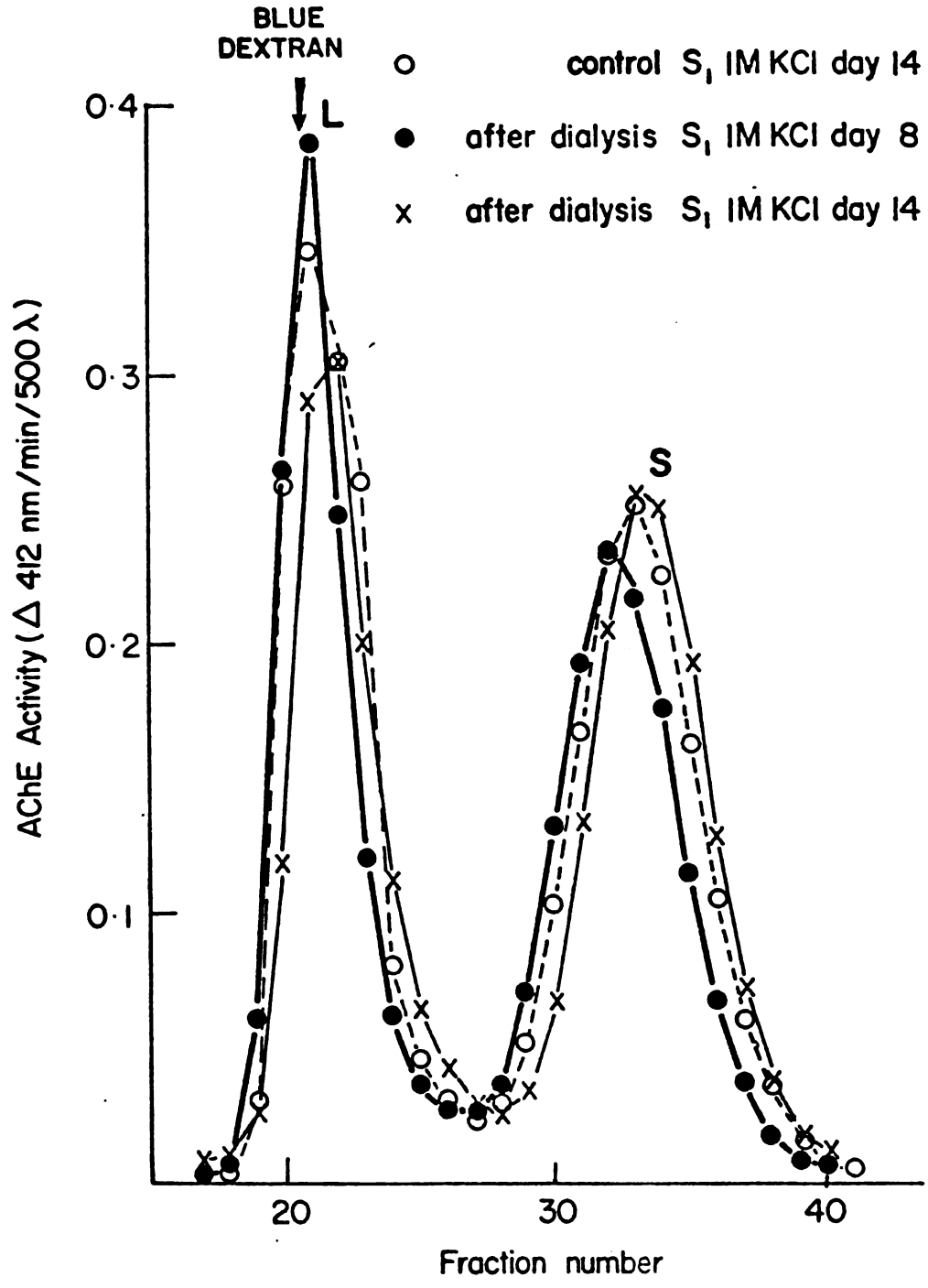

Figure 23. Sephadex G-200 Chromatography of AChE Forms Present in KCl-treated S1, 1st Method, Before (Control) and After Dialysis.

The addition of KCl and dialysis (for 3 h, against 30 mM phosphate buffer, pH 8.0) were performed at days 0 and 8, respectively. The dialysed material was chromatographed again, at day 14. Aliquots: AChE = 189 units; protein = 9 mg.

or NaCl. The purpose of this experiment was to obtain an S1 preparation less contaminated with membrane-bound enzyme.

Figure 24 shows the results from an S1 (1^{st} method) preparation carried out in the presence of 0.25 and 0.5 M KCl, respectively. The enzymatic content (6%) was less than that obtained with the usual conditions of extraction (10-14%). On the other hand, the proportions of S and L forms were greatly changed. The percentage of total enzyme activity was 23 and 32 for the S form, and 74 and 63 for the L form, in the enzymes prepared in the presence of 0.25 and 0.5 M KCl solutions, respectively. In addition, the amount of enzyme present in the I form was very small (3-4%).

Another interesting point observed with S1 (1^{st} method) was a drastic decrease of enzyme activity with time (almost a 90% decrease within a 13-day period), as compared to only a 15% decrease with S2 (1^{st} method), at the same period of time. The addition of KCl, at day 0, to S1 tended to reduce this loss in enzyme activity to a 65% decrease in the presence of 0.25 M KCl, and to only a 20% decrease in the presence of 1 M KCl. There was also a decline in precipitation of particulate material in S1, with time, which paralleled the increase in salt concentration.

Molecular Weights of Multiple Forms of Brain AChE

Molecular weight determinations were carried out on Sephadex G-150, Sephadex G-200, and Sepharose 6B, according to Andrews' method (3). Figures 25 and 26 show the molecular weights of the AChE forms and the calibration proteins for the columns used. Table 12 presents the general results.

As far as the molecular weight determinations on gel filtration are concerned, some important considerations have to be taken into account,

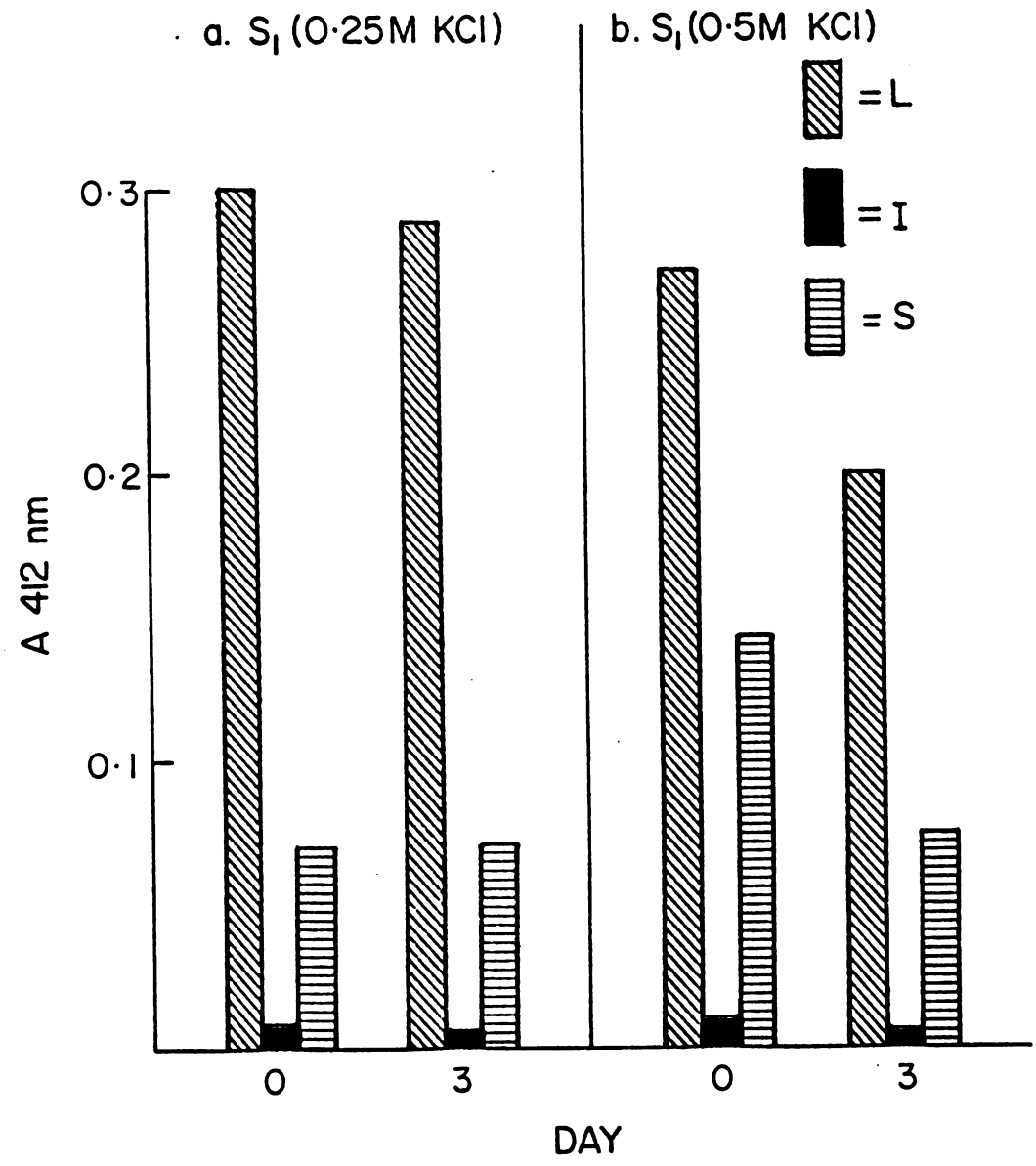

Figure 24. Sephadex G-200 Chromatography Pattern of AChE Forms (Peak Heights) in S1, 1st Method, Obtained in the Presence of 0.25 and 0.5 M KCl.

Aliquots for chromatography: AChE = 166 and 149 units; protein = 15 mg. Aliquots for enzyme assay: 250 λ.

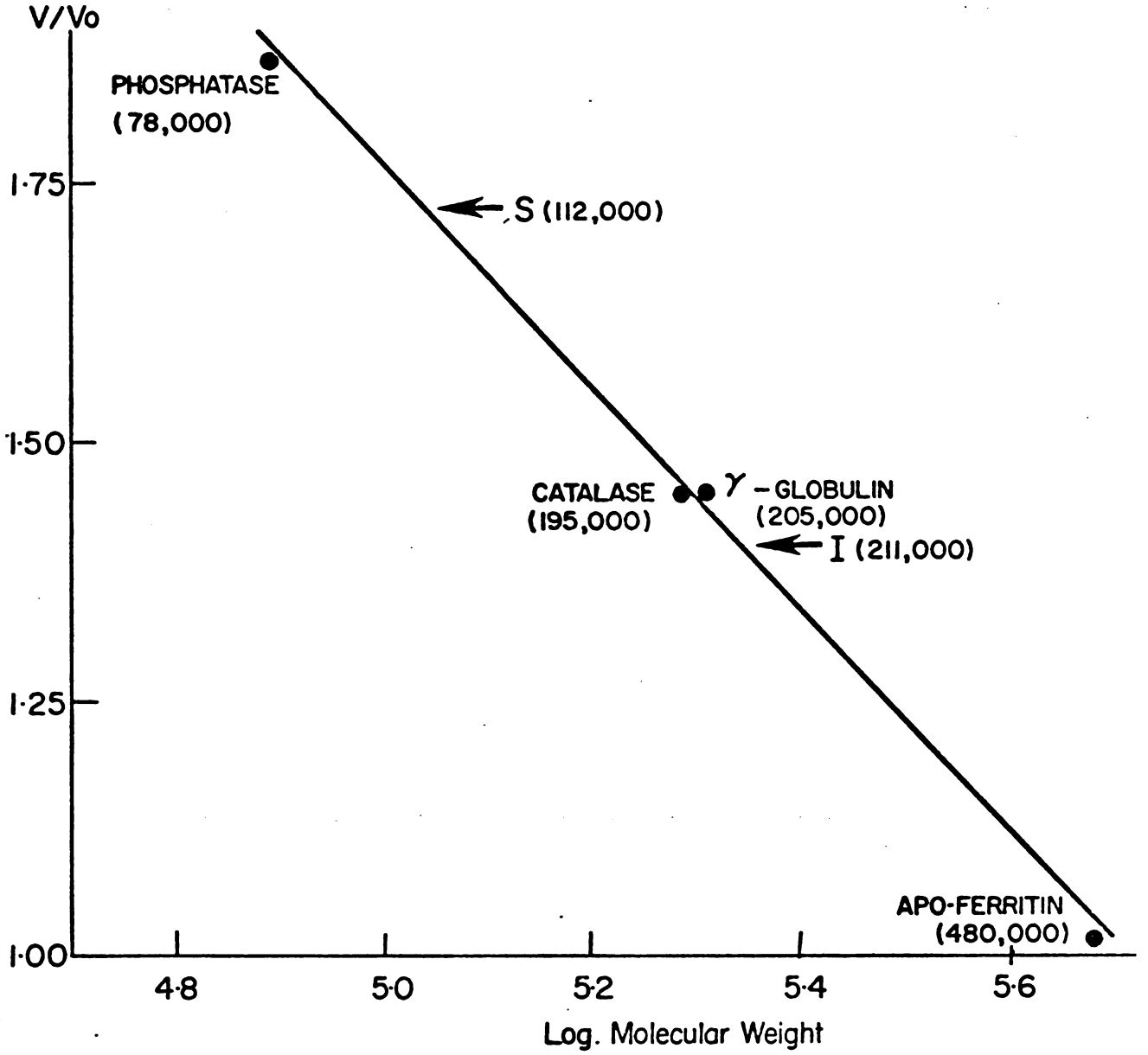

Figure 25. Molecular Weight Determination on Sephadex G-200 of AChE Forms Present in S2, 1st Method.

The column (1.5 x 90 cm) was standardized with proteins of known molecular weights, shown. The molecular weights of AChE forms S and I were obtained from the plot of V/Vo against log M.W. V: elution volume; Vo: void volume of the column.

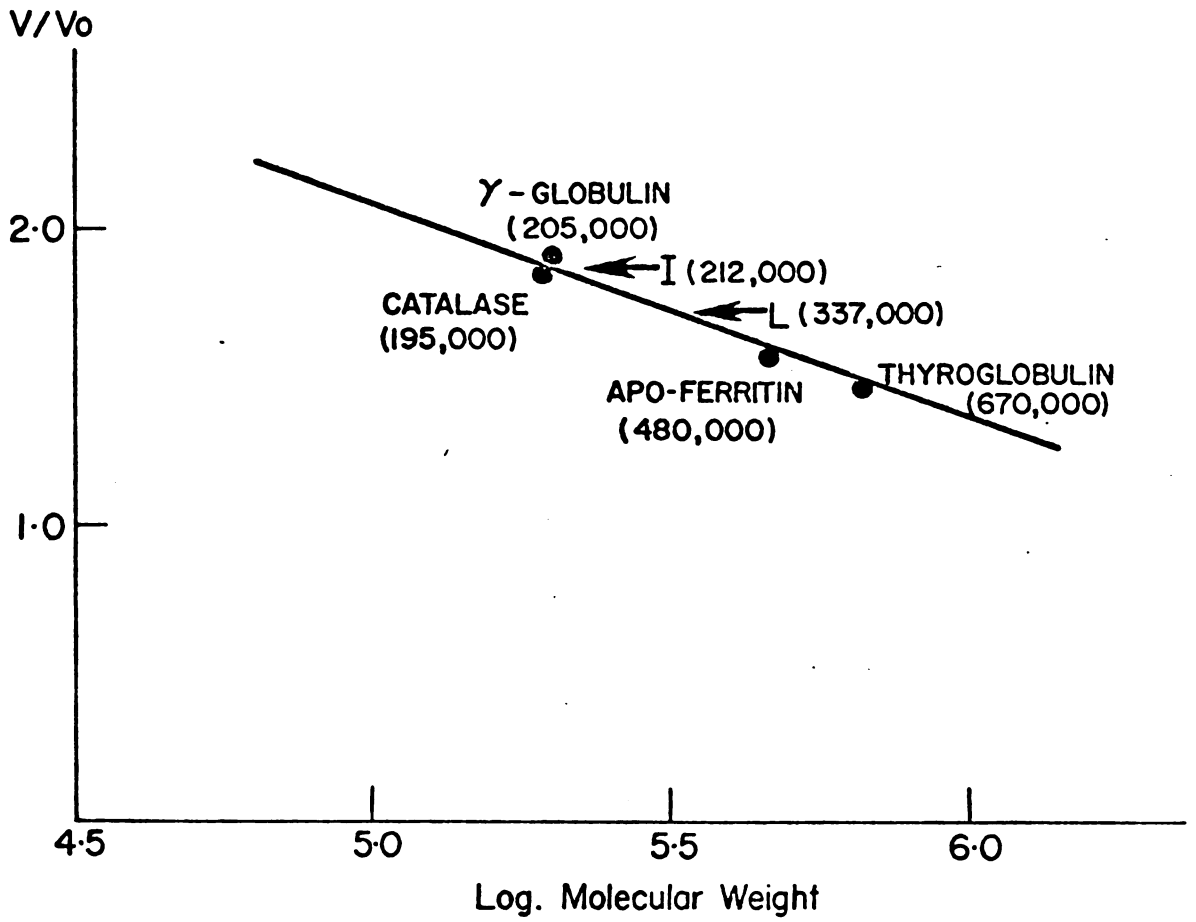

Figure 26. Molecular Weight Determination on Sepharose 6B of AChE Forms Present in S2, 1st Method.

The column (1.5 x 90 cm) was standardized with proteins of known molecular weights, shown. The molecular weights of the I and L forms were obtained from the plot of V/Vo against log M.W. V: elution volume; Vo: void volume of the column.

such as the choice of the gel and the type of column that best fulfills the required experimental purposes. In the present situation, Sephadex G-150 and G-200 would be adequate for the molecular weight determination of the S form, whereas Sephadex G-200 would be a better choice for the molecular weight determination of the I form. Finally, because of reasons which will be explained below, Sepharose 6B would be more adequate for the molecular weight determination of the L form.

The results of the molecular weight determinations of the S form, when estimated on Sephadex G-150 and Sephadex G-200, were similar, as also were the results for the I form when estimated on Sephadex G-200 or Sepharose 6B. However, the molecular weight of the L form was overestimated when Sephadex G-150 or Sephadex G-200 columns were used. This fact was due to the contamination of the L form with a very large aggregated complex, which was eluted together with the L form on Sephadex G-150 or Sephadex G-200 columns, but separated from it on Sepharose 6B column.

Most of the molecular weight determinations were carried out with S2 (1^{st} method). The molecular weight determinations of AChE forms on S2 (2^{nd} method) were done only on Sephadex G-200 for a comparative study; therefore, no conclusive results can be drawn concerning the molecular weight determination of the L form, using this 2^{nd} method.

The results suggested that the molecular weight of the I form was similar in both methods (Table 12). However, the value for the S form was lower with the 2^{nd} method (90,000); and comparison between the average elution volumes of the S form in the two methods, by means of Student's t-Test, showed that they were statistically different at a 99% confidence level (Table 13).

TABLE 12. MOLECULAR WEIGHT DETERMINATION* OF AChE FORMS IN SUPERNATANT FRACTIONS 2

AChE Form	Sephadex G-150 1st method	Sephadex G-200		Sepharose 6B 1st method
		1st method	2nd method	
S	106 ± 9 (3)	112 ± 6 (15)	90 ± 2 (29)	--
I	--	212 ± 29 (19)	219 ± 12 (16)	212 ± 12 (8)
L	--	--	--	337 ± 35 (6)

*Mol. wt. x 10^3. The molecular weight values were obtained from the plot of V/Vo against log Mol. Wt., as detailed in Materials and Methods. Number of experiments is indicated in parentheses. Values are means ± S.E.M. 1st method: Chan et al. (24), modified; 2nd method: Hollunger and Niklasson (75), modified.

TABLE 13. STUDENT'S t-TEST: COMPARISON BETWEEN THE AVERAGES OF ELUTION VOLUMES OF AChE FORMS ON SEPHADEX G-200

AChE Form	Method	Mean (ml)	S.D.	d.f.	t	Result*
S	1st (15)	76.4	3.6	42	3.1	R
	2nd (29)	81.0	5.2			
I	1st (19)	62.0	7.3	33	0.1	A
	2nd (16)	62.1	4.1			

*R: Reject the hypothesis of equal means, at $\alpha = 0.01$
A: Accept the hypothesis of equal means, at $\alpha = 0.01$

Number of experiments is indicated in parentheses. 1st method: Chan et al. (24), modified; 2nd method: Hollunger and Niklasson (75), modified.

Reconstitution Experiments

The basic aim of these experiments was to attempt an identification of the factor(s) responsible for or involved with form conversion, and to ascertain how this process would occur and also be altered. Two types of enzyme preparation were used: after DEAE Sephadex A-25 treatment, and after affinity chromatography.

1. DEAE Sephadex A-25 preparations: Supernatant fractions 2 (S2, 1^{st} and 2^{nd} methods) were treated with DEAE Sephadex A-25, at day 0. The enzyme adsorbed on the gel surface and further eluted with 0.25 M KCl, containing 0.025 M Tris at pH 8.0, was designated Seph E. Supernatant not adsorbed was denominated as Seph Sup 1. This contained on average 1 or 10% of the original enzyme activity, when prepared with the 1^{st} or 2^{nd} methods, respectively.

After enzyme release the gel was washed with 1 M KCl, containing 0.1 M Tris at pH 8.0, and the wash was called Seph Sup 2. An additional 14% (1^{st} method) or 9% (2^{nd} method) of the total enzyme activity present in the starting material was released in Seph Sup 2.

a. Results from enzyme prepared with the 1^{st} method: Figure 27 shows that the addition of Seph Sup 1 to Seph E did not affect very much the stability of the S form, under both experimental and control conditions. Likewise, the addition of Seph Sup 2 to Seph E did not affect the stability of the S form, in the experimental, but there was a decrease of the S form, followed by a build up of the I and an increase of the L forms, in the control.

b. Results from enzyme prepared with the 2^{nd} method: The addition of Seph Sup 1 to Seph E did not change the general isozymic

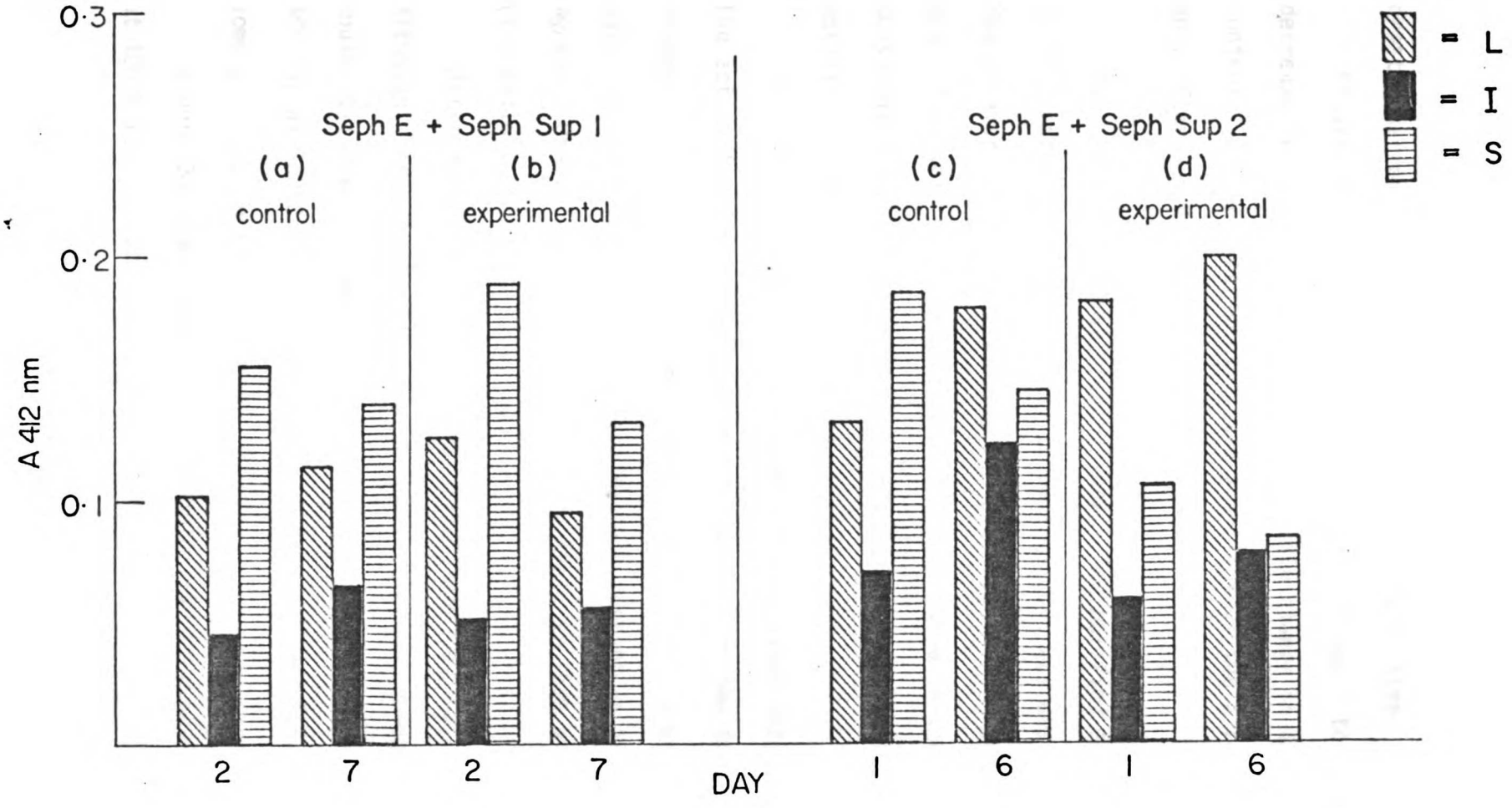

Figure 27. Reconstitution Experiment with Seph E from S2, 1st Method.

Sephadex G-200 chromatography pattern of AChE forms (peak heights) present in Seph E plus Seph Sup 1 or 2, under control and experimental conditions (see text). Seph E (2.5 ml) was added to Seph Sup 1 or 2 in a 1:2 ratio. Aliquots for enzyme assay: a,b,c = 500 λ; d = 250 λ.

pattern in the control (Figure 28); in the experimental, however, a drastic decrease in the S form was observed with time.

Figure 29 shows that the addition of Seph Sup 2 to Seph E caused a decrease in S, an increase in I, and little change in the L form, in the control. In the experimental, the stability of the S form was affected only after day 4.

2. Affinity enzyme preparations: The purified enzyme separated from S2 (1^{st} method), after affinity chromatography, was designated Aff E. It had an average specific activity of 49 mmoles of ATC hydrolysed per milligram of protein per hour. The material not bound in the affinity column constituted the Aff Sup. It represented only about 4% of the total enzyme activity of the starting material.

The purified enzyme (Aff E) was a concentrated enzyme preparation. The active fractions (1-3) contained about 90% of the total recovered enzyme activity. γ-globulin was added to the Aff E preparation in order to have a protein concentration close to that present in the Aff Sup (1.17 mg/ml). One volume of Aff E was added, then, to five volumes of Aff Sup (1:5 ratio).

After affinity chromatography, the enzyme (Aff E) did not aggregate. Although there was predominance of the I form, the presence of the S form could still be detected at day 3. Denatured Aff Sup was obtained by heating Aff Sup at 100°C for five minutes to determine the heat sensitivity of some aggregation factor present, if any.

Figure 30 shows that the addition of Aff Sup (before and after heating at 100°C for 5 minutes) to Aff E had little effect on the AChE forms when run on Sephadex G-200. In all situations, a predominance of I and smaller L and S forms were observed.

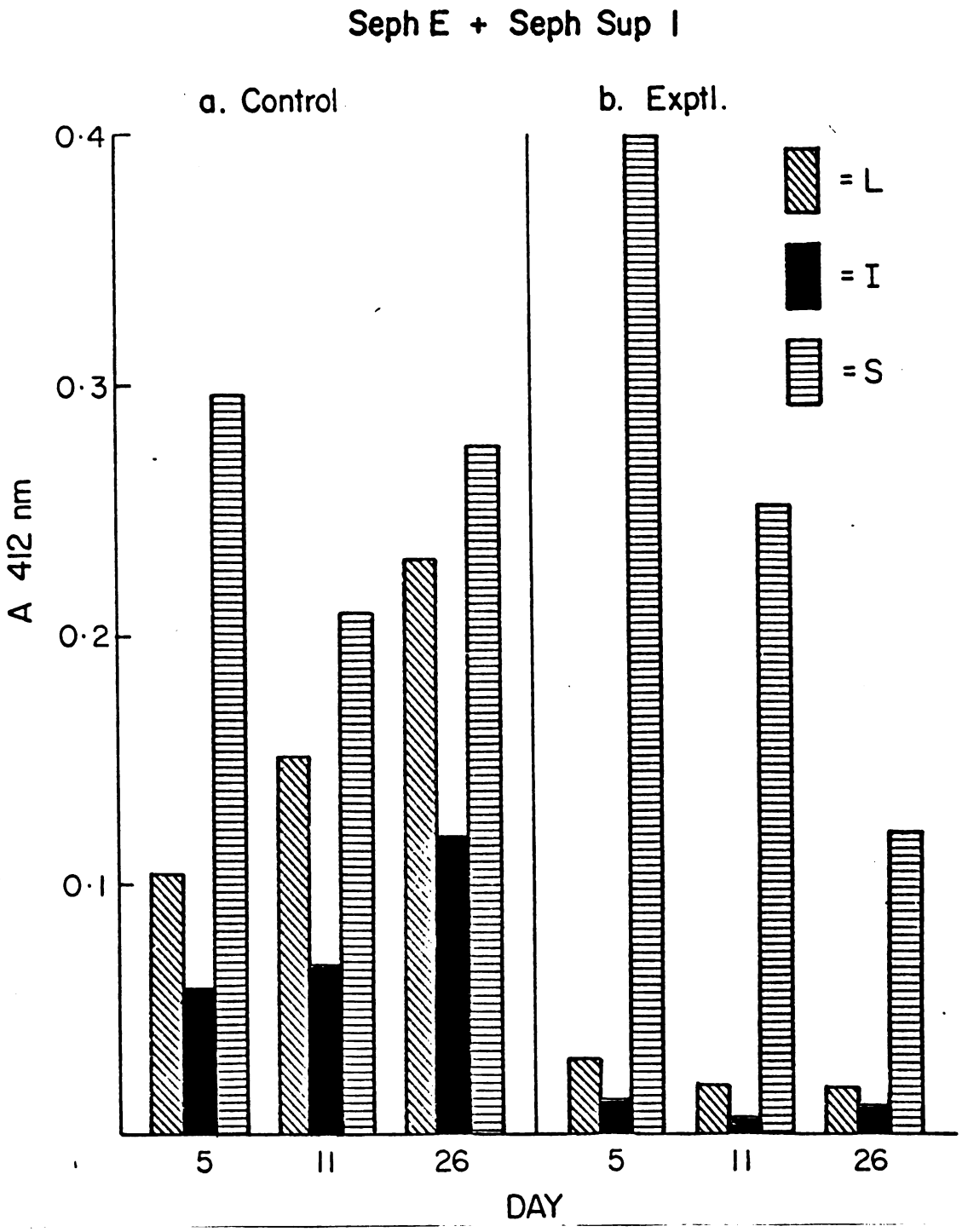

Figure 28. Reconstitution Experiment with Seph E plus Seph Sup 1 from S2, 2nd Method.

Sephadex G-200 chromatography pattern of AChE forms (peak heights) present in Seph E plus Seph Sup 1, under control and experimental conditions (see text). Seph E (2 ml) was added to Seph Sup 1 in a 1:2 ratio. Aliquots for enzyme assay: a = 300 λ and b = 150 λ.

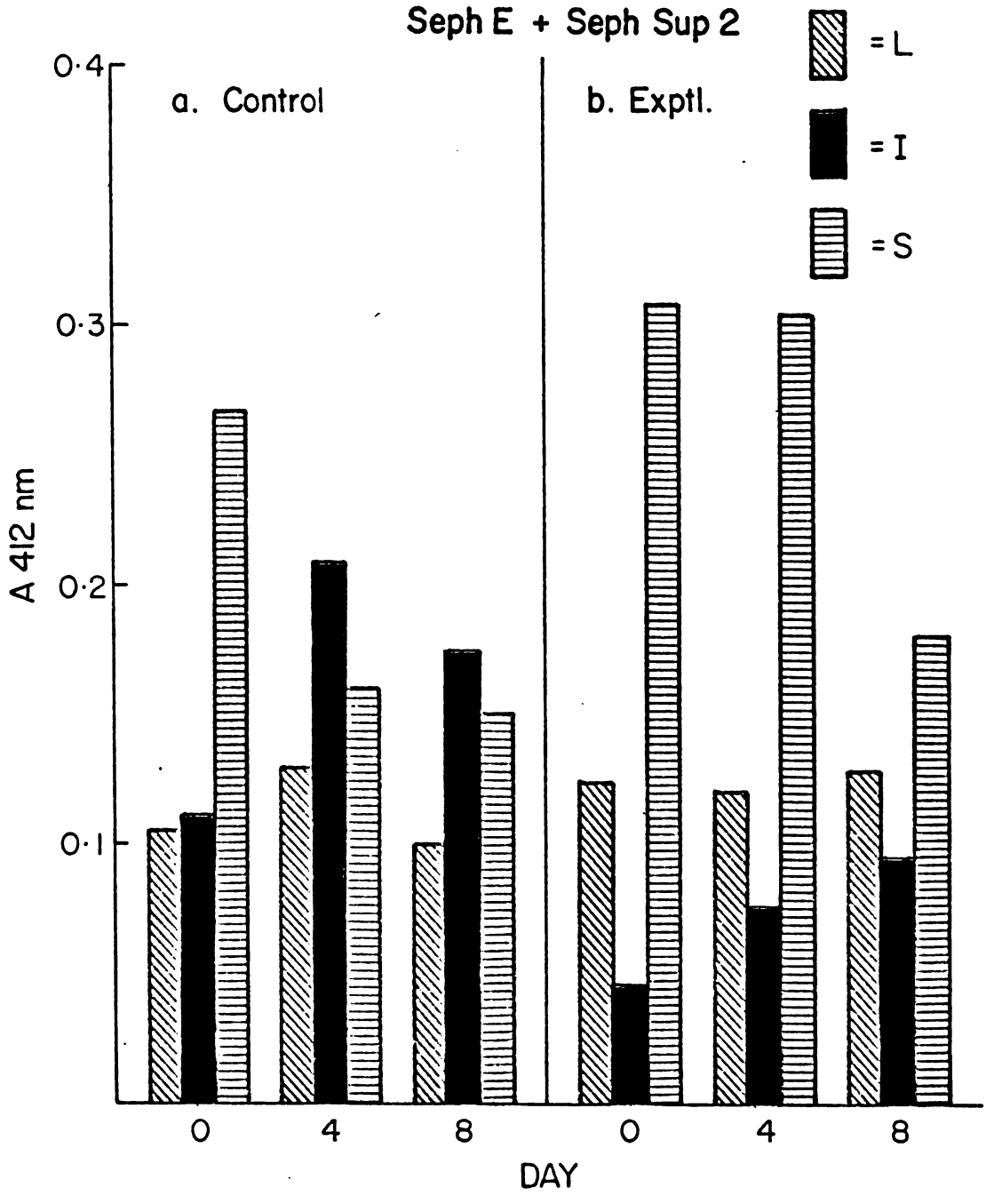

Figure 29. Reconstitution Experiment with Seph E plus Seph Sup 2 from S2, 2nd Method.

Sephadex G-200 chromatography pattern of AChE forms (peak heights) present in Seph E plus Seph Sup 2, under control and experimental conditions (see text). Seph E (2 ml) was added to Seph Sup 2 in a 1:2 ratio. Aliquots for enzyme assay: a = 300 λ; and b = 200 λ.

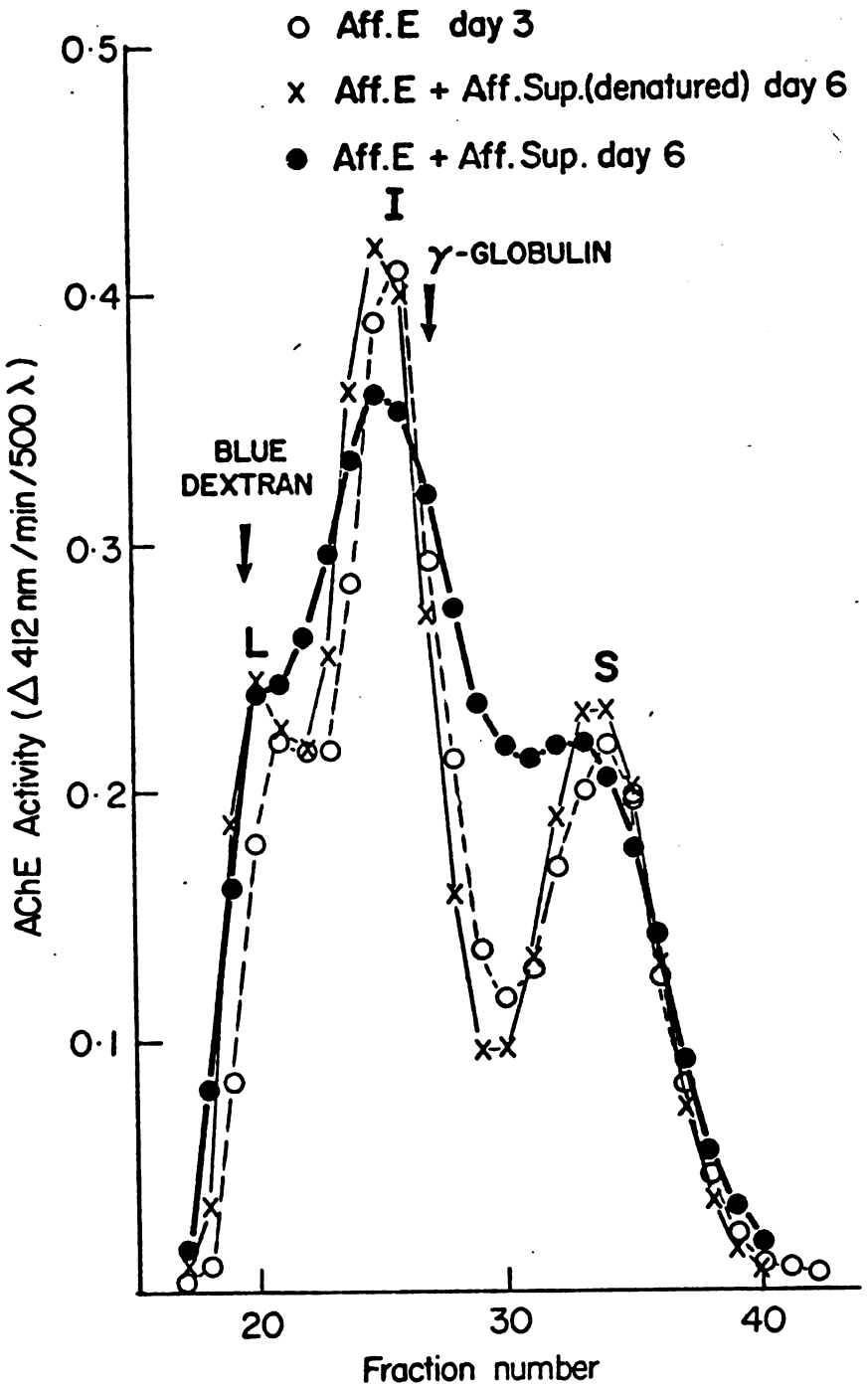

Figure 30. Reconstitution Experiment with Aff E from S2, 1st Method.

Sephadex G-200 chromatography of Aff E and Aff E plus Aff Sup, at different times. Denatured Aff Sup was obtained by heating the Aff Sup at 100 °C for 5 min. The addition of Aff E (3 ml) to Aff Sup in a 1:5 ratio was carried out at day 3.

Effect of $(NH_4)_2SO_4$ Treatment on AChE Form Conversion

Treatment of this type is widely used in protein purification studies. The present objective was to see if any protection could be achieved or if the enzyme behavior was different after $(NH_4)_2SO_4$ treatment. The experiment was done with S2 (1^{st} method).

Figure 31 shows that, at day 0, the usual picture was detected with predominance of S, almost no I, and some L form (nearly 50% of S). At day 2, the S form decreased sharply, there was a trace of I and an increase in L; whereas, two days later, there was only a trace of both the I and S forms. The change was drastic and took place in a short time. The I form here, as after KCl or DEAE Sephadex A-25 treatments, was less stable and the L form was predominant after day 2.

Chromatography of Seph E and Aff E on Sepharose 6B

The purpose of this experiment was to detect the presence of the void volume enzyme peak, obtained after Sepharose 6B chromatography, in S2 (1^{st} method) submitted to different treatments. At day 5, Seph E was chromatographed on Sepharose 6B column together with catalase and alkaline phosphatase as internal markers. Figure 32 shows the three AChE forms (with predominance of the S form) plus the void volume component. However, Aff E, at day 7, showed only L and S forms, with predominance of L, and no I form. The void volume component was absent here.

Rechromatography of the S Form after Separation on Sepharose 6B

The aim of this experiment was to check how stable the S form was after separation and semi-purification on gel filtration, using S2 (1^{st} method) and Seph E (1^{st} method) as S form sources.

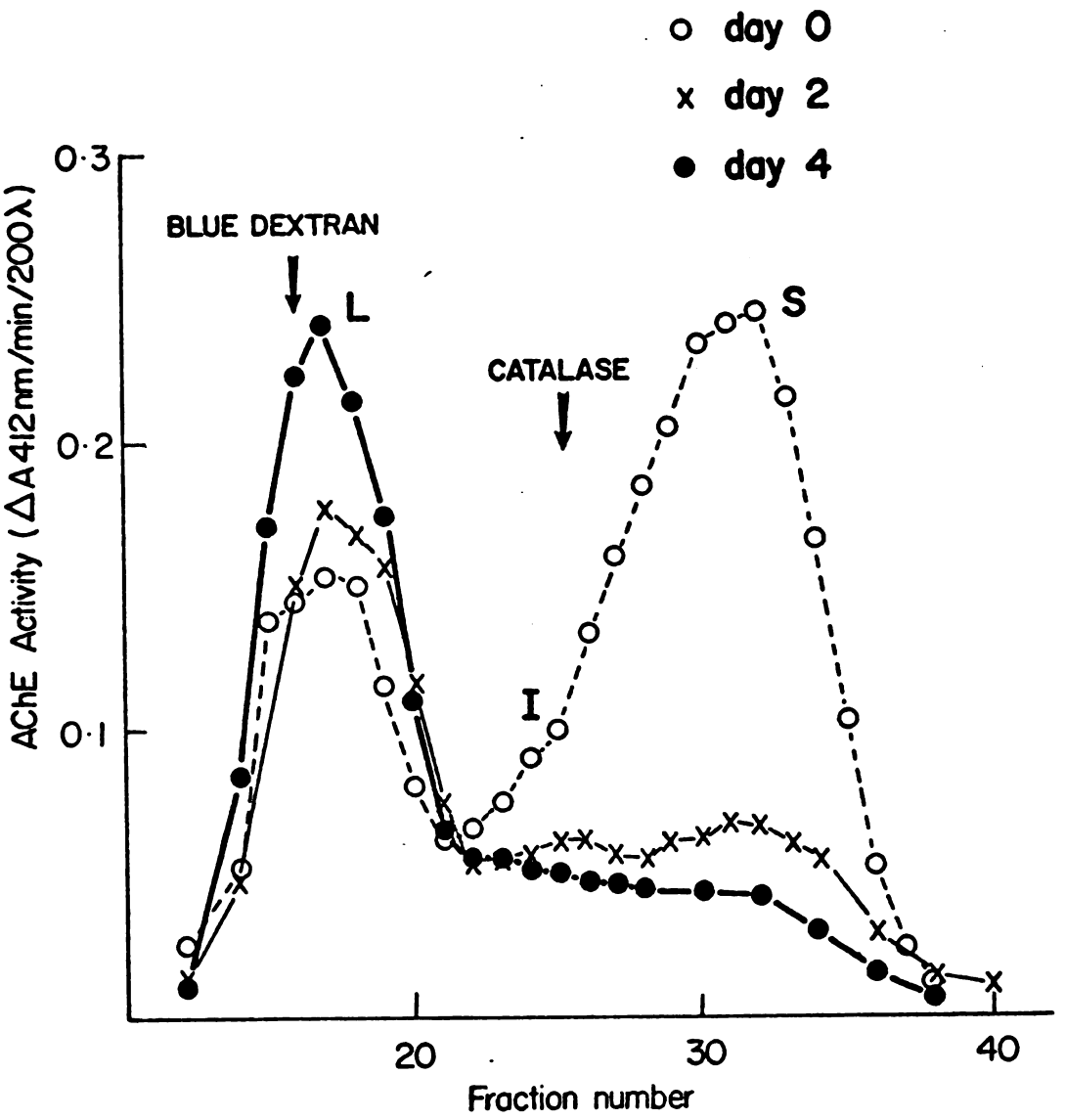

Figure 31. Sephadex G-200 Chromatography of AChE Forms Present in S2, 1st Method, at Various Times after $(NH_4)_2\ SO_4$ Treatment.

The treatment was performed at day 0. Aliquots: AChE = 305 units; protein = 3 mg.

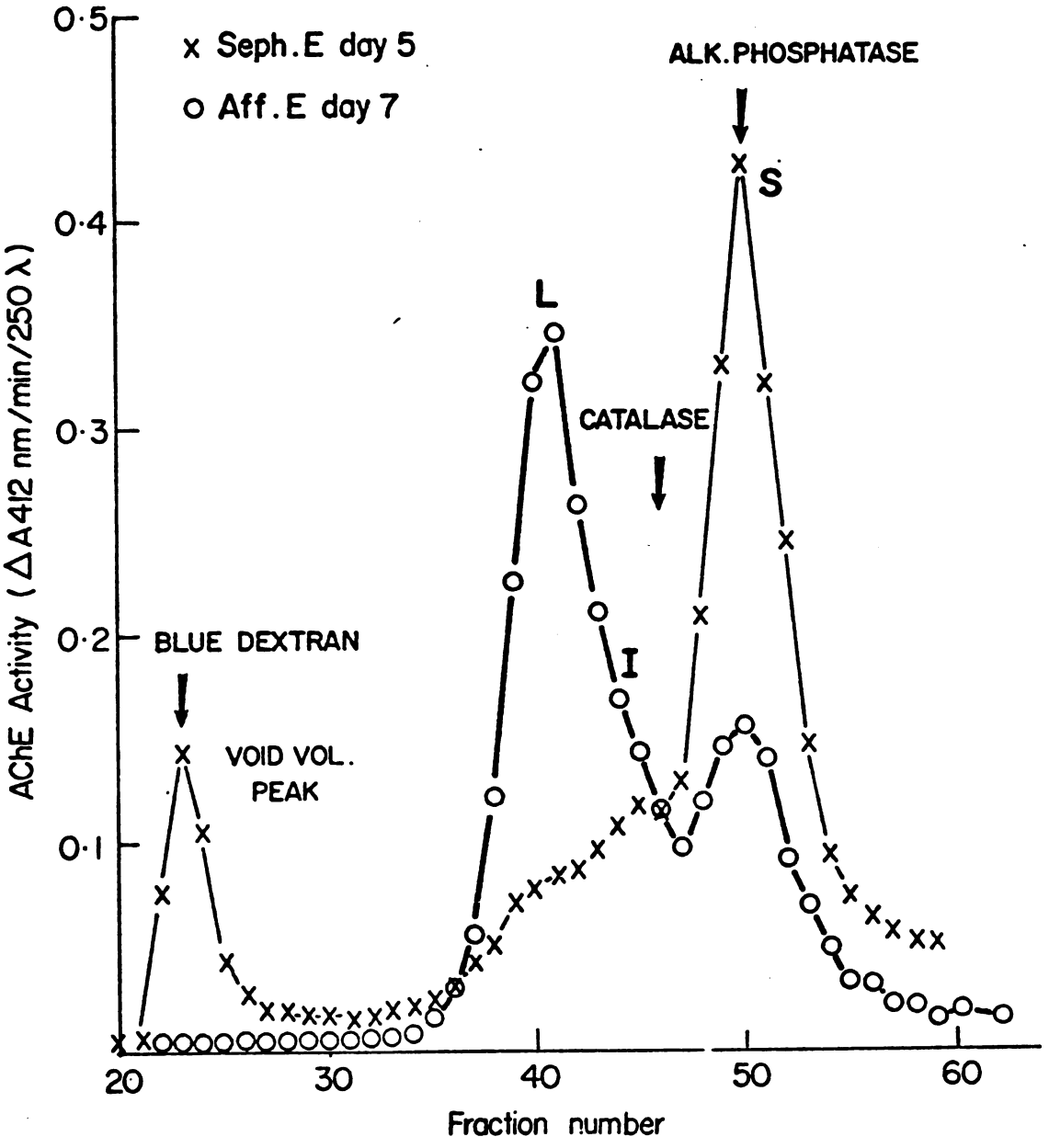

Figure 32. Chromatographic Separation of AChE Forms Present in Seph E and Aff E on Sepharose 6B Column.

Respective aliquots for Seph E and Aff E: AChE = 761 and 418 units; protein = 12 and 0.2 mg. Enzyme source was S2, 1st method.

At day 0, S2, 1st method (AChE: 875 units; protein: 15 mg), was chromatographed on Sepharose 6B column. The active fractions (46-55) corresponding to the S form were pooled together, concentrated, and re-chromatographed on Sephadex G-200, at day 2. Figure 33 shows predominance of the S form (80%), but some contamination with I (15%) and L (5%) was also observed. The enzyme recovery in this rechromatography was 67%.

Essentially the same procedure was repeated using Seph E (AChE: 610 units; protein: 9.8 mg). The results showed only minor contamination with I (9%) or L (5%) forms, and predominance of the S form (87% of the total recovered enzyme activity), although the form separation was done at day 5 and the rechromatography at day 8. The enzyme recovery, however, was only about 41%.

Chromatography of S2 (1st method) on DEAE Sephadex A-25

The purpose here was to detect any difference in charge properties among the AChE forms. S2 was loaded, at day 0, on a DEAE Sephadex A-25 column. Elution was performed with a linear gradient of KCl ranging from 0.0 to 2.0 M or from 0.25 to 1.0 M (Figure 34).

The results showed a major peak of AChE activity eluted at 0.41 M KCl. There was also a suggestion of another peak, which appeared as a shoulder and was eluted at 0.34 M KCl, and two large protein peaks with very little enzyme activity, corresponding to 0.54 and 0.73 M KCl, respectively.

The rechromatography of the enzyme peak obtained from the linear gradient of KCl (0.25 to 1.0 M) on Sephadex G-200 showed a predominance of L (37%) as compared with the I form (28%). However, the S form contained

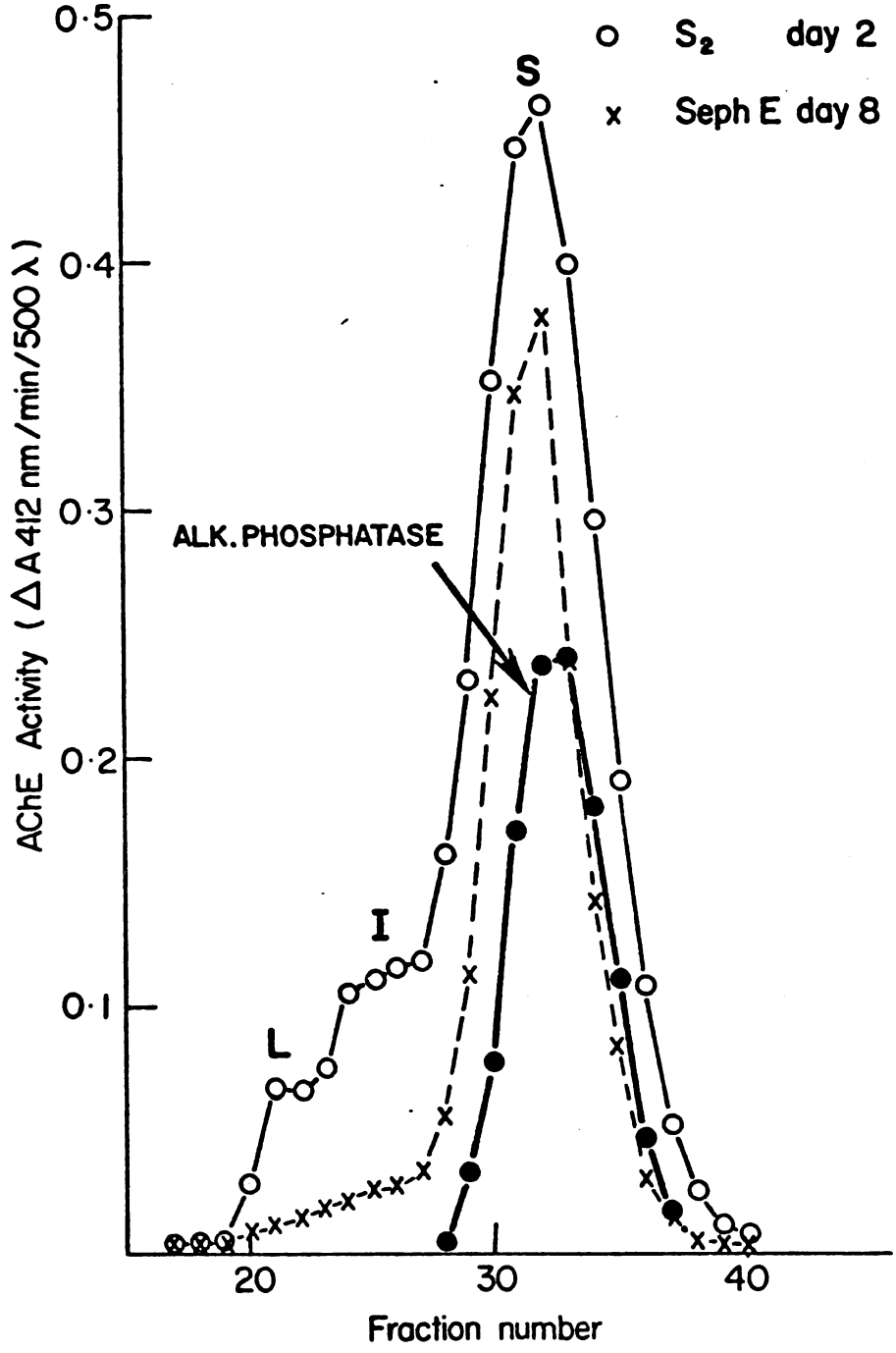

Figure 33. Sephadex G-200 Rechromatography of S2 and Seph E, after AChE Form Separation on Sepharose 6B Column.

The source of enzyme was S2, 1st method. The separation of forms from S2 and Seph E was carried out at days 0 and 5, respectively. The rechromatographies were done with aliquots from the active fractions, corresponding mainly to the S form.

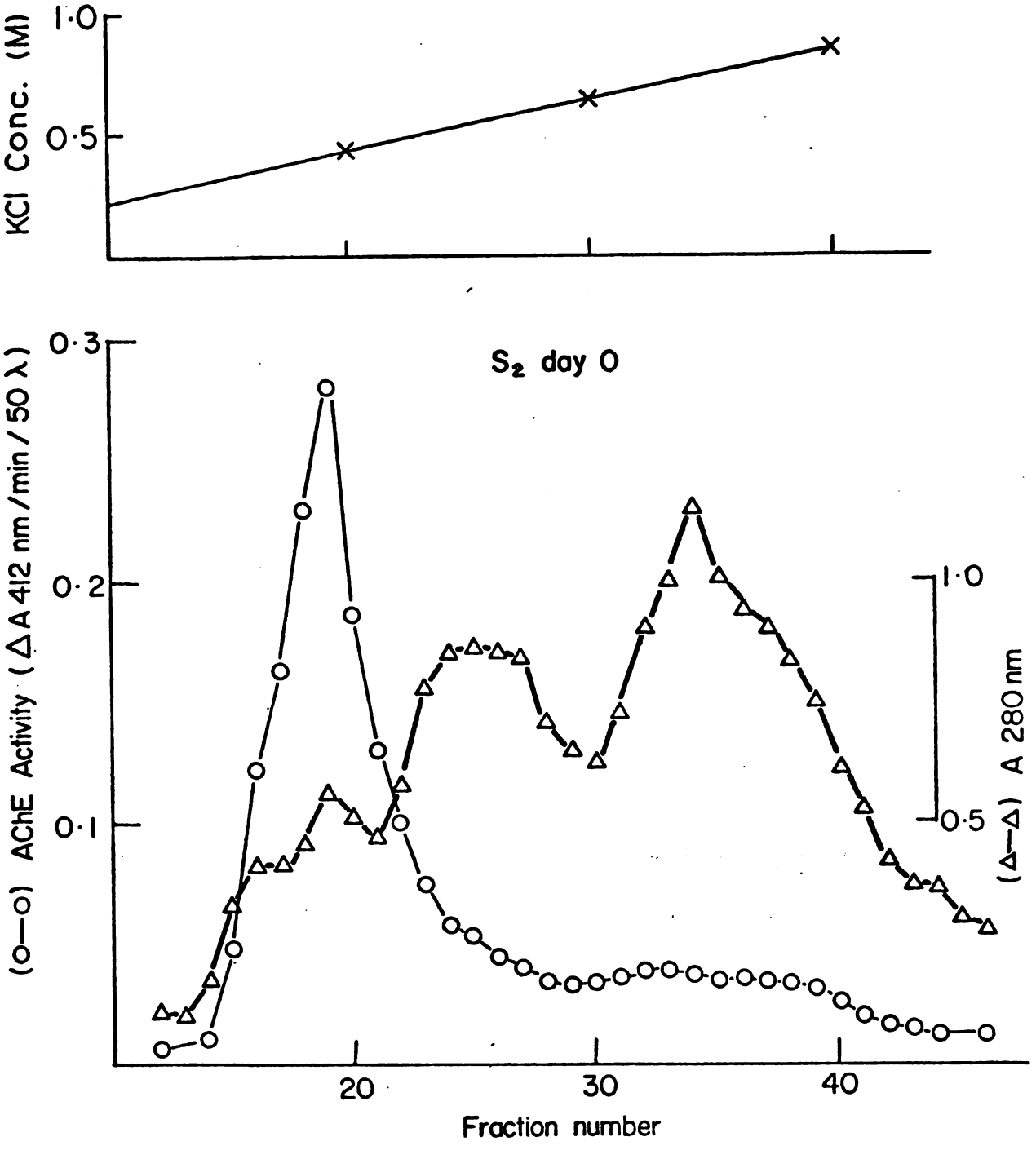

Figure 34. Chromatographic Separation of AChE Forms Present in S2, 1st Method, on DEAE Sephadex A-25 Column.

Elution was performed with a linear gradient (upper, ×—×) of KCl (0.0-2.0 M) containing Tris (0.03-0.2 M), at pH 8.0. Fractions of 2.8 ml were collected and assayed for AChE activity (o—o) and protein content (Δ—Δ).

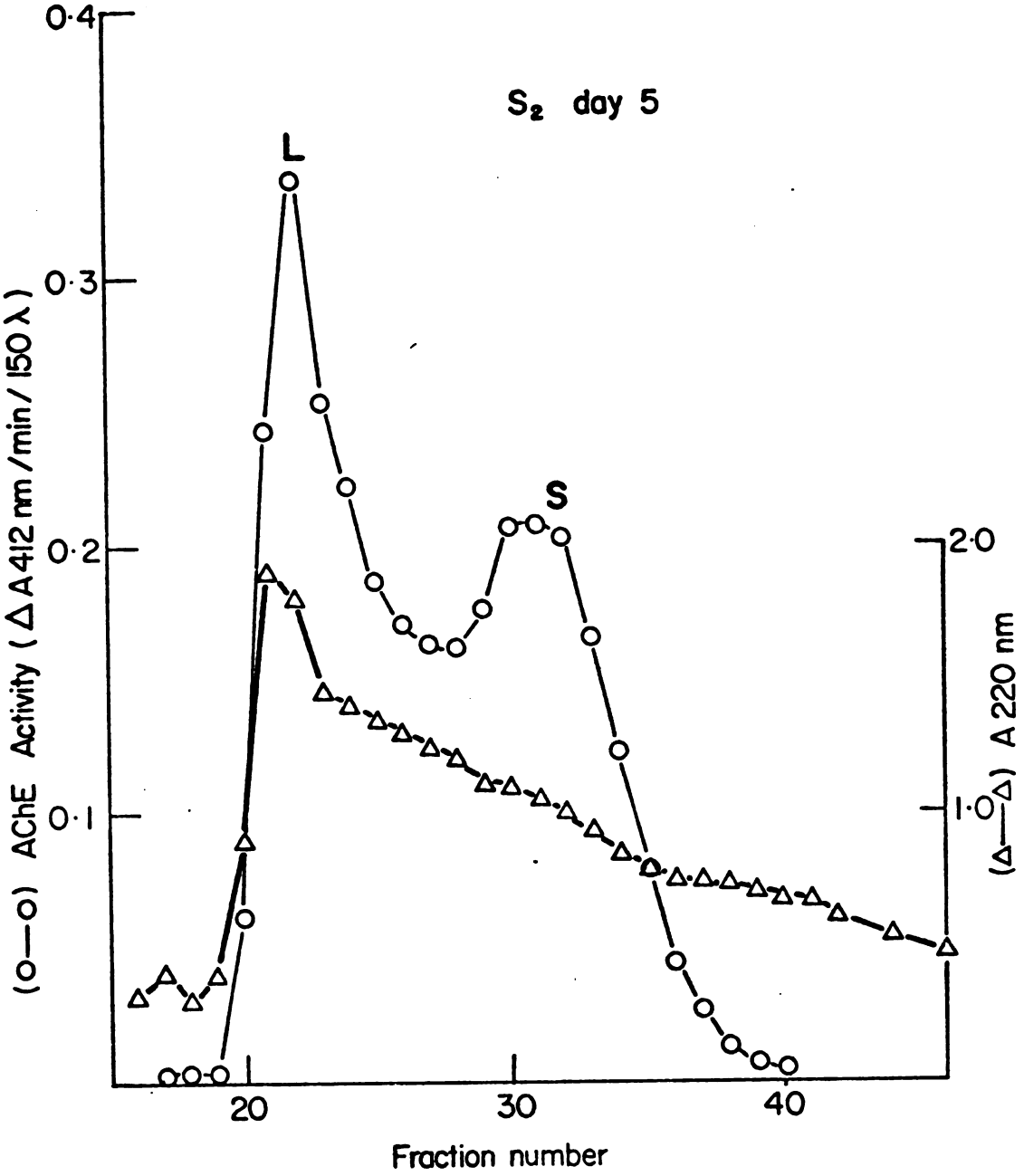

Figure 35. Chromatographic Separation of AChE Forms Present in S2, 1st Method, on Sephadex G-200 Column.

Active fractions from DEAE Sephadex A-25 chromatography (linear gradient of KCl: 0.25-1.0 M, performed at day 0) were pooled and used as enzyme source. AChE activity (o—o); protein content (Δ—Δ).

nearly as much enzyme (about 35% of the total enzyme activity) as the L form (Figure 35). Enzyme recovery was about 76%.

Reversion of AChE Form Aggregation

The purpose of the following experiments was to find whether or not the AChE aggregation process or form conversion could be reversed, by using the enzyme forms after gel filtration and studying the effect of storage on these isolated AChE forms.

1. Sepharose 6B preparations: After separation of enzyme forms present in S2 (1^{st} method), at day 0, on Sepharose 6B column, fractions were designated A, B, C, D and E, as shown in Figure 36. Fraction C represented enzyme mainly in the small molecular weight form (S), containing some intermediate form (I) as contaminant. Enzyme recovery ranged from 76 to 83% when the fraction was rechromatographed at day 1. However, it decreased with time of storage, and the recovery at day 7 ranged from 50 to 72%.

Figure 37 shows that addition of fraction A or B to the enzyme C did not change the enzymatic forms previously present in the mixture, at day 1. However, at days 4 and 5, the I form disappeared and there was an increase in the S form (Figure 38).

2. Sephadex G-200 preparations: At day 0, AChE forms in S2 (1^{st} method) were separated by conventional gel filtration procedures. Although the separation of fractions here was similar to the one indicated above, the composition of some of the fractions was different. For instance, fraction A now represented a combination of void volume peak and L form.

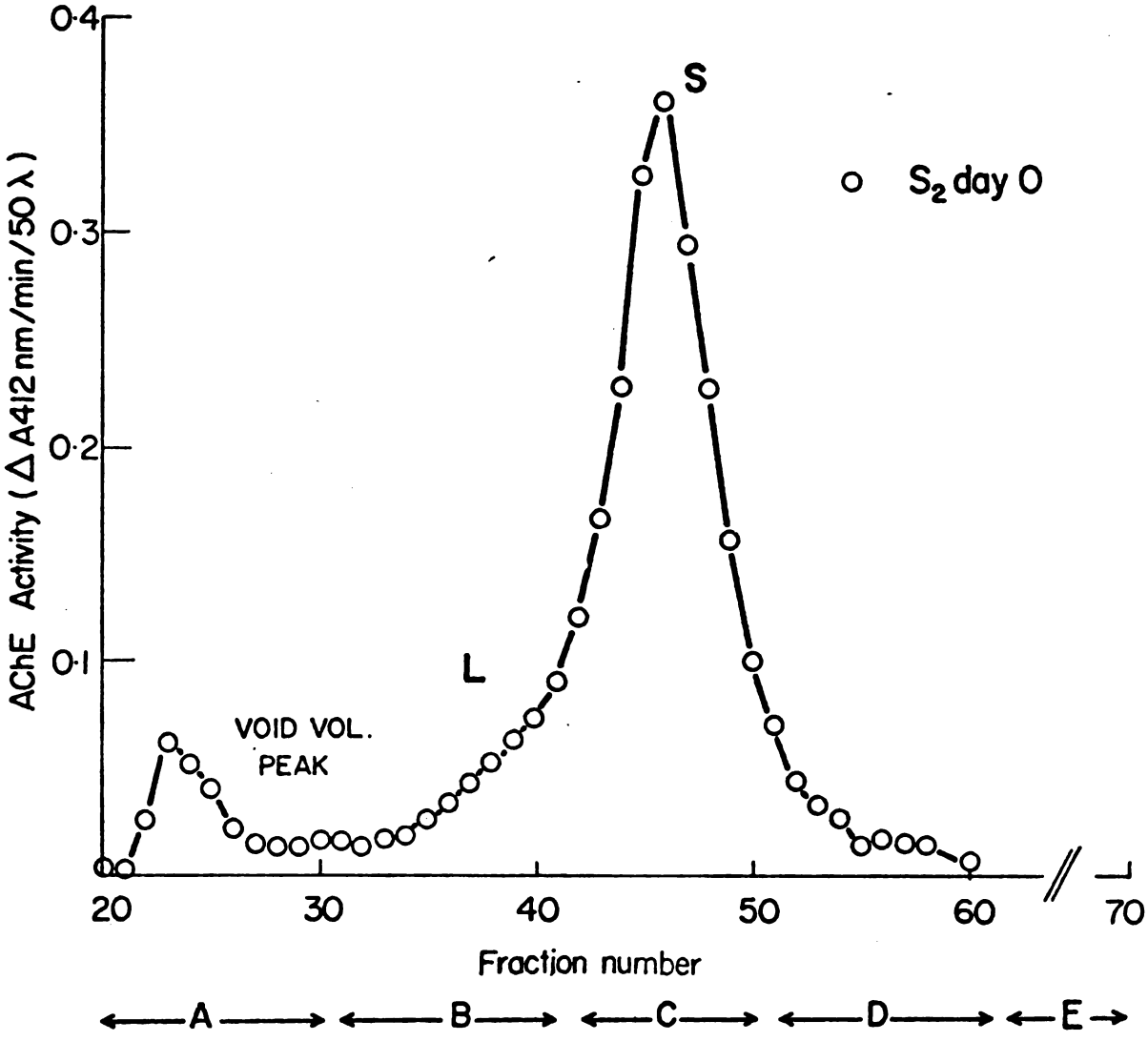

Figure 36. Chromatographic Separation of AChE Forms Present in S2, 1st Method, on Sepharose 6B Column.

Active fractions were divided into A, B, C, D, and E as specified in text, pooled together, and further used in the reversion experiments.

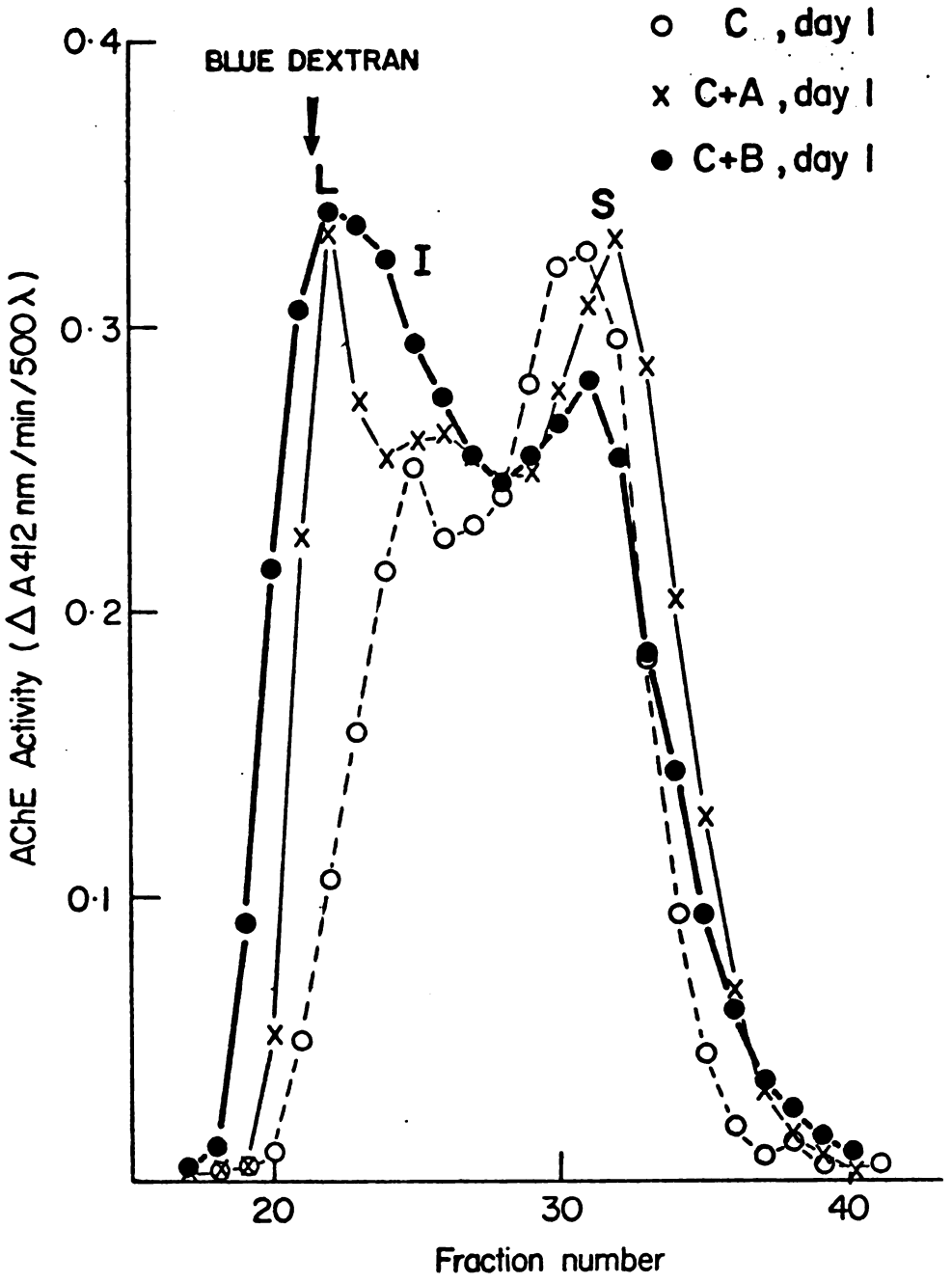

Figure 37. Sephadex G-200 Rechromatography of AChE Forms Present in Fractions C, C+A, and C+B, at Day 1.

The separation of forms was performed at day 0, on Sepharose 6B column. Fraction C (4 ml) was then chromatographed, alone or added to fraction A or B in a 1:2 ratio.

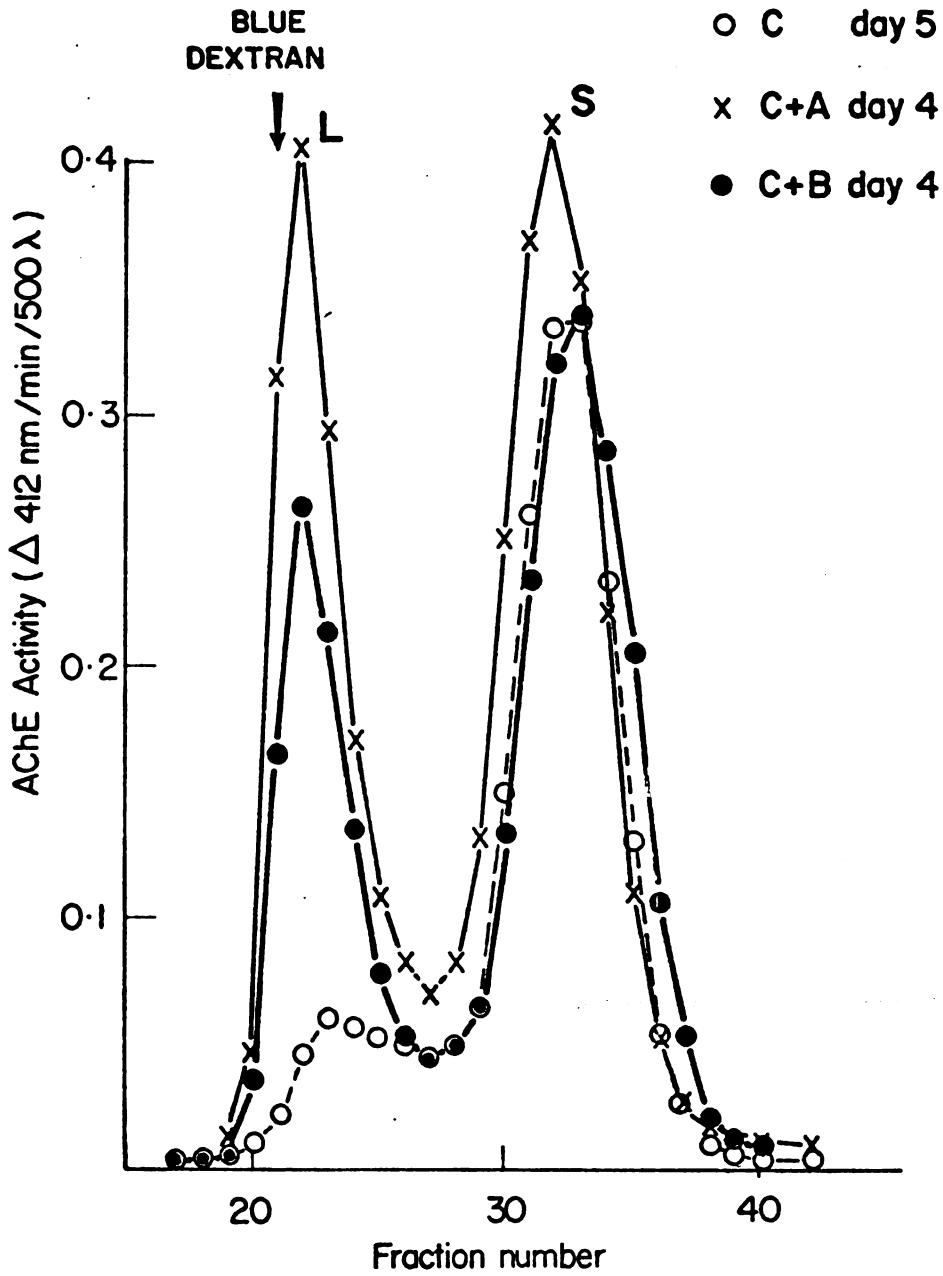

Figure 38. Sephadex G-200 Rechromatography of AChE Forms Present in Fractions C, C+A, and C+B, at Days 4 and 5, Showing Form Reversion.

Experimental conditions were those described in Figure 37.

Fractions were designated A, B and C, with major enzyme peak (S and I forms) present in fraction B. Enzyme recovery ranged from 71 to 84%, when the rechromatography was performed on the day of fraction isolation (day 1). It also decreased with time of storage, the recovery at day 15 ranging from 45 to 79%.

The results showed that fraction B, alone, initially started to decrease in S form content. At two weeks, S became bigger than originally and the I form disappeared almost completely. On the other hand, fraction A alone, at day 16, showed a predominance of L, no visible sign of I, and some S form (Figure 39).

From days 1 to 7, fractions B plus A and B plus C showed also a small increase in S, with consequent decrease in the L and I forms (Figure 40). Fraction B (plus A and C) showed evidence for conversion of the I form to the S form, with time (Figure 41).

3. Reversion of I to S form: While the previous reversion experiments were carried out with fresh enzyme preparation (where the S form is the main component), and the separation of the forms was done at day 0, the present one was performed with old enzyme preparation. The purpose of this experiment was, then, directed towards the deaggregation of AChE forms on an aged enzyme preparation, where I is the predominant form.

The experiment was done as follows: at day 6, 25 ml of S2, 1^{st} method, (AChE: 2180 units; protein: 37 mg) were loaded, after concentration, on a Sephadex G-200 column. Enzyme recovery was 77%. The initial fractions (discarded) were a combination of L and I forms, representing 10% of the total enzyme activity. The active fractions, corresponding mostly to the I form and containing 81% of the total enzyme activity

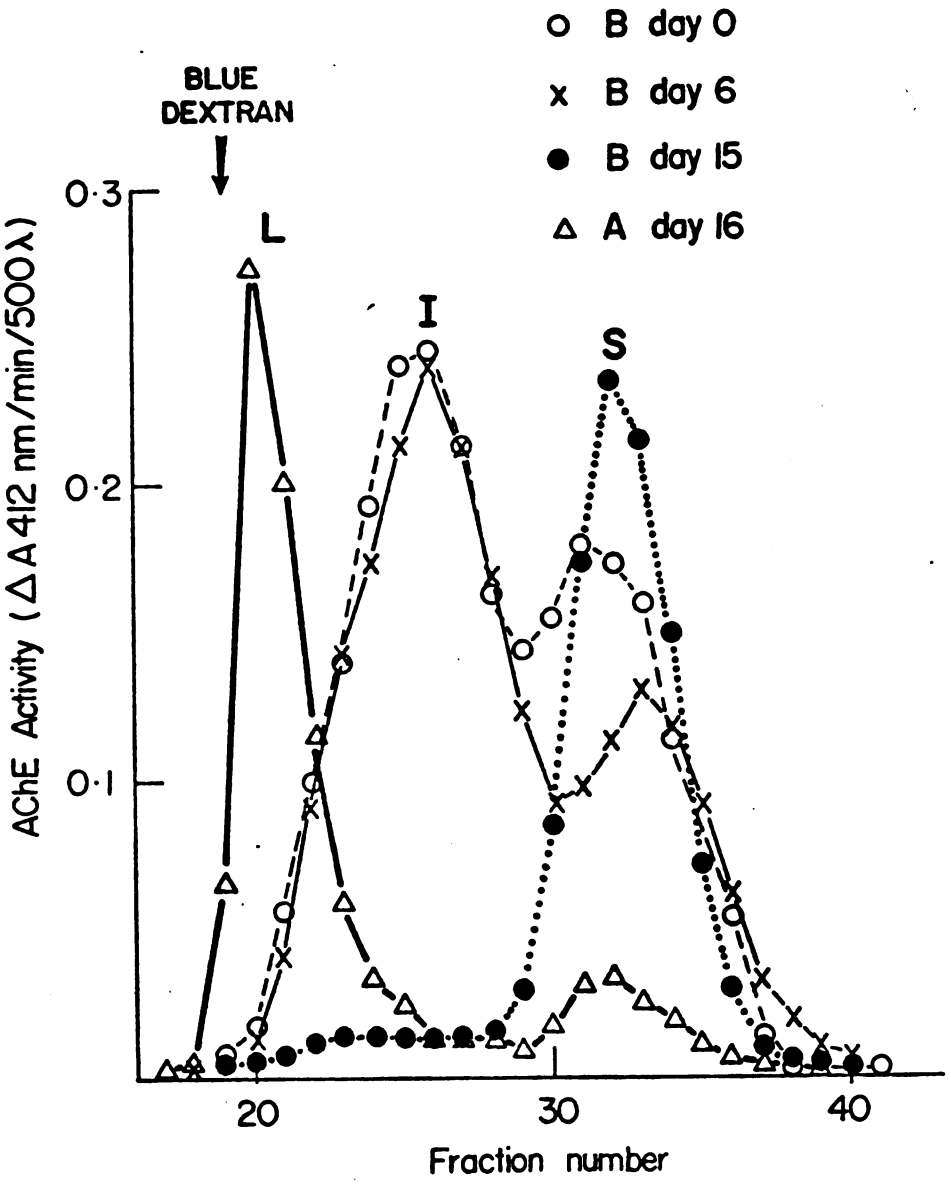

Figure 39. Reversion of AChE Forms: Sephadex G-200 Rechromatography of AChE Forms Present in Fractions B and A, at Different Times.

The separation of forms (from S2, 1st method) was performed at day 0, on Sephadex G-200 column. Active fractions were designated A, B, and C.

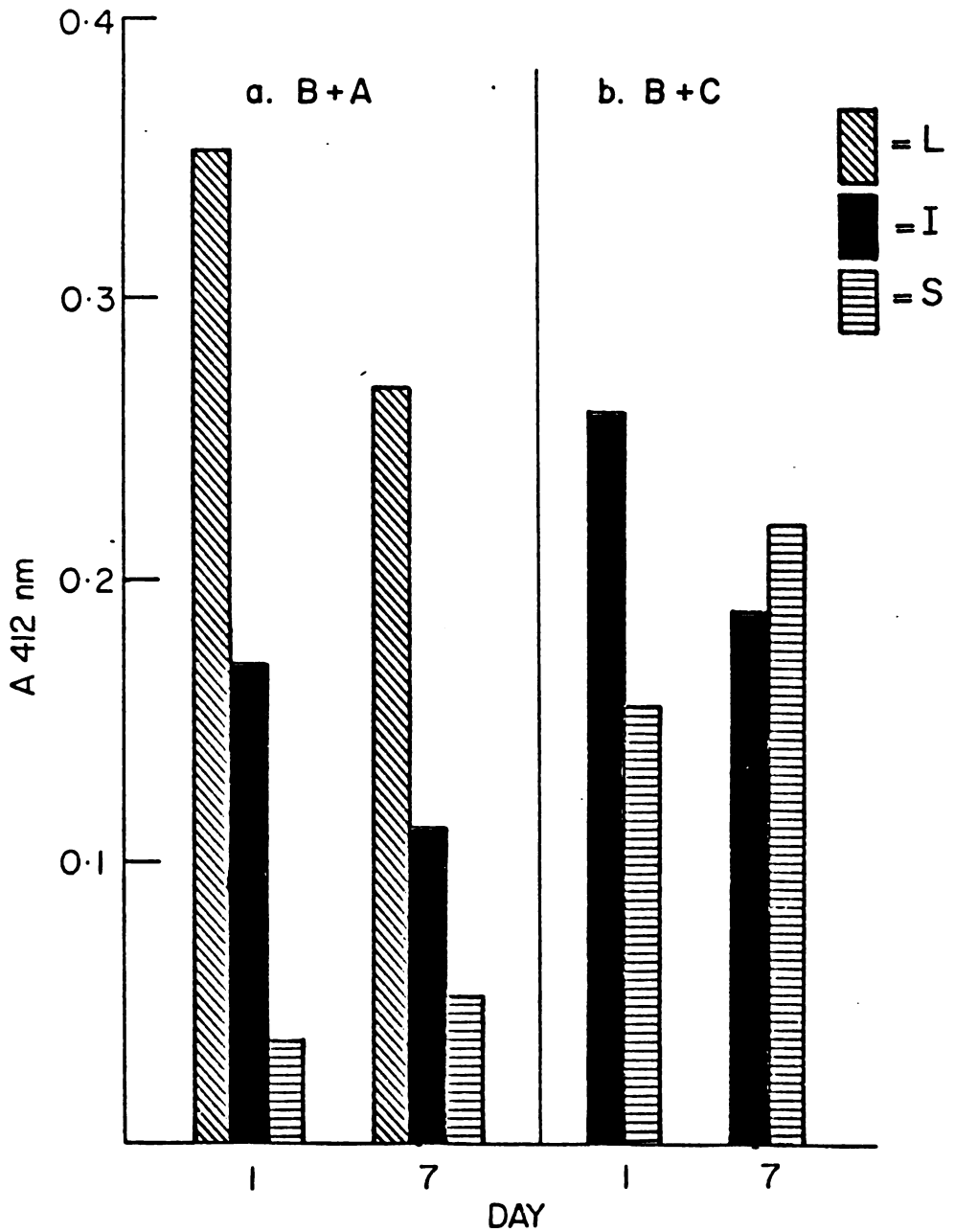

Figure 40. Reversion of AChE Forms: Sephadex G-200 Rechromatography Pattern (Peak Heights) of AChE Forms Present in Fractions B+A and B+C, at Days 1 and 7.

Fraction B (3.7 ml) was added to fractions A or C in a 1:2 ratio. Aliquots for enzyme assay: a = 250 λ; b = 500 λ. Experimental conditions were those described in Figure 39.

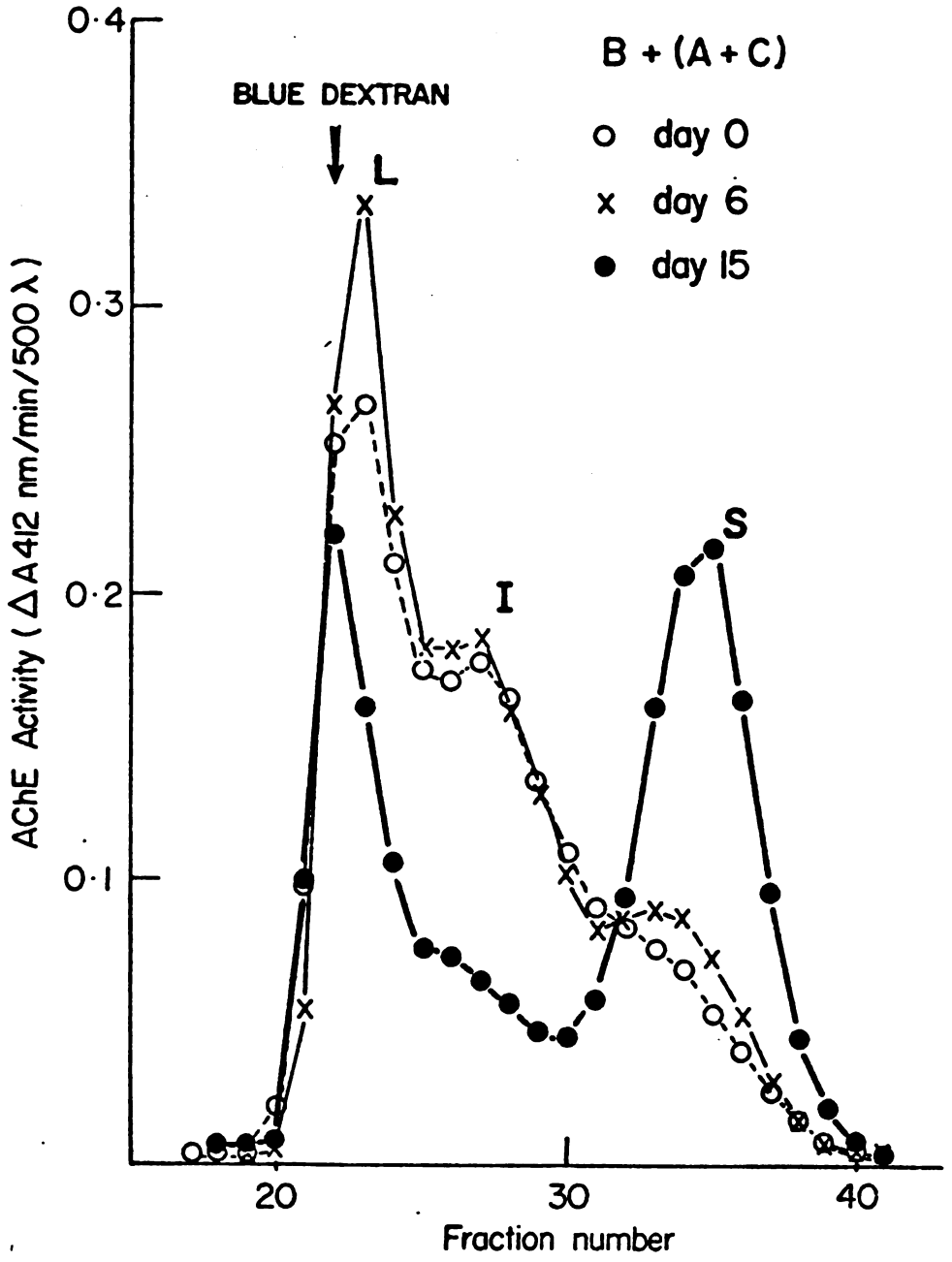

Figure 41. Reversion of AChE Forms: Sephadex G-200 Rechromatography of AChE Forms Present in Fractions B+(A+C), at Different Times. Fraction B (3.7 ml) was added to fractions A and C in a 1:2 ratio. Experimental conditions were those described in Figure 39.

recovered were pooled together, as were the fractions that would correspond to the S form (almost absent). This form represented only about 9% of the total enzyme activity. With time, the I form was progressively reconverted to S. This reversion was almost complete when the experiment was done with a combination of I and "S" forms (Figure 42).

Neuraminidase Treatment

The action of neuraminidase (or sialidase) is defined as the hydrolytic cleavage of the glycosidic bond joining the keto group of neuraminic acid to D-galactose or D-galactosamine and, possibly, to other sugars. It catalyses the release of N-acetylneuraminic acid (NANA or sialic acid) residues from glycoproteins and glycolipids. If glycoprotein residues are involved with AChE aggregation, it is possible that neuraminidase treatment could act as a means of form stabilization.

The first type of experiment was done with S2 (1^{st} method) at day 0 and pH 6.0 previously adjusted. Neuraminidase was added then to S2, and the mixture was incubated overnight at room temperature. The effect of neuraminidase in the experimental was not noticeable at pH 6.0, as compared to the control. The aggregation process was very slow at this pH (not shown).

Overnight incubation at pH 6.0, but readjusted to pH 8.0 afterwards, indicated some neuraminidase effect. Under these conditions the treatment of S2 with neuraminidase slowed AChE aggregation. The picture of the experimental (Figure 43), at day 4, was very similar to that obtained at day 1, while under the control conditions the enzymatic pattern tended to change, with time, toward the L form.

Perhaps the effect of neuraminidase would be better demonstrated at a pH closer to 5.0 (and readjusted to pH 8.0 afterwards), which is the

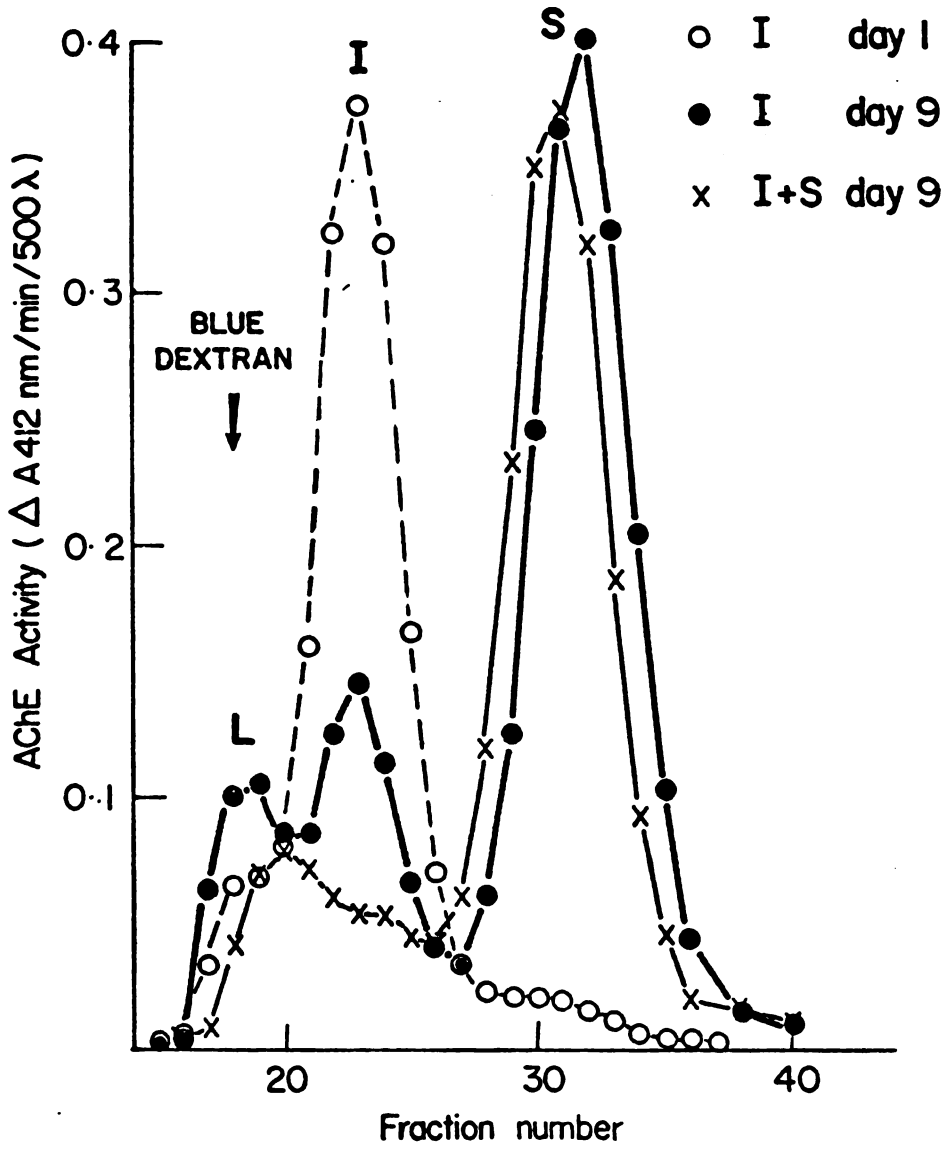

Figure 42. Reversion of AChE Forms: Sephadex G-200 Rechromatography of AChE Forms L, I and S, at Days 1 and 9.

The separation of forms (from S2, 1st method) was performed at day 6, on Sephadex G-200. Active fractions corresponded mainly to the I form, with L form as contaminant. I (4 ml) was then chromatographed as such or added to the S form (almost absent) in a 1:2 ratio.

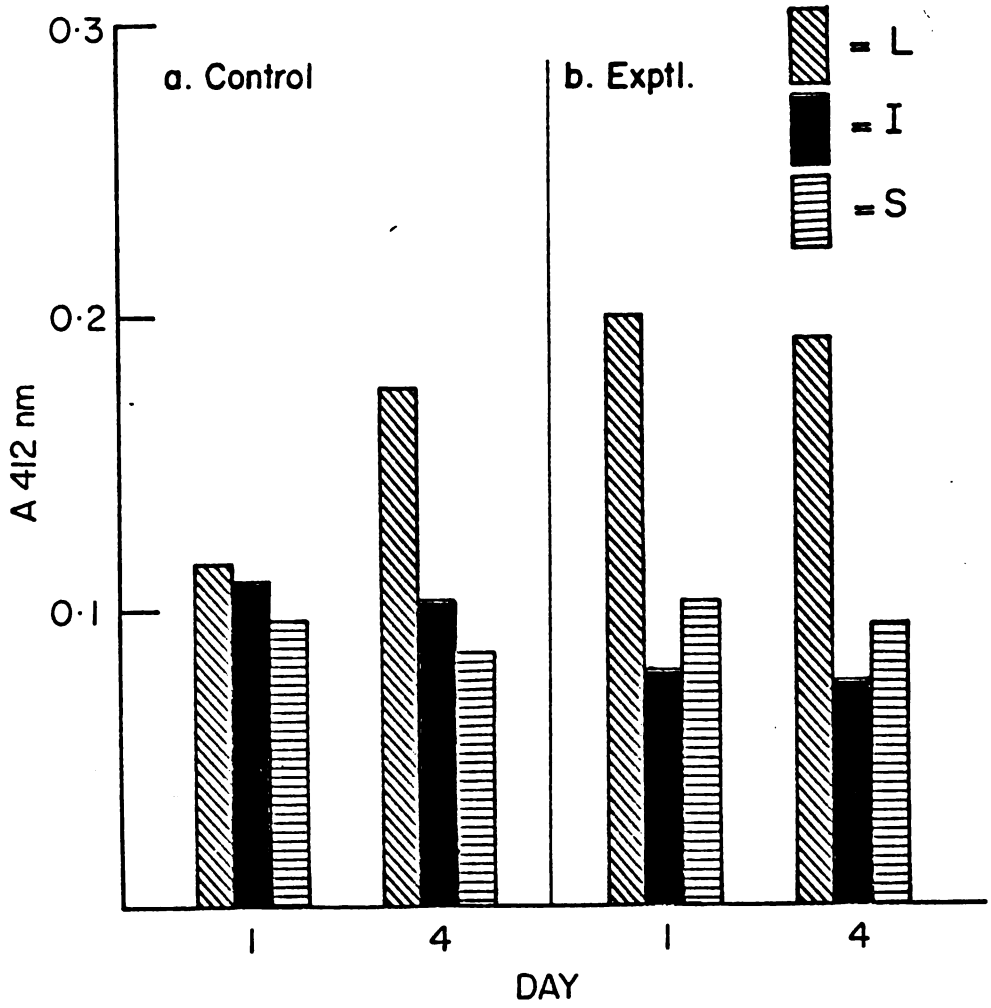

Figure 43. Effect of Neuraminidase Treatment on AChE Forms at Different Times.

Sephadex G-200 chromatography pattern of AChE forms (peak heights) in S2, 1st method, in the absence (control) and presence (experimental) of 0.13 unit of neuraminidase. Aliquots for chromatography: 5 ml (AChE = 421 units; protein = 7 mg). Aliquots for enzyme assay: 125 λ.

optimum pH for enzyme activity. However, in order to avoid AChE precipitation, it is not advisable to go below pH 6.0.

Concanavalin A (Con A) Experiments

Con A is a lectin, from jack beans, used as a tool to detect the presence of carbohydrate residues in biopolymers. The interaction between Con A and the carbohydrate moieties, which appear to be a constituent of the AChE macromolecule, was studied here. The enzyme forms from S2 (1^{st} method) were used after their separation on Sephadex G-200 and Sepharose 6B.

The results showed that Con A formed a sedimentable complex with the AChE forms. Enzyme assays on the supernatants showed a decreased enzyme activity in all AChE forms in the experimentals, as compared to the controls (Table 14). The decrease was greater in the void volume peak (separated on Sepharose 6B), and became progressively smaller towards the I form. The decrease of enzyme activity in L was also great, as compared to that in the I or S forms.

The Con A effect was at least partially reversed by α-methyl-D-mannoside, in all situations tested. α-methyl-D-mannoside had no effect per se on AChE activity. Figure 44 shows the effect of increasing concentrations of Con A on the L form, separated on Sepharose 6B, and the reversibility of its effect in the presence of α-methyl-D-mannoside (10 mM), added after enzyme incubation with Con A.

TABLE 14. INTERACTION BETWEEN MULTIPLE AChE FORMS AND CONCANAVALIN A (CON A)

AChE Form	% of AChE activity in the presence of Con A (μM)		
	0.2	0.4	1.0
Void Vol. Peak (4)	54.1 ± 2.3	38.1 ± 1.9	22.2 ± 1.5
L (2)	70.0 ± 2.4	65.5 ± 1.4	56.2 ± 1.0
I (3)	97.2 ± 1.3	85.5 ± 2.0	83.0 ± 2.9
S (5)	71.1 ± 2.4	66.1 ± 1.3	71.8 ± 1.5

AChE forms from supernatant fraction 2, 1st method [Chan et al. (24), modified], were obtained after gel filtration on Sepharose 6B (void vol. peak and L) or Sephadex G-200 (I and S). All forms (with the exception of I form, which was separated at day 6) were separated at day 0. Number of experiments is indicated in parentheses. Values are means ± S.E.M.

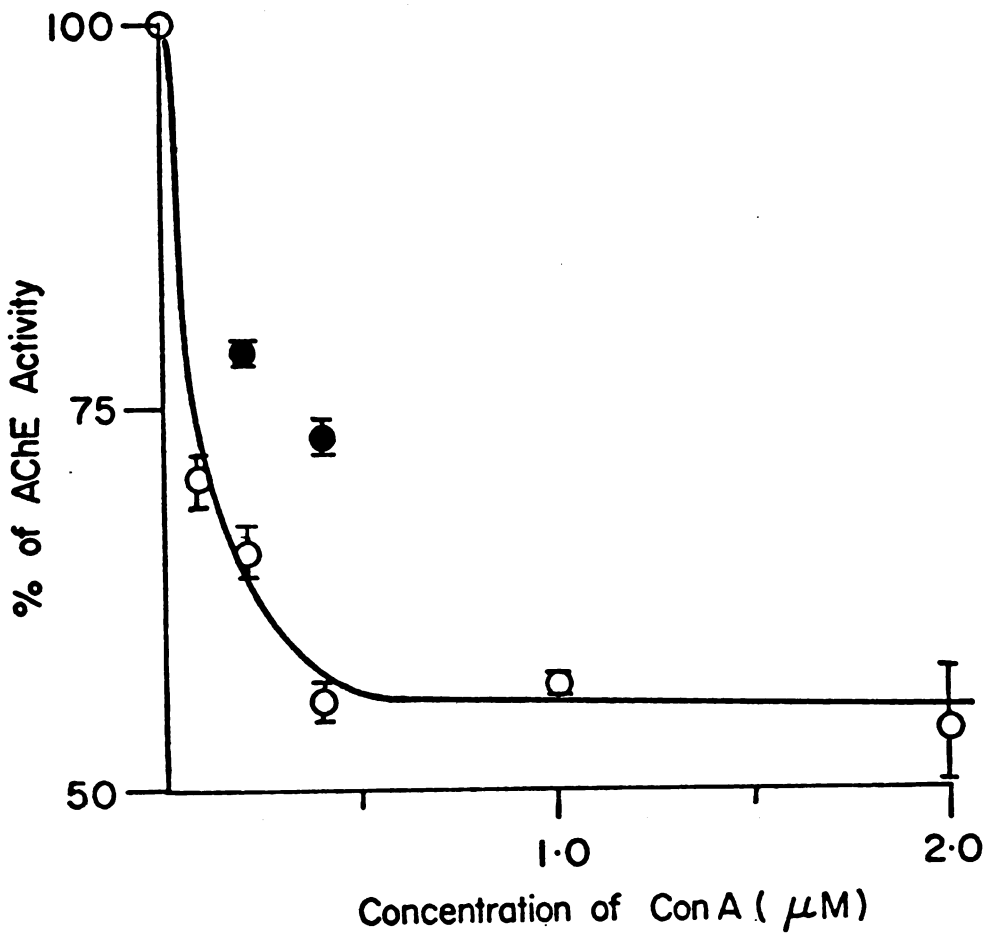

Figure 44. Titration of AChE Form L from S2, 1st Method, with Con A.

●: means extent of aggregation when 10 mM of α-methyl-D-mannoside was added to the AChE form previously incubated with Con A. Points are means ± S.E.M. of 2 experiments.

DISCUSSION

The following subjects will be discussed in this section:

1. The comparative study of brain AChE obtained by two different methods of solubilization;
2. The effect of storage time on AChE forms (aggregation) and the influence of pH and ionic strength on this process;
3. The determination of factor(s) which could alter the enzyme aggregation such as DEAE Sephadex A-25 treatment, and form separation by gel filtration techniques;
4. The study of the presence of carbohydrate moieties in the brain AChE preparation by means of neuraminidase and Con A treatments.

Solubilization of Brain AChE

Studies on the regional distribution of AChE activity in the brain have shown that the caudate nucleus tissue contains very high concentrations of enzyme, the bulk of which can be shown to be bound to membrane components. This fact makes the tissue a good source for studying AChE in the CNS. Compared to studies carried out on eel enzyme, however, the problem of releasing brain AChE from membrane components has been more troublesome as detailed in the introduction of this thesis.

Recently, somewhat milder extraction procedures have been used to attempt the solubilization of AChE from membrane components of brain tissue, by means of a chelating agent such as EDTA (25,75). With these methods, the amount of soluble enzyme obtained was approximately 70 and 90%, respectively, of the original homogenate. These procedures can be anticipated to expose the membrane components to milder conditions, as compared to those of enzyme treatment, solvent extraction or detergents.

Thus one would expect to minimize any physico-chemical changes of the enzyme macromolecule, which could alter its characteristics and/or properties (128).

In the present study, it was hoped that the use of EDTA as an agent for AChE solubilization would decrease the possibility of attaining artifactual forms of the enzyme, that could lead to misleading interpretations of those forms actually occurring *in vivo*. Although the mechanism of effect of EDTA is not clear it may be related to chelation of divalent ions such as Ca^{2+} or Mg^{2+} involved with the binding of the enzyme to membrane components. In addition, by using a compound known to compete with calcium at the membrane level such as tetracaine, the chelating effect of EDTA on the AChE release would also be increased, as had been already demonstrated (75). Finally, it was expected that by using two different methods of enzyme extraction a better comparison of the number of AChE isoenzymic forms, their molecular weights, and aggregation behavior would be achieved.

For these reasons, in the present study the modified procedures of Chan *et al.* (24), 1st method, and that of Hollunger and Niklasson (75), 2nd method, were used. Under the present experimental conditions, the percentage of AChE solubilization in S2 was relatively low, being 15 to 18% with the 1st method and 25% with the 2nd one (Tables 3 and 4). This fact was probably due to the short time of enzyme extraction (1 hour), and also to the low temperature of extraction chosen (4°C) to avoid enzyme denaturation. The yield and specific activity of AChE in S2 from fresh caudate nucleus tissue were always greater following the 2nd method of solubilization.

This may be due in part to the combined effects of EDTA and tetracaine and to pH differences. Previous investigations (75) have shown that in the presence of either EDTA or tetracaine 45 and 50%, respectively, of AChE

activity were released. The release was increased to 60% by the addition of the two compounds together.

Local anesthetics including tetracaine seem to block conduction in nerve, by competing with calcium at some receptor site that controls the membrane permeability (14). Blaustein and Goldman (14) showed, for several local anesthetics, a fairly good correlation between their effects as inhibitors of nerve conduction and their actions on calcium-binding to phosphatidylserine. However, in preliminary experiments, Hollunger and Niklasson (75) were not able to find any stimulation of AChE release caused by the local anesthetic lidocaine, one of the drugs examined by them.

In view of the fact that EDTA appears to solubilize the enzyme via Ca^{2+} chelation (75) it seems reasonable to assume that the local anesthetic functions also by some interaction with this cation. The mechanism of action of tetracaine on membranes to release AChE is not well understood. It is possible that the displacement of calcium from its binding to phospholipids and/or proteins (50) by perturbation of the membrane structure might reduce the binding forces of the enzyme to the membrane.

The percentage of more easily solubilized AChE, which could also represent soluble or cytoplasmic enzyme, ranges from 10 to 14% in the present study, which agrees with previous investigations (25,75). However, the results on the solubilization of the membrane-bound enzyme (Tables 3 and 4) are not strictly comparable to those observed by Chan et al. (25) or Hollunger and Niklasson (75), due to different experimental conditions. Chan et al. obtained a 35% AChE activity in S2, but their extraction was carried out overnight at 4°C. On the other hand, Hollunger and Niklasson obtained a 60% solubilization after two hours extraction at room temperature.

Multiple Forms of AChE

In the present study, two forms of brain AChE appeared to exist in a fresh extract, at the day of enzyme preparation (day 0): the larger, L, and the smaller, S, molecular weight forms (Figures 3 and 4). Under such conditions, the presence of a predominant amount of S over the L form was a characteristic of all supernatant fractions, except for S1 obtained with the 1st method, where there was a predominance of the L form, instead (Figure 17). With aging, an intermediate form, I, appeared in supernatant fractions 1 and 2, from both methods, that seemed to result from an aggregation of the S form which diminished gradually with time.

It is of interest that the preparation of S1 by the 1st method involves less likelihood of contamination by membrane-bound enzyme. This is because it involves exposure only to sucrose and not to chelating agent or tetracaine. The implication is that cytoplasmic AChE may exist mainly in the L form.

The existence of more than one active form of brain AChE from several species has been suggested from purification studies, which employed protease solubilization (74) and butanol extraction (81). It has been reported that AChE from human brain consists of three isoenzymes (12,13, 116). Bajgar and Zizkovisky (5) found at least two different AChE isoenzymes in the rat brain, which differ in physicochemical and enzymatic properties. More recently, Bellanger et al. (8) detected as many as five isoenzymes of AChE in soluble extracts of adult brain of baboons, after electrophoretic separation. The concentration of certain of these isozymic forms decreases in the newborn brain, and one of them was absent in the brain of a five-month old fetus.

McIntosh and Plummer (108), by using a 6% (w/v) gel and continuous buffer system, observed two forms of AChE in pig brain after solubilization of the enzyme in Triton X-100. Further, by changing the experimental conditions, between two and six forms were detected depending on the method used for extraction. The average molecular weight of the five forms most frequently found were: 60,000; 130,000; 198,000; 266,000 and 350,000.

Using milder procedures, Chan et al. (25) found multiple forms of the enzyme in bovine brain. The molecular weights of the three peaks were estimated as 390,000; 270,000 and 130,000, respectively. According to these investigators, these forms of brain AChE represent dimers, tetramers and hexamers, with the smaller molecular weight forms predominating.

Several forms of the enzyme were also found by Hollunger and Niklasson (75). By their method, they were able to obtain a form with an estimated molecular weight of 80,000 which is the lowest value hitherto reported for the brain enzyme. They also suggested that the form of the enzyme with molecular weight of 80,000 was the monomeric form of the mammalian brain enzyme. The molecular weights of about 250,000 and 510,000, according to them, suggested that these aggregates were composed of respectively three and six monomers of 80,000 molecular weight.

Very recently, Wenthold et al. (143) studied the properties of purified AChE from rat brain, after affinity chromatography. The enzyme had a specific activity of 4.5 mmoles of ATC hydrolysed per milligram of protein per hour. Gel filtration yielded oligomers with molecular weights of 320,000; 500,000 and 650,000 with 60% of the activity in a 150,000 molecular weight fraction. In the present investigation the molecular weight values of the brain AChE forms were: 110,000; 214,000 and 340,000, as determined by gel filtration experiments (Table 12). These values are slightly smaller than

those reported by Chan et al. (25), but are probably more reliable since they were conducted on various types of columns. The discrepancy among the molecular weight figures estimated for the brain AChE isoenzymes may well be explained by differences in the isolation procedures which could lead to artifacts and aggregated forms of the enzyme. Alternatively, differences in form may relate to the animal species used.

In this study besides the three main peaks, an aggregated complex was also found (molecular weight greater than 4 million), which was only separated from the larger component, L, on Sepharose 6B column. This form probably contributed to the increase of the molecular weight of the L form seen on Sephadex G-150 and Sephadex G-200 chromatography. A similar void volume peak has also been detected for erythrocyte AChE after Sepharose 6B gel filtration, at low ionic strength. This large form was converted to intermediate species in the presence of low $CaCl_2$ concentrations (123). However, under the present experimental conditions, in the absence of calcium, no conversion of the void volume peak to smaller AChE forms with time was detected.

The molecular weight of the S form obtained by the 2^{nd} method, as determined by Sephadex G-200 (Table 12), was smaller than and statistically different from that of the S form, 1^{st} method (Table 13). So far, it is difficult to establish unequivocally what factor is responsible for this difference. Since the 2^{nd} method involved exposure to tetracaine it is possible that this compound has an action resulting in some change in molecular size or shape. Alternatively, the particulate fraction used as starting material for enzyme extraction in the 2^{nd} method was more impure and contaminated with nuclear components among others. Since proteolytic enzymes are present in such fraction these substances could affect the

molecular weight of AChE forms, by breaking peptide linkages on the enzyme molecule. If this occurs, however, it is detectable only in terms of the change in molecular weight of the S form.

In the enzyme preparation from fresh tissue, the higher molecular weight component, L, appeared to be most evident in supernatant fraction 1 (S1 = soluble enzyme), whereas the smaller enzymatic form, S, was predominant in supernatant fraction 2 (S2 = membrane-bound enzyme). These observations are based on data obtained by chromatography of the supernatant fractions at day 0 (Figures 3 and 17). The literature reports investigations suggesting that soluble and insoluble AChE may originate from different biological structures. Thus, Tennyson et al. (135), in an electron microscopic-cytochemical and biochemical study, demonstrated AChE activity in the myotube and in extrajunctional skeletal muscle of the rabbit fetus. The insoluble AChE, according to them, probably corresponded to the cytochemical end product which is bound to the elements of the reticulum and some elements of the Golgi complex. The soluble AChE appeared to be derived from the AChE-containing mononuclear cell and the Schwann cell which accompanies the spinal nerve to the motor end plate.

Although studies on electroplax of Electrophorus indicated that the bulk of the AChE activity was associated with an insoluble fraction (83), soluble AChE has been reported as well (25,35,75,88). Very recent investigations showed the presence of soluble and membrane-bound AChE in rat brain (143) with some difference in properties (e.g., isoelectric points). Since the enzyme came from different origins it perhaps undergoes a conformational change when becoming attached to the membrane, or carries membrane components in its molecule when in soluble state, as has been suggested by Wenthold et al. (143).

It must be realized that S1 completely freed of contamination with insoluble or membrane-bound enzyme is not easy to obtain, and even homogenization in isotonic sucrose solution may contribute, to a certain degree, to enzyme solubilization. In the present investigation, it has been shown that homogenization of caudate nucleus tissue in isotonic sucrose solution containing 0.25 M and 0.5 M KCl decreased the proportion of the S form as compared to the L form (Figure 24). It is tempting to suggest that the S form is the predominant AChE active form in the membrane-bound enzyme. Evidence for this is based on the following results obtained with fresh tissue: (a) the S form is the predominant form present at day 0 following solubilization; (b) there is mainly L form under conditions of low membrane solubilization (e.g., S1); (c) S converts to the L form with time when in solution.

The proportion of enzyme forms in soluble or insoluble AChE may be different according to the type of tissue, its storage condition (fresh or frozen), and the species considered. The role performed by the two types of AChE isoenzymes is not fully understood. It would be worthwhile to study the AChE forms in vivo, from an immunological approach, and to test which form would be the most reactive under certain conditions. Antibodies to AChE forms have been already produced with purified enzyme from electric eel (10,119,139).

In this investigation, it was observed that the intermediate form I was nearly absent in the fresh extract. It appeared to arise, perhaps, from the isolation procedures as an artifact and with aging of the brain enzyme preparation. It is suggested that the I form does not exist as such in the biological system. On the other hand, S and L may represent the AChE

forms occurring *in vivo* since they are the ones present at day 0. Form I appears to develop at the expense of the S form, as a consequence of an aggregation process.

Aggregation Phenomenon on AChE Macromolecules

Aggregation phenomenon has been observed not only with cholinesterases from several sources, but also with other enzymes such as placental alkaline phosphatase and *E. coli* beta-galactosidase (57). Studies by LaMotta *et al.* (91,92) showed an interconversion of the molecular forms of serum cholinesterase. A stepwise process of aggregation or polymerization was proposed as the most likely interpretation of the relationship between the multiple molecular components. They suggested that the various components of serum cholinesterase, regardless of their molecular form, i.e., aggregates or conformers, arise from a single enzymatically active polypeptide unit.

In 1965, Grafius and Millar (61) investigated the sedimentation coefficients of AChE from *Electrophorus electricus*, using the sucrose gradient centrifugation technique. Three major components could be observed, depending upon the ionic strength of the medium. The rapidly sedimenting component appeared only after dialysis against buffers of low ionic strength. Its formation was at the expense of the two slower components and was reversed by raising the ionic strength of the medium. Subsequently, Grafius *et al.* (64) studied the effect of several hydrolytic enzymes on the fast AChE component. It was found that phospholipase C and D, and pancreatic lipase dissociated the fast to the slow AChE component. Their results indicated that the aggregated or fast AChE was a complex between "monomer" and

a matrix of lipoproteins, which in itself is an aggregation of molecular species similar in sedimentation value to the "monomer" AChE.

Dudai et al. (45) showed that AChE from the electric organ of electric eel was present in 1 M NaCl extracts of frozen or fresh tissue as three main components, which could be distinguished by their sedimentation coefficients (about 18, 14 and 8 S) on sucrose-gradient centrifugation at high ionic strength. The 18 S and 14 S components, which comprised the major part of the total AChE activity, aggregated at low ionic strength. The purified AChE, obtained from toluene-treated electric organ tissue, had a sedimentation coefficient of about 11 S and did not aggregate at low ionic strength.

Aggregation was also observed in the molecular species of AChE from Torpedo marmorata electric organ (102). Two species, rapidly migrating in a sucrose gradient, showed the phenomenon of aggregation in low salt concentration (0.01 M), whereas a slowly sedimenting species did not. The buoyant density of the active species in their extract was the same as that of the purified enzyme; a high content of lipids was therefore excluded.

Such aggregation phenomenon, so far, has been well studied in AChE forms from E. electricus, but the process in the mammalian brain enzyme had not been explored until recently. Hollunger and Niklasson (75) have shown that the aggregation behavior of the brain enzyme differs from that of the electric eel enzyme. The rate of aggregation can be reduced at high ionic strength but, unlike the eel enzyme, the process is irreversible. They suggested that probably a hydrophobic binding was preceeded by an ionic interaction. With in vitro aging, their enzyme preparation changed its chromatographic pattern. The activity in the peaks representing the larger forms of the enzyme increased at the expense of the activity in those representing the smaller forms. However, their study did not show a

build-up of the I form with time as was observed here. Their chromatographic technique (e.g., type of column used) was probably not suitable for a good separation of the enzymatic forms. As they noted, in some chromatographic separations there was an indication of the presence of a molecular weight form between that of 80,000 and that of the enzyme at the exclusion border. This intermediate form would correspond, here, to the I form.

In the present research, the observed aggregation of brain AChE forms appeared to be dependent, to some extent, on the pH of the medium. The process was slowed by decreasing the pH in the range of pH 6.0 to 7.0, and above pH 7.0 the aggregation was accelerated. At the lowest pH examined (6.0), the enzyme was very stable and little build-up of I was observed (Figures 7, 8 and 9). Effects of pH are assumed to be due to changes in the state of ionization of the system. Since enzymes are proteins containing many ionizable groups, they exist in a whole series of different states of ionization. The distribution of the total enzyme among the various forms will depend on the pH and on the ionization constants of the different groups present on the enzyme macromolecule (44).

Perhaps, at lower pH there is less interaction among groups on the outside of the AChE macromolecules, which are then ionized. Studies by Chan et al. (25) have shown that the isoelectric points of AChE forms range from 4.7 to 5.1. An alternative explanation of this pH effect concerning the action of neuraminidase is given later.

The results on the linear gradient of KCl (0 to 2 M) on DEAE-Sephadex A-25 column presented some indication of difference in charges among AChE forms (Figure 34). These observations agree in part with those of Chan et al. (24). They also found two peaks of AChE activity after a linear gradient of NaCl (0.03 to 0.45 M) on DEAE cellulose column. They suggested that the two major AChE species might have different charges. More recently,

the same group of investigators (25) has shown four active forms of AChE that were separable by isoelectric focusing of sucrose-EDTA extracts of brain tissue, a procedure which depends mainly on net ionic charge rather than on molecular volume or shape. The present results, however, are not absolutely comparable with theirs. They used a different range of NaCl concentration and DEAE cellulose as ion exchanger. But, most importantly, fresh enzyme preparation at day 0 presented, here, a predominance of S, almost no I, and little content of the L form. However, in aged preparations, the L and I forms were predominant, which might well be their case. Finally, it is always possible that, because of differences in experimental conditions, the major peak of AChE activity detected by them may not correspond to the major enzymatic peak observed in this investigation.

The treatment of the enzyme preparation with high salt concentrations protected the AChE forms against aggregation. Under these conditions there was a decreased stability of the I form, which did not build up in the usual manner. The effect was concentration dependent and best demonstrated at lower pH, such as pH 6.5 (Figure 13). At a given pH (e.g., pH 7.0) the salt effect was more pronounced in S2 obtained by the 2^{nd} method than by the 1^{st} method (Figures 14 and 15). On the other hand, the soluble enzyme appeared to be more sensitive to the effect of ionic strength than did the membrane-bound enzyme. These results are similar to those of Crone (35) on the effects of inorganic salts on the activity of mammalian AChE.

Above pH 7.0 the salt effect was minimal. This finding was also observed by other investigators (75) who showed that the addition of 0.4 M KCl to the solubilized AChE, at pH 7.0, protected their enzyme preparation against form conversion, and this effect did not appear at pH 8.0. However, even at this high concentration of KCl and pH 7.0, their form protection

was less efficient than that shown in the present study where lower salt concentration (0.25 M KCl) was used.

As far as the ionic strength is concerned, a simple explanation might be that, in the presence of high salt concentration, less interaction between AChE macromolecules would take place, due to the known disruptive effect of salts on protein-protein interactions and on enzyme structures (141). Ionic strength plays a most important role, in regulating enzyme activity, through its effect on protein conformation, in general, and on the state of AChE aggregation, in particular (28,62).

On the other hand, it is known (44) that, with enzymes existing in two forms in equilibrium with one another, the addition of ions can affect the enzymatic equilibrium in favor of one form. This, however, seems to be unlikely here because the further decrease in the ionic strength of the medium did not actually reverse the salt effect, what would be expected in such situations.

Dialysis against buffer of lower ionic strength (Figure 16) did not reverse the effect of salt. S1, 1^{st} method, was more resistant to dialysis than S2, 1^{st} method (Figure 23). This suggests that the disruptive effect of salts on enzyme structures, in general (141), and/or the dissociation of ionic bonds caused by high salt concentration on AChE aggregates, in paricular (61,62), may not be reversed by a consequent lowering of the ionic strength of the medium (e.g., with dialysis).

Studies by Crone in 1973 (35), on AChE from bovine erythrocytes and rat brain, showed aggregation of all enzymatic forms, at low ionic strength. An increase to I = 0.15 dissociated AChE to a single molecular form. Alterations in the NaCl concentration, which produced effects on the activity of the AChE, also caused changes in the molecular aggregation of the enzyme. It was not possible to decide whether the two phenomena were directly

linked or whether the aggregation was a homogeneous association of AChE-molecules or a random association of the various proteins present in Crone's impure preparations. He suggested that AChE existed in two extreme forms, the properties observed in practice being a resultant of the particular equilibrium established between them. In media of low ionic strength or in hydrophobic areas of membranes, AChE was present in the form of large complexes. The second form of AChE existed as the disaggregated molecule of molecular weight 260,000, when the ionic strength of the medium was high. Rapid equilibrium occurred between the two states, so that species of intermediate average size could be observed on exclusion chromatography. The affinity for ACh was different in these two forms and a regulatory role for the enzyme was discussed (35).

The treatment of the AChE preparation with DEAE Sephadex A-25, also, prevented enzyme aggregation under the experimental conditions of the present research. The protection was more easily observed with S1, from both methods (Figures 19 and 22), and S2, 2^{nd} method (Figure 11), as compared to S2, 1^{st} method (Figure 10). Perhaps the lower pH (6.5 and 7.0, respectively) of the first two enzyme preparations would contribute in part to the magnitude of DEAE Sephadex A-25 action. Alternatively, it is possible that some factor responsible for, or involved with, the aggregation process was less concentrated in S1, 1^{st} method, and S2, 2^{nd} method, or it was better adsorbed on the gel surface because of the lower pH's of these enzyme preparations.

Non-aggregating forms of brain AChE after DEAE Sephadex A-25 treatment were observed for the first time by Hollunger and Niklasson (75). They suggested that the enzyme released in the presence of the gel did not aggregate, because some factor responsible for the aggregation was adsorbed on the ion-exchanger and not eluted together with the enzyme. The treated

enzyme seemed neither to precipitate, at low pH, nor to aggregate if added to a preparation of aggregating enzyme. They concluded that, perhaps, the factors responsible for aggregation and precipitation were liberated together with the enzyme in stoichiometric amounts. If so, the aggregation phenomenon would seem to be specific and, despite its slow rate and irreversible character, it might be related in some manner to the physiological binding of the enzyme to the membrane.

An aggregating protein was also found in AChE preparation from E. electricus by Kremzner and Fei (90). The protein was strongly cationic and adsorbed to gel systems such as agarose or dextran. They concluded that an "aggregating protein" of membrane origin and molecular weight 1.5×10^5 was responsible for AChE aggregation in their biological system.

Dudai et al. (45), studying AChE from electric eel by sucrose-gradient centrifugation technique, showed that purification of the 14 S and 18 S isoenzymes by affinity chromatography did not alter their sedimentation coefficients or their tendency to aggregate at low ionic strength. Thus, the aggregation did not result from the presence, in the crude extract, of some other components that interact with the enzyme at low ionic strength and that dissociate at high ionic strength. Obviously components such as lipids, polysaccharides or polynucleotides, whether bound to the major polypeptide chain or to minor ones, could be involved in the aggregation process. Their observations led them to propose that lipids were not involved in the aggregation phenomenon. However, they could not exclude the possible participation, in the process, of small quantities of any of the components mentioned above.

At this stage, it is worthwhile to point out some differences between AChE from Torpedo or Electrophorus electric organs and mammalian brain AChE. The former ones are easily extracted from electric tissue in the presence

of high concentrations of salt, while such extraction conditions decrease the yield and solubilization of brain AChE. Salt-extracted enzymes from Torpedo or Electrophorus have the tendency to aggregate in low ionic strength media, and this process is reversible. On the other hand, AChE aggregation in brain enzyme is not easily reversible by manipulations of the ionic strength of the medium.

In the present investigation, attempts to cause AChE aggregation, by means of reconstitution experiments (Figures 27 to 30) or by lowering the ionic strength of solutions of a stabilized enzyme preparation (Figure 16) were unsuccessful. As far as the reconstitution experiments are concerned, perhaps any possible aggregation factor previously present in the enzyme preparation was adsorbed on the gel, or retained in the affinity column and not further eluted with the Seph Sup 2 or with the Aff Sup, under the experimental conditions used. Alternatively, the elution from DEAE Sephadex A-25 required 1 M KCl which has already some stabilizing effect per se on the AChE forms. Attempts to deaggregate an in vitro aged enzyme preparation by means of DEAE Sephadex A-25 treatment were likewise unsuccessful. The enzyme was usually chromatographed within a 24-hour period, after the treatment, which perhaps was not long enough to detect changes in the isoenzymic pattern.

However, deaggregation was achieved with an older preparation, by increasing the ionic strength of the medium or after AChE form separation on gel filtration. The I form was progressively "reconverted" to S after its isolation and storage. With time, in enzyme preparations containing a combination of L, I and S, or only the I and S forms, I gradually disappeared and S built up instead at the expense of I (Figures 37 to 41). This phenomenon was observed not only with fresh enzyme preparation where I was

present at low concentration, but also with an older enzyme preparation where I was the predominating form (Figure 42).

As far as it could be ascertained, this was the first time in which a deaggregation process occurred at low ionic strength (I = 0.09) with brain AChE. Perhaps some component responsible for, or involved with the aggregation process was separated on the gel from the aggregated isoenzymes, thus decreasing the stability of the I form. Based on the molecular weight calculations of AChE forms, I is assumed to be a multiple form of S. Under the present experimental conditions, deaggregation was, however, a slow process and required a longer time to completion (1-2 weeks), compared with aggregation which could be already observed within a 24-hour period.

There is evidence (143) indicating the glycoprotein character of brain AChE. Glycoproteins, as part of the AChE molecules and/or as free compounds in solution, appear as a possible candidate to be involved with the aggregation process since they might promote or facilitate the interaction between AChE macromolecules. After gel filtration glycoproteins could be separated from AChE macromolecules, due to molecular weight differences, or perhaps would be retained in the gel, to a certain extent. If the carbohydrate moieties of the individual AChE forms are involved in aggregation, gel filtration may cause some alterations in their ability to interact.

Structurally Complex Carbohydrates: Presence in the Brain and Relationship with AChE

Glycoproteins have been detected in brain and in nerve endings, and have been suggested to participate in memory processes and in the transmission of nerve impulses (132). Soluble glycoproteins account for only a small proportion of the total brain glycoprotein material; 80% of the

glycoprotein carbohydrate of brain tissue is bound to membraneous material (16). Five to twelve per cent of all cerebral proteins are glycoproteins (99).

Investigations have shown that synaptosomes, axons and other cell membrane fragments contain glycoprotein as structural elements. The highest glycoprotein content has been found in the membraneous material associated with the synaptosomes subcellular fraction (99), which is also rich in AChE (40). High amounts of glycoproteins have been also found in the non-purified microsomal fraction. All the carbohydrate segments present in these substances contain N-acetylneuraminic acid and glucosamine (99). Some evidence indicates that the soluble glycoproteins have a great tendency to aggregate, so that they appear to have very large molecular weights by Sepharose gel filtration (43).

In the present investigation, stabilization of AChE forms after neuraminidase treatment was observed. The enzyme form distribution at day 1 and day 4 was not changed if the preparation was treated with neuraminidase. However, in the absence of neuraminidase the enzymatic pattern was changed toward the L form via I, with time (Figure 43). It is generally accepted that fucose and NANA occupy peripheral positions, farthest from the polypeptide chain in glycoproteins. Thus neuraminidase could have a more easily accessible substrate, and may release NANA residues from AChE macromolecular surfaces to prevent aggregation of AChE to larger forms. On the other hand, acetylglucosamine and galactose are often found nearest the polypeptide chain in glycoprotein and often form part of the carbohydrate-peptide linkage (132).

The presence of neuraminidase in the brain tissue has been confirmed by many investigators (136). Enzyme prepared from calf brain was reported to be particle-bound, containing forty times more particulate neuraminidase

than soluble enzyme. The optimum pH for most neuraminidases, including calf brain neuraminidase, is close to 5.0 (136). Gielen and Harpprecht (58) reported the occurrence of a higher activity of particulate neuraminidase in grey matter than in white matter; this was confirmed by other investigators (114,124). Among CNS structures, the enzyme activity in the caudate nucleus tended to be higher than in the lenticular nucleus and in the thalamus (114). The available data strongly suggested the hypothesis that brain particulate neuraminidase acts on gangliosides and is linked to nerve tissue membranes (136).

The possible involvements of neuraminidase with brain metabolism and function may be anticipated from its proposed roles (136): (a) degradation of gangliosides:oligosialogangliosides which appear to act as the physiological substrates for "major" particulate and soluble neuraminidase; (b) regulation of the negative charge of neuronal membranes by this action on gangliosides. Gangliosides are specific components of nervous membranes. They contribute, by the dissociation of sialic acid residues, to the net negative charge of the membrane. The release of NANA from such molecules, catalyzed by neuraminidase, causes a decrease of the negative charge of the membrane. Conversely, an increase of the negative charge follows the action of sialyltransferase which restores the content of NANA on the ganglioside molecule. Both neuraminidase and sialyltransferase are located in the brain particulate fractions richest in gangliosides. This suggests the hypothesis that the two enzymes play a role in the modulation of the net negative charge of the ganglioside containing membranes, and are therefore involved in the correlated functional events; (c) intermittent modification of neuronal membrane permeability. Gammack (55) observed that in aqueous media oligosialogangliosides form micelles of greater size than monosialogangliosides. Considering that gangliosides are fundamental constituents

of some nerve membranes, the observed *in vivo* degradation of oligosialo- into monosialogangliosides could be followed by dramatic changes in the architecture and, consequently, in the permeability of membranes. Nothing is known on the specific mechanisms by which brain neuraminidase starts acting in biological systems (136).

As far as AChE is concerned, there is evidence that indicates an involvement of carbohydrate moieties in the AChE macromolecule in aggregation phenomenon. Gaffney (54) observed that human serum cholinesterase, presented seven major components on polyacrylamide gel electrophoresis, six of which survived the purification procedure and these were reduced in number to a single rather diffuse component following prolonged treatment with neuraminidase. According to him, NANA might play an important role in maintaining the stability of this multicomponent system. It was observed that the treatment of human serum with neuraminidase produced a decrease in the electrophoretic mobility of cholinesterase (4). Serum cholinesterases from other species also seem to be glycoproteins (71).

Powell *et al.* (117) reported that the main components of AChE from electric eel showed a positive stain for both proteins and carbohydrates after polyacrylamide gel electrophoresis. This could be particularly significant since many membrane proteins have been proved to be glycoproteins: the glycoprotein nature of AChE would correlate well with its anticipated position at the surface of the synaptic membrane. Since neuraminidase did not modify the activity of the enzyme, those authors concluded that the charged NANA was not critically involved in maintaining the ionic environment of the active sites, although it was involved in antigenic recognition sites and low ionic strength aggregation.

Human erythrocyte AChE is also considered to be a glycoprotein (31). Erythrocyte membrane is known to contain glycoproteins and to be rich in

NANA. The erythrocyte AChE was considered as one of the glycoproteins of the erythrocyte membrane, since a considerable amount of periodic acid Schiff stainable material was observed.

Several forms of AChE from electric eel have been found, differing by their sedimentation constant and by their structure: globular forms (11.8 S) and elongated forms, which electron microscopy showed to be composed of a rod-like structure (called the "tail") and a cluster (called the "head") of varying number of tetramers (119). These elongated forms had been separated into A (8.5 S), C (14.2 S) and D (18.4 S). Antibodies to AChE have been produced with globular and elongated forms of AChE. It was found that the elongated forms were more reactive than the globular forms, with each antiserum tested. After treatment with neuraminidase, there was a progressive decrease in the affinity of the antiserum for the D and C forms, and no effect on the globular forms. This would mean that polysaccharides might be important for the structure of the elongated forms. Neuraminidase hydrolyses NANA residues. Therefore, NANA and presumably other polysaccharides give an important contribution to the structure pattern of almost all antigenic determinants of the elongated forms.

Very recently, Wenthold et al. (143) have shown that AChE from rat brain is a glycoprotein and contains a single subunit with a molecular weight of 80,000. The preliminary treatment of the enzyme with neuraminidase did not change the isoelectric patterns of the enzyme fractions. According to them this was an indication of the absence of sialic acid residues in terminal positions. However, the experiment was performed with the crude enzyme product at pH 8.0. It is known (19) that neuraminidase is not active at this pH. In addition, the incubation was carried out at 37°C for short periods (15-60 min), which perhaps were not sufficient for completion of the neuraminidase activity.

However, the literature also reports investigations which deny the participation of carbohydrate residues in AChE structure and lead one to suggest the need for a more thorough study on this question. Thus, neuraminidase had no effect on the aggregated form of AChE from eel electric organ (64). Also, treatment with neuraminidase had no effect on the electrophoretic pattern of AChE forms from human erythrocytes (148); therefore NANA did not appear to determine or affect the ratios of the AChE multiple forms. Iqbal and Talwar (80) examined the isozymic forms of AChE preparations from 18-day-old chick embryo brain, before and after treatment with neuraminidase. No change in the isozyme pattern was observed. The four bands corresponding to AChE activity were not due to the attachment of different amounts of NANA residues to the polypeptide chains, as the treatment of the enzyme preparation with neuraminidase did not alter the differential migration pattern of the isozymes.

Another approach to study involvement of carbohydrate moieties in macromolecules is by means of their interaction with Concanavalin A. Concanavalin A (Con A), a globulin from the jack bean (Conavalia ensiformis), has been shown to interact to form a precipitate with biopolymers containing multiple α-D-glucopyranosyl (or its 2-acetamido-2-deoxy derivative), α-D-mannopyranosyl, or β-D-fructofuranosyl residues as non-reducing termini. These include polysaccharides, glycoproteins and lysopolysaccharides, among other compounds (1).

Recently, Con A has been shown to interact with bacterial and animal cells, to distinguish between normal and tumor cells on the basis of membraneous carbohydrate-containing structures, and to initiate differentiation and cell division. Thus, Con A may serve as a versatile model for studies of protein-carbohydrate and antibody-antigen interaction, as a

tool for investigating the glycosyl composition and structure of certain biopolymers, and as a probe for studying the carbohydrate-containing constituents of the cell surface (1).

Experiments carried out with rat synaptic plasma membranes (SPM) showed the presence of glycoproteins with mannose as the terminal sugar of their carbohydrate moieties. These glycoproteins appeared to be responsible for the agglutination of the synaptosomes in the presence of Con A. This effect was inhibited by α-methyl-D-mannoside, which inhibits the binding of Con A to mannose (60).

Recent studies have shown the presence of carbohydrate residues attached to the ACh receptor protein as evidenced by its ability to bind plant lectins (submitted for publication, see 79). This constitutes an interesting observation, considering the similarities existent between AChE and AChR.

Torpedo AChE contains 7.9% of carbohydrates present as hexoses, hexosamines, and sialic acid, and at least some of these residues are exposed on the outer surface of the molecule (134). Titration of the enzyme with increasing quantities of Con A resulted in the formation of a sedimentable complex, which was partially reversed by α-methyl-D-mannoside. Prior to sedimentation, AChE activity in the saturated complex was diminished by 70%, indicating that the enzyme-Con A aggregate showed a reduced catalytic efficiency. Con A possesses four identical subunits, and each 25,200 subunit species contains a single carbohydrate binding site. Apparent 1:1 stoichiometry was observed between the 82,000 AChE and the 25,000 Con A subunits. The protection by Con A from trypsin-induced solubilization of membrane-associated AChE, according to Taylor et al. (134), could well arise from the binding of Con A to the same oligosaccharide chains shown to be present on the purified AChE molecule. Con A predominates as a 100,000

molecular weight tetrameric species, above pH 7.0; thus, being of size comparable to an AChE subunit, it could prevent access of trypsin to peptide bonds in the enzyme susceptible to cleavage. Alternatively, protection could be afforded by Con A binding to the presumed AChE fragment which is cleaved, or to a neighboring saccharide-containing macromolecule on the membrane surface (134).

The tendency of the salt-extracted enzymes to aggregate in low ionic strength media is lost upon lytic conversion to the 11 S enzyme (102,103). The moieties responsible for the aggregation phenomena may be involved in the association of AChE with the membrane. Thus, a portion of the native AChE which is cleaved or reoriented upon lytic treatment could well serve, in conjunction with the saccharide residues, to orient the enzyme molecule with respect to the membrane surface (134).

In the present investigation, the multiple forms of brain AChE constituted a sedimentable complex in the presence of Con A. The determination of enzyme activity in the supernatants showed a decrease of AChE activity in the experimentals compared to the controls. More interesting was the fact that the void volume peak, separated on Sepharose 6B column, was the most sensitive to the Con A effect. The decrease in enzyme activity was progressively lessened towards the S and I forms (Table 14). This effect of Con A was at least partially reversed by α-methyl-D-mannoside, in all situations (Figure 44). The content of carbohydrate appeared to become proportionally important with the increase in molecular weight of the AChE form. This would reinforce the presence of carbohydrate moieties in the brain AChE macromolecule and their involvement with the aggregation phenomenon. Further evidence for the presence of carbohydrates in the highly purified brain AChE preparation was shown by means of polyacrylamide gel

electrophoresis stained by periodic acid Schiff, according to recent information from Chan et al. (personal communication). These investigators report that all three forms examined appear to contain carbohydrate residues.

The results of the present study suggest that carbohydrates and neuraminidases and/or other specific glycosidases are involved with the AChE aggregation-deaggregation process of the mammalian brain enzyme (Figure 45). The rationale for this hypothesis is based on the possibility that glycoproteins play a role in the association between AChE molecules (aggregation) and, perhaps, with the binding of the enzyme to membrane components. Aggregation would be the consequence of unfavorable conditions for neuraminidase activity such as neutral or alkaline pH and/or low ionic strength. In this situation any glycoprotein present would act without having its effect counteracted.

Neuraminidases, on the other hand, by releasing sialic acid residues from glycoproteins, would dissociate AChE macromolecules (deaggregation) and, perhaps, stimulate AChE release from the membrane cell surface. Deaggregation would occur under optimum conditions for neuraminidase activity (e.g., pH 5.0, 37°C, etc.) and/or acidic pH and high ionic strength. Alternatively, under conditions not favorable to neuraminidase activity, followed by the removal or inactivation of glycoproteins (e.g., physical separation from the AChE molecule on gel filtration or their adsorption on the gel surface), deaggregation would also occur.

Of course, the general phenomenon is probably much more complex and the involvement of other components, besides those, cannot be ruled out. Finally, the equilibrium between AChE forms ($S \leftrightarrow L$) by means of an aggregation-deaggregation process (Figure 45) would be, perhaps, operative

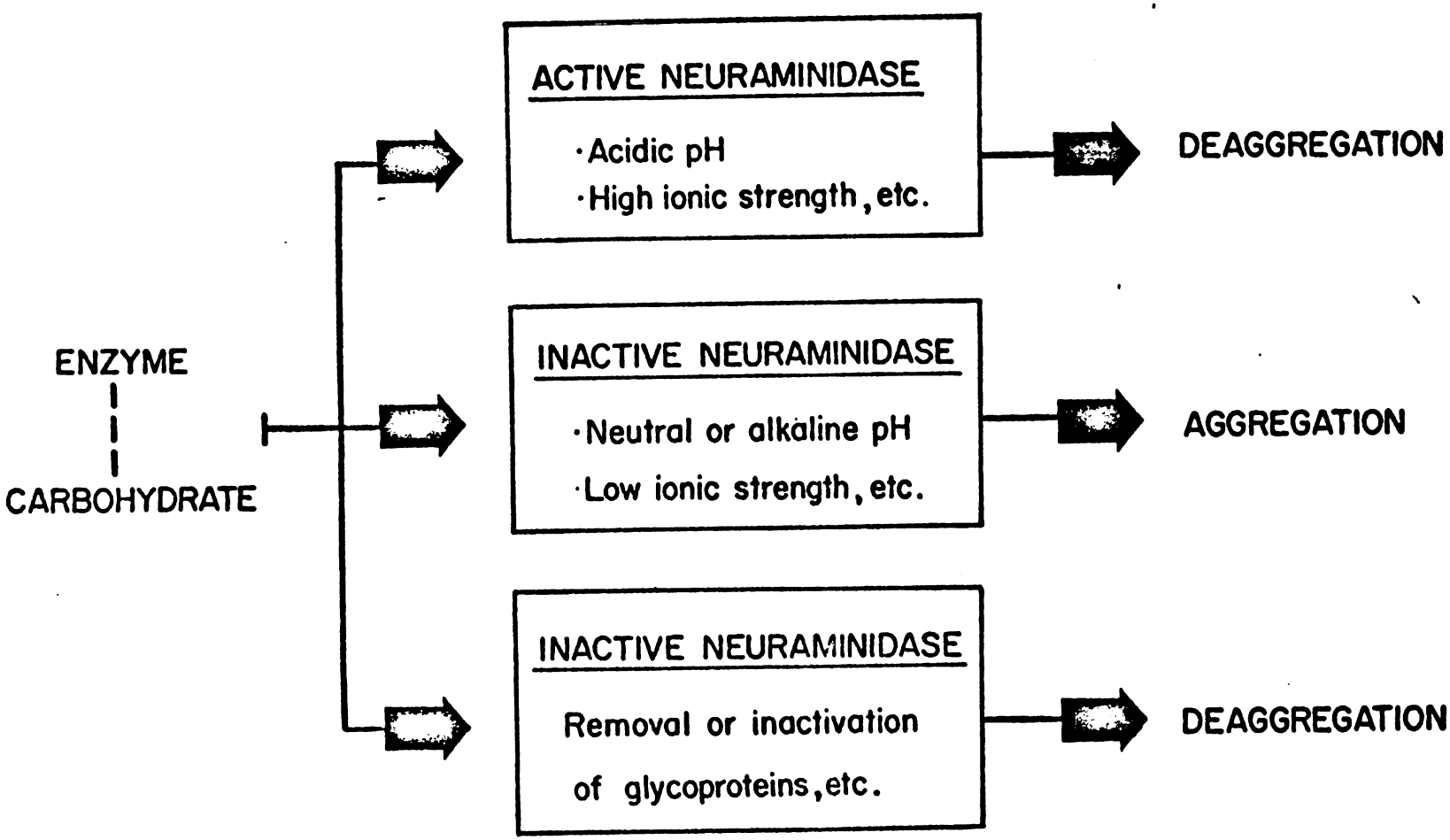

Figure 45. Proposed Hypothesis.
Probable mechanisms explaining the AChE aggregation-deaggregation process.

in vivo, could well represent the dynamic state of the cell, and might be involved in functional events taking place in the cell membrane.

Conclusions and Directions for Future Studies

The main findings of the present work can be summarized as follows:

1. Under the experimental conditions used, the 2nd method of solubilization was statistically superior to the 1st one, as far as AChE specific activity and percentage of recovery of the solubilized enzyme are concerned;
2. In a fresh enzyme preparation, two forms of brain AChE existed: a large, L, and a small molecular weight form, S;
3. With time, an intermediate molecular weight form, I, was originated at the expense of the small form, S (aggregation);
4. After the separation of the forms present in a fresh or old enzyme preparation, by gel filtration techniques, the I form was reconverted to the S form (deaggregation);
5. The treatment of the enzyme preparation with DEAE Sephadex A-25 slowed the aggregation process, as did decrease in pH or increase in the ionic strength of the medium;
6. Some protection against aggregation was also attained by treatment of the enzyme with neuraminidase. This suggested the presence of sialic acid residues in the brain preparation;
7. The presence of carbohydrate moieties was detected by the formation of a sedimentable complex between the AChE forms and Con A. The interaction was partially prevented by α-methyl-D-mannoside;

8. Based on the presence of glycoproteins and neuraminidases in the brain enzyme preparation, a hypothesis is proposed to explain the mechanisms of the AChE aggregation-deaggregation process.

From these findings some suggestions for future studies may be presented:

1. Analysis of the effect of neuraminidase on AChE solubilization, per se, to determine how it would affect the yield, enzyme aggregation and the molecular weights of brain AChE forms;
2. Analysis of the effect of tetracaine on AChE solubilization to determine how it would affect the yield (in the presence and absence of divalent cations) and the molecular weights of AChE isoenzymes;
3. Electrophoretic study of complex carbohydrates and AChE isoenzymes in the purified enzyme preparation, before and after neuraminidase treatment, to detect possible changes in electrophoretic mobilities. The presence of sialic acid groups is known to increase the electrophoretic mobility of proteins (130). In addition, other investigations showed that neuraminidase treatment resulted in a decreased anodal migration of some alkaline phosphatase isoenzymes (20,121);
4. Determination of the neuraminidase content that might be present in the solubilized enzyme preparation, in an attempt to establish the involvement of this enzyme with the AChE aggregation process;
5. Study of AChE isoenzyme and glycoprotein patterns in mammalian species of different ages (developmental study). The activity of AChE is known to increase progressively with age. It is assumed to serve as an index of the development of synapses (80). In addition, gangliosides and glycoproteins are also involved with new synapse formations (43). This suggested study would contribute to a better definition

of the multiple forms of brain AChE and their relationship with glycoproteins in the different stages of development of the CNS;

6. Study of isoenzymes from synaptosomal origin using a more purified enzyme preparation to attempt to determine the topographical distribution of complex carbohydrates in the "labeled" synaptosomal membrane, and their relationship to the enzyme and/or ACh receptor.

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