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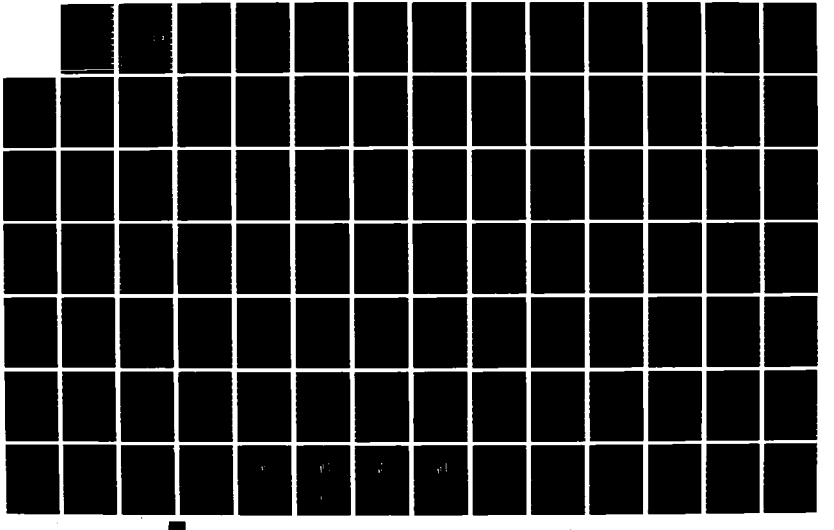

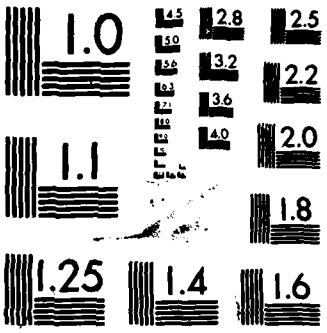

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Abstract

The Regional Variability of Enzymes in the Brain

David A. Armbruster, Ph.D.

Medical College of Virginia-Virginia Commonwealth University, 1986

Advisor: Dr. Hanns-Dieter Gruemer

Enzyme levels in cerebrospinal fluid (CSF) have been used as prognostic markers in cases of brain injury with equivocal results. It is hypothesized that CSF enzymes are derived from the brain and that the destruction of brain tissue will result in the release of enzymes which will in turn elevate the CSF levels. Elevated CSF enzymes may thus correlate with the degree of tissue destruction and ultimate patient outcome. But if the concentration of an enzyme varies substantially in different regions of the brain, equal amounts will not be released per unit of tissue and the CSF enzyme level may not correlate with prognosis. In this study, the question of regional variabilty of enzymes in the brain is addressed.

The concentrations, expressed as U/g wet tissue weight, of seven enzymes, acid phosphatase (ACP), aspartate aminotransferase (AST), creatine kinase (CK), glutamate dehydrogenase (GDH), NADP-linked isocitrate dehydrogenase (ICDH), lactate dehydrogenase (LD), and malate dehydrogensase (MDH), were determined in seven different brain regions in the rat and cat. Tissue specimens weighing about 50 mg were dissected from the cerebellum, medulla, hypothalamus, striatum, midbrain, cortex, and hippocampus. The fresh tissue was either: homogenized in a 50 mmol/L

Tris-HCl, 150 mmol/L NaCl buffer, pH 7.4; frozen in liquid N_2, thawed at 37 C, and homogenized in the Tris buffer; or homogenized in a 1% solution of non-ionic detergent made up in the Tris buffer.

The enzyme concentrations are much higher when freeze-thawing or detergent treatment were used to increase enzyme solubilization in comparison to simple homogenization. Little regional variability exists for ACP, AST, ICDH, LD, or MDH in the rat and cat as each enzyme is present in nearly equivalent concentrations in all regions. CK and GDH exhibit marked regional variability in both animals with enzyme concentrations being two- to three-fold greater in some regions than in others.

THE REGIONAL VARIABILITY OF ENZYMES IN THE BRAIN

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Virginia Commonwealth University

This is to certify that the dissertation prepared by David Allyn Armbruster entitled "The Regional Variability of Enzymes in the Brain" has been approved by his committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy.

Director of Dissertation

Committee Member

Committee Member

Committee Member

Committee Member and Director of Graduate Study

Department Chairman

School Dean

4 August 86
Date

Dedication

I dedicate this dissertation to my parents, who gave me the opportunity and will to pursue an education, to my wife, who supplied the love, patience, and understanding to see me through all of my graduate programs, and to my children, a source of great satisfaction and inspiration to me.

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List of Abbreviations

ACP	Acid phosphatase
AK	Adenylate kinase
AST	Aspartate aminotransferase
BRIJ	Brij 35
CK	Creatine kinase
CNS	Central nervous system
CS	Clearing Solution
CSF	Cerebrospinal fluid
CV	Coefficeint of variation
DTT	Dithiotheitol
Ea	Activation energy
F-T	Frozen-thawed
GDH	*Glutamate dehydrogenase*
H	Heavy mitochondrial fraction
ICDH	Isocitrate dehydrogenase
L	Light mitochondrial fraction

LD	Lactate dehydrogenase
MDH	Malate dehydrogenase
N	Nuclear fraction
NWA	New Wetting Agent
P	Microsomal fraction
PDH	Pyruvate dehydrogenase
RIA	Radioimmunoassay
RSA	Relative Specific Activity
S	Supernatant fraction
SD	Standard deviation
X-100	Triton X-100
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INTRODUCTION AND LITERATURE REVIEW

This research seeks to answer the question: Is there regional variability of enzymes in the brain? The question of regional variability of enzymes in the brain, that is, whether or not enzymes are found in higher or lower concentrations in some regions of the brain than in others, is of interest for at least two reasons. First, it is relevant to the study of brain metabolism in general and to understanding embryological and neurophylogenetic enzyme development as it relates to metabolism specifically. Second, it has a direct impact on the use of quantitative cerebrospinal fluid (CSF) enzyme determinations as diagnostic and prognostic indicators of central nervous system (CNS) disorders.

Before proceeding, it is essential to define the term "enzyme concentration" as used in this work. Enzyme concentration is expressed as units of enzyme activity per gram wet tissue weight. This is at variance with the traditional concept of concentration according to which the amount of analyte present is expressed as g/mL, mol/L, etc., and the concentration value is ultimately related to the number of molecules of analyte present. Here, enzyme activity, an expression of the enzyme molecule rather than the molecule itself, is measured. Thus enzyme concentration is a description of activity rather than a strict quantitative expression in mass units of the number of enzyme molecules.

BRAIN ENZYMES AND BRAIN METABOLISM

When examining brain metabolism, it is desirable to correlate enzymatic activity with morphological, functional, and developmental changes in the CNS. It has been shown that the biological activity of the brain varies with

respect to age and the region of the brain studied (1). However, while enzymes must be associated with metabolic activity, little is known about the levels of enzymes present in different regions of the brain and how they vary with age. Leong and Clark studied the development of several enzymes associated with glucose metabolism and energy metabolism in general in rat brain from birth to adulthood (2-4). They found that the enzymes developed in six different regions at different rates and that adult concentrations of some enzymes in these regions varied, with up to two-fold differences in concentrations. It was concluded that the neurological maturity of the brain may be correlated with enzyme development and that this development recapitulates the embryological and neurophylogenetic development of the brain regions. Presumably, neurological differences in animals at various points along the evolutionary scale should be reflected by enzyme levels in the nervous tissue. Sugden and Newsholme have argued that the comparison of the maximum flux in vivo through a metabolic pathway with the maximum activities of enzymes in that pathway can be used to identify reactions that are near-to or far-displaced from equilibrium (5). A non-equilibrium reaction is indicated if the maximum activity of the enzymes are similar to the maximum flux.

Lai et al. found that mitochondrial NAD-linked isocitrate dehydrogenase (ICDH) from rat brain showed regional variability and age-related variability with the enzyme concentrations decreasing in senescent animals (6). It was noted that a diet high in manganese chloride opposed the age-related decreases in ICDH. These authors suggested that disturbances of brain energy metabolism seen in the senescent rat might be alleviated by manganese treatment. Others have also studied the patterns of brain enzymes linked to energy metabolism and the changes observed with senescence (7).

Pyruvate dehydrogenase and NAD-linked malate dehydrogenase (MDH) concentrations increase with ageing while NAD-linked ICDH decreases.

Not only does regional variability for some enzymes exist in whole rat brain tissue but this heterogeneous distribution of enzymes extends to the subcellular level. Leong et al. assayed a variety of enzymes from non-synaptic (free) and synaptic mitochondria from different brain regions and found that enzyme activities differed in the two types of mitochnondria and differed from region to region in the two types of mitochondria (8).

In general, the regional distribution of the enzymes mediating energy metabolism parallel differences in the rate of energy metabolism as shown by comparison of enzyme patterns with data on regional differences in rates of tissue respiration (9). Investigators have considered the measurement of enzyme activities as a useful approach to relating metabolic potential to tissue function, particularly when related to a variable physiological state (2). As an example, during hypoxia there is a rapid loss of neuronal function in the brain. Under these conditions, the brain's adaptations include significant modifications of some enzyme activities, including those of cytochrome c oxidase and MDH (10). Head injury which results in brain damage also causes physiologic changes, in some cases resulting in a metabolic acidosis due to an accumulation of lactic acid, which may be due to secondary ischemia or hypoxia (11). It is not expected that enzyme activities may be altered under these conditions.

Accurate knowledge of enzyme concentrations in the brain, and really in specific regions of the brain as it is a complex and heterogeneous organ, are desirable and useful when studying brain metabolism. This type of information is particularly relevant to: (1) phylogenetic investigations when the metabolism of nervous tissue in animals at different levels of

evolutionary development are to be compared, (2) studies of the rate of neurological development from birth to adulthood in a given species of animal, (3) research on the effects of ageing on neurological function, and (4) any metabolic investigation of the brain when the normal physiological conditions are altered, as in neurological disease processes and traumatic head injury.

CSF ENZYMES AND NEUROLOGICAL DISEASE

The CSF is formed by the choroid plexuses of the ventricles in the brain and it circulates in the subarachnoid space around the brain and spinal cord, cushioning and supporting the CNS. The CSF normally contains a small amount of protein, including enzymes, which originate in the brain and cross the brain-CSF barrier, which is relatively weak in comparison to the blood-brain barrier as fairly large molecules such as enzymes and globulins can cross it. It has been assumed that enzyme levels in the CSF correspond to the extent of tissue injury in central nervous system disorders in much the same way as serum enzymes reflect tissue injury, e.g., the relationship between creatine kinase (CK) and lactate dehydrogenase (LD) total activities and isoenzyme activities to myocardial infaction. Savory and Brodie have reviewed the measurement and diagnostic value of CSF enzymes (12). CSF enzymes have been studied in cases where tissue injury occurs due to cerebrovascular accident, meningitis, central nervous system leukemia, hydrocephalus, epilepsy, brain tumors, increased intracranial pressure, and subarachnoid hemorrhage. Three enzymes, CK, LD, and aspartate aminotransferase (AST), have been most frequently assayed. CSF levels of one or more of these enzymes seem to be useful for diagnosis or prognosis in some conditions, e.g., CSF LD in cases of viral and bacterial meningitis, but

in other conditions, conflicting or inconsistent reports have tempered the utility of CSF enzyme determinations (13,14,15,18,20).

CSF ENZYMES AND BRAIN INJURY

In controlled experiments in which cold lesions of differing severity were produced in cat brains, Maas found that higher CSF enzyme levels were found in animals with more severe injuries, but considerable variation in CSF enzyme activity in relation to the degree of trauma was noted (16.17). Maas produced cold lesions in the brains using methanol at -40 C and found that CK, LD and AST were all increased in the CSF. Sherwin found that CSF CK activity was independent of spinal fluid protein and serum enzyme activity so that it appears to be derived from the brain but in 70 out of 185 patients who had elevated CSF CK values the CK elevation was not diagnostic of any particular disease (18). Using liquid nitrogen to induce cold injuries in rabbit brains, Somer et al. found increased CK-BB in the peripheral blood (19). Since it is generally accepted that the brain contains only the CK-BB isoenzyme, it was suggested that serum CK-BB might offer a new criteria for assessment of the severity of brain damage. In a study of 28 patients with head injuries of differing severity, Florez et al. found a correlation between the severity of brain lesion and the serum CK and AST values and the CSF CK, LD, AST, and MDH values (20). But it was concluded that the prognostic value of enzyme determinations was doubtful. Bell et al., using a CK-BB radioimmunoassay, found that CK-BB was elevated in the serum and CSF of patients with a variety of neurological disorders, presumably as a result of tissue destruction (21). They suggested further work to determine if quantification of actual tissue damage can be made using the CK-BB RIA assay. Vaagenes et al. studied CSF CK in patients who suffered cerebral ischemia and later died (22). They concluded that CSF CK was a valid way of

measuring the extent of permanent brain damage but noted that the best conformity between CK values and tissue destruction were observed in patients with diffuse ischemia damage and that regional variations in brain damage might explain the individual discrepancies between CK values and damage that were observed. CSF AST levels were found to increase with worsening prognosis in patients with head injuries in a study by Sood et al. (23). Longstreth et al. looked at CSF CK-BB values in patients resucitated from out of hospital cardiac arrests and demonstrated a relationship between CSF CK-BB and neurologic outcome after the arrest. They called for a study of CSF CK determinations in a larger number of patients before the actual prognostic value of the assay could be determined (24). Using an RIA assay for CK-BB, Cooper et al. felt that serum CK-BB levels were a reliable marker of cerebral parenchymal injury, but noted that 35% of patients with a "safe level" of CK-BB nevertheless died and that 29% of patients with a value above the safe level made a good recovery (25). Bakay et al. examined serum and CSF CK, LD, and AST in patients with head injuries whose severity was rated on the Glascow Coma Scale (26). They concluded that serum enzyme determinations had inadequate sensitivity and specificity to be useful as an index of neurological trauma. CSF enzymes were found to correlate directly with the coma scale scores and CK-BB was touted as a specific marker for neurological trauma. In a retrospective study, Wevers et al. found CK non-M activity (CK-BB and mitochondrial CK) to correlate with damage to the central nervous system and felt that this determination could have prognostic value, but called for a prospective study to provide more details on the behavior of CK non-M in the CSF (27). In a prospective study again involving patients who suffered out of hospital cardiac arrest, Longstreth et al. examined CSF CK (28). Their results show that increased CSF CK

activity is associated with poor neurologic recovery after cardiac arrest. But they caution that different brain insults may have different relationships to CSF CK and, as an example note that head trauma may lead to increased CSF CK but that the prediction of recovery may be influenced by the fact that brain damage may be concentrated in functionally silent areas or because dynamics of enzyme release may be different for trauma than for ischemia.

The literature cited is not intended to represent an exhaustive review of reports dealing with serum and CSF enzymes and brain injury but it does cover the major conclusions and points of contention and allows certain general observations to be made. First of all, there is clearly an interest in relating enzyme levels to brain tissue destruction. By and large, serum enzymes are not reliable for this purpose, CK-BB showing the greatest promise but still lacking as a prognostic marker, in part because it is not totally brain specific and general body trauma can release it from other tissues. CSF enzymes, providing that the blood-brain barrier is intact, show the greatest correlation between severity of damage and enzyme elevations. The effect of factors such as the rate of turnover of the CSF, the type of brain injury incurred, the degree of integrity of the blood-brain barrier, and, of course, the question of regional variability of enzymes, is still unclear. While some authors are quite confident and optimistic about using CSF enzymes as prognostic indicators, others are less so and all seem to agree that more work is required relating CSF enzymes to tissue damage in various neurological conditions to determine the sensitivity and specificity of CSF enzymes as markers of the extent of cellular destruction in these conditions.

ENZYME REGIONAL VARIABILITY AND BRAIN INJURY

Two assumptions are generally made when enzyme activities measured in body fluids such as serum or CSF are used to gauge the extent of tissue

damage: (1) that an enzyme is distributed fairly homogeneously throughout the tissue of interest, and (2) that there is no marked inter-individual variation in enzyme concentration in the tissue. Clearly, if certain regions within a tissue are relatively rich in an enzyme while other areas are poor, how much enzyme will ultimately be measured in a body fluid such as serum or CSF depends in part upon what region of the tissue has suffered damage. Enzyme elevation cannot be directly related to a given amount of tissue destroyed if this is the case. Similarly, differences in enzyme concentration between individuals, for example, due to sex, race, or age, means that enzyme values may have to be interpreted carefully, attempting to take into account this inter-individual variability. These assumptions were made when serum enzymes began to be used in the diagnosis of myocardial infarction and liver disease. These assumptions were systematicallty tested by Visser et al. who examined canine heart and liver to determine if the variation in enzyme content between different sites in an organ was small and if variation of enzyme content among individuals was likewise small (29). It was found that the coefficient of variation (CV) for CK, LD, and AST was about 10% in the heart and liver and that most of the variation in enzyme values was due to interindividual differences among the animals. This study was ultimately concerned with calculating the quantity of tissue destroyed based upon the total enzyme released into the circulation. In the case of CSF enzymes, rather than determining the absolute quantity of brain tissue damaged or destroyed, an elevation in a CSF enzyme is used as a prognostic tool to make some prediction about the chances of the patient surviving and/or the extent of recovery that can be anticipated. Interpretation of CSF enzyme values will be at least complicated if considerable regional variability of enzymes in the brain exists. A rigorous proof that the assumptions of negligible or

acceptable regional variability and interindividual variability hold for brain enzymes has not been undertaken.

It is appropriate to investigate the regional variability of brain enzymes because of its relevance to metabolic studies of the brain and to the clinical usefulness of CSF enzyme determinations as prognostic indicators in neurologic disorders of the CNS.

PREVIOUS WORK ON ENZYME VARIABILITY IN THE BRAIN

In 1954, Strominger and Lowry used histochemical methods to look at the regional distribution of LD, MDH, and glutamate dehydrogenase (GDH) in rabbit brain in order to study regional metabolic differences (30). This work involved assaying different histological layers from various brain regions. Using these histochemical techniques and reporting results as moles per kilogram dry weight per hour, regional variability was noted for these enzymes. In 1958, Miyazaki measured AST in one human brain obtained eight hours after death and saw activities that ranged from 0.52 to 0.83 micromoles per hour per milligram of tissue in eight different regions (31). Tyler in 1960 examined LD and MDH activity in human brains obtained within three hours after death and reported results using units defined as a change of 0.01 optic density unit/minute/cc of solution (32). He noted greater activity in gray matter compared to white matter and regional variations in concentrations, particularly for LD. In 1962, van der Helm used electrophoresis to study the isoenzymes of LD, MDH, and GDH in six different brain regions and found no qualitative or quantitative differences in the isoenzyme composition of the regions examined (33). Unfortunately, he did not report total activities. In contrast, Gerhardt and Petri in 1965 did find regional differences in LD isoenzyme distribution although most

regions showed a fairly uniform distribution with the highest activities in the first three anodic isoenzymes (34). They also found regional differences in LD total activity with activity being expressed as the change in extinction at 366 nm per second per microgram of protein. Tissue from human brains obtained 12-20 hours post mortem was used.

The work from th' 50's and 60's is generally suggestive of regional variability of brain enzymes, including the clinically useful enzymes LD and AST. At least four weaknesses are evident though: (1) often autopsy material was used without regard to changes in enzyme activity with time after death, either in the whole brain or different regions, (2) the methods used were not optimized assays and the various definitions of a unit of enzyme activity are obsolete, (3) the disease states leading to death were not addressed, and (4) how accurately the measured enzyme "activity" reflects the actual "concentration" of enzyme is not always apparent.

In more recent work, Rabow examined LD activity and LD isoenzyme patterns in four regions of rabbit brain and in human surgical brain specimens (35). LD isoenzyme patterns differed with region, which is not unexpected as the regions with higher oxygen consumption showed more anodal (LD-1) activity, consistent with aerobic metabolism. LD activity in the rabbit was distinctly higher in the brainstem compared to the cerebral hemisphere, basal ganglia, and cerebellar hemisphere, which were essentially equivalent. In man, the activities in the gray and white matter differed. LD activity was recorded in Wroblewski-LaDue units, defined as a decrease in optical density of 0.001 per minute per ml. Yasmineh et al. worked with fresh specimens from the cerebrum and cerebellum of baboons and found mean values of 178 and 182 IU/g wet weight respectively for CK, indicating little variability between these two regions for this enzyme (36). Baboons

were used because they are phylogenetically close to man and the importance of using fresh tissue to obtain valid results was stressed in this work. In 1980, Petronia et al. examined CK isoenzyme patterns in various regions of the human brain using autopsy material and found essentially only CK-BB and mitochondrial CK (37). They concluded that the distribution of these CK isoenzymes depended strongly on the region of brain studied; unfortunately they did not measure total CK activity either. In 1981 Leong et al. studied changes of energy-metabolising enzymes in six different regions of the aging rat brain (38). It was found that the activities of the following enzymes in both male and female rats showed no meaningful biochemical differences in different brain regions: hexokinase, phosphofructokinase, aldolase, pyruvate kinase, pyruvate dehydrogenase, citrate synthase, fumarase, NAD- and NADP-linked ICDH, MDH, and LD. Lai reported in 1982 that mitochondrial NAD-linked ICDH showed regional variability in the rat brain with the concentration in the cerebral cortex being 60% greater than in the medulla oblongata (6). In three related papers, Leong and Clark in 1984 studied the development of a number of metabolically important enzymes in prenatal, neonatal, and adult rats in six different brain regions (2-4). While their major interest was in the rate of development of these enzymes, their work shows that glucose-6-phosphate dehydrogenase, NAD-linked ICDH, citrate synthase, pyruvate dehydrogenase, and fumarase are found in essentially the same concentrations in the regions they studied while LD, GDH, hexokinase, and aldolase exhibited regional variability. These findings were not considered unusual because of the complexity and heterogeneity of the brain and the fact that different regions of the brain utilize glucose at different rates. It is of interest to note that in the 1981 paper of Leong, LD, hexokinase, and aldolase were considered not to show regional variability

(38). Also in 1984, Leong et al. sought to determine if a heterogeneous distribution of brain enzymes was reflected at the subcellular level by looking at mitochondrial enzymes from nonsynaptic (free) and synaptic mitochondria from different regions (8). Citrate synthase, MDH, and CK were found to be equivalent in mitochondria from all regions while pyruvate dehydrogenase, fumarase, and NAD- and NADP-linked ICDH showed regional variability. Fumarase had been shown not to exhibit regional variability in whole brain by Leong and Clark previously but it did vary in the mitochondria.

Directly related to the issue of CSF enzymes is the report of Chandler et al. in 1984 which stated that CK exhibited regional variability (39). Using autopsy tissue, specimens were removed from 22 different regions of human brain. Total CK activity ranged from 5.6 to 76 IU/g tissue. It was concluded that cerebral infarcts of similar size may release different amounts of CK activity into the CSF depending on the location of the infarct, which would make interpretation of the CSF-CK results difficult. In a related paper, the same group reported the CK extracted from autopsy brains showed a significant difference in activity between the cerebral cortex and the putamen (40).

CONCLUSION AND OBJECTIVES

Considering the complexity and heterogeneity of the brain in terms of the cell types it contains and the metabolic differences that exist from region to region, it would follow that enzyme activity, might well reflect this regional variability. Regional variability of some metabolically important enzymes has recently been shown, although the same authors reporting this variability had previously suggested that these same enzymes were homogeneously distributed in the brain. A review of the literature suggests that the enzymes

most frequently investigated as CSF markers of brain tissue damage, CK, LD, and AST, do exhibit regional variability. The existing reports may, however, be questioned in retrospect, since they were based upon enzyme assays that had not been optimized, while others are for some reason open to question, e.g., autopsy tissue in which enzyme inactivation may have occurred was used. It is of interest to address the question of enzyme regional variability in a study using fresh animal tissue, modern enzyme assays, and a well defined tissue processing protocol. Such studies should focus on enzymes generally considered to be of clinical interest.

Specifically, the objective of this research is to examine a number of enzymes, including the clinically useful and commonly assayed AST, CK, and LD, in several different brain regions to determine if significant regional variability exists. These findings will help to determine whether the measurement of CSF enzymes is a reasonable approach in assessing the extent of brain tissue damage and which enzymes, if any, are good candidates for use as CSF prognostic markers.

METHODS

ENZYME ASSAYS

CK, LD, and AST were chosen to be assayed because they are the enzymes of clinical interest that have been most frequently measured in the CSF. Acid phosphatase (ACP) represents a lysosomal marker enzyme and cytochrome c oxidase, bound to the inner mitochondrial membrane, as well as fumarase, GDH, NAD- and NADP-linked ICDH, and MDH, all found in the mitochondrial matrix, are mitochondrial marker enzymes. To gauge the effectiveness of the homogenization process, these marker enzymes were chosen to determine to what extent subcellular organelles were disrupted. For the enzyme assays, CVs of up to 20% were considered to be acceptable.

ACP (EC 3.1.3.2) Assay : The Dupont ACA (Dupont, Wilmington, DE.) method, an adaptation of the method of Roy et al., employing the ACP reagent packs, was used (41). The reaction is as follows:

Thymolphthalein monophosphate ---->

Thymolphthalein + Inorganic Phosphate

Thymolthphalein + Base ----> Thymolthphalein Anion

Activity is calculated by measuring the absorbance at 600 nm. The assay has a day-to-day CV of 22.2% and is linear to 20 U/L as shown with dilutions of brain homogenates.

AST (EC 2.6.1.1) Assay : The Beckman (Beckman, Carlsbad, CA.) Plus Chem Optimized AST Reagent, adapted to the method of Karmen, was used (42,103). The reaction is as follows:

Aspartate + alpha-Ketoglutarate ----> Oxaloacetate +Glutamate

Oxaloacetate + NADH ----> Malate + NAD

Activity is determined by monitoring the decrease in absorbance at 340 nm. AST was assayed using the Cobas Bio centrifugal analyzer (Roche, Nutley, N.J.). This assay has a day-to-day CV of 4% and was found to be linear to 900 U/L as determined using dilutions of brain homogenates.

CK (EC 2.7.3.2) Assay : The Beckman Plus Chem Optimized CK-NAC Reagent, adapted to the method of the Scandinavian Society for Clinical Chemistry, was used (43). The reaction is as follows:

Creatine Phosphate + ADP ----> Creatine + ATP

ATP + Glucose ----> Glucose-6-phosphate + ADP

Glucose-6-phosphate + NADP ----> 6-Phosphogluconate + NADPH

Activity is calculated by measuring the increase in absorbance at 340 nm. CK was assayed using the Cobas Bio. This assay has a day-to-day CV of 4.5% and was found to be linear to 1300 U/L using dilutions of brain homogenates.

Cytochrome c Oxidase (EC 1.9.3.1) Assay : Cytochrome c oxidase was assayed by the method of DeDuve (44). The reaction is as follows:

Cytochrome c (reduced) ----> Cytochrome c (oxidized)

One mL of 0.2 mol/L KH_2PO_4 (Mallinckrodt, St. Louis, MO.) buffer, pH 7.4, was added to nine mL of deionized water and 6 mg of cytochrome c (Sigma, St. Louis, MO.) was added. The cytochrome c was reduced by addition of sufficient sodium dithionite, $Na_2S_2O_4$ (Matheson Colemam + Bell, East Rutherford, N.J.) to result in at least a 98% reduction of cytochrome c as determined by a ratio value of absorbances at 550 nm and 565 nm (550 nm reduced/565 nm oxidized) equal to 8-9. Sample is added to the reduced cytochrome c oxidase reaction mixture and activity is determined by following the decrease in absorbance with time at 550 nm at 25 C. This method was adapted to the Cobas Bio centrifugal analyzer and was found to have a within-run CV of 18.5%.

Fumarase (EC 4.2.1.2) Assay : Fumarase was assayed by the method of Stitt (45). The reaction is as follows:

L-Malate ----> Fumarate + H_2O

The reaction mixture consists of 84 mmol/L K_2HPO_4 (Mallinckrodt) buffer, pH 7.5, and 46 mmol/L malate (Sigma). Activity is determined by following the increase in absorbance at 240 nm with time at 30 C. The assay was performed using a Cary 118 double beam recording spectrophotometer (Varian, Palo Alto, CA.). The assay was found to have a within-day CV of 6.7% and to be linear to 500 U/L.

GDH (EC 1.4.1.3) Assay : The method of Ellis and Goldberg was used initially (46). The reaction is as follows:

2-Oxoglutarate + NADH + NH_3 ----> L-Glutamate + NAD + H_2O

Reagent I consists of 50 mmol/L triethanolamine, pH 7.4, 50 mmol/L ammonium acetate, 0.95 mmol/L ADP, and 0.19 mmol/L NADH. Reagent II consists of 10 mmol/L 2-Oxoglutarate. All chemicals were obtained from Sigma. Activity is calculated by measuring the decrease in absorbance at 340 nm. The assay was adapted to the Cobas Bio and was found to have a within-run CV of 2.8% and to be linear to 300 U/L as determined using dilutions of brain homogenates. Later, the method of Jung et al. was used (47). The reaction mixture consists of 50 mmol/L triethanolamine buffer, pH 7.6, 125 mmol/L ammonium acetate, 320 micromolar NADH, 8 mmol/L L-leucine, 400 micromolar ADP, and 12 mmol/L 2-oxoglutarate. This assay was performed on the Cobas Bio centrifugal analyzer using the same parameters previously worked out. This method was reported to yield activities about 20% higher than those found using the method of Ellis and Goldberg which was used previously in this work. The new GDH assay was found to be linear to at least 350 U/L and to have a within-run CV of 8.2% at about 20 U/L, a

relatively low value. It was found to give results about 50% higher than those obtained using the Ellis and Goldberg assay and for this reason was chosen to replace that procedure.

NAD-Linked ICDH (EC 1.1.1.41) Assay : NAD-linked ICDH was assayed by the method of Wilson and Tipton (48). The reaction is as follows:

D,L-Isocitrate + NAD ----> 2-Oxoglutarate + NADH + CO_2 + H^+

The reaction mixture consists of 0.1 mol/L triethanolamine buffer (Sigma), pH 7.5, 10 mmol/L $MgCl_2$ (Mallinckrodt), 1 mmol/L NAD (Sigma), 2 mmol/L ADP (Sigma), and 67 mmol/L D,L-isocitrate (Sigma). Activity is determined by following the increase in absorbance at 340 nm at 37 C. The assay was adapted to the Cobas Bio centrifugal analyzer and was found to have a within-run CV of 5.7% and to be linear to 30 U/L.

NADP-Linked ICDH (EC 1.1.1.42) Assay : NADP-linked ICDH was assayed by the method of Goldberg and Ellis (49). The reaction is as follows:

D,L-Isocitrate + NADP ----> 2-Oxoglutarate + NADPH + CO_2 + H^+

The reaction mixture consists of 65 mmol/L triethanolamine buffer, pH 7.3, 1.67 mmol/L $MnCl_2$ (Mallinckrodt), 0.42 mmol/L NADP, and 6.7 mmol/L isocitrate. Activity is determined by following the increase in absorbance at 340 nm at 37 C. The assay was adapted to the Cobas Bio centrifugal analyzer and was found to have a within-run CV of 4.9% and to be linear to 130 U/L.

LD (EC 1.1.1.27) Assay : LD is assayed using the method of Buhl et al. (50). The reaction is as follows:

L-lactate + NAD ----> Pyruvate + NADH

Reagent I consists of AMP-lactate (0.83 mol/L 2-amino-2-methyl-1-propanol buffer, pH 9.0, containing .116 mol/L DL-lactate). Reagent II consists of 6.4 mmol/L NAD in a 0.83 mol/L AMP buffer. All chemicals

were obtained from Sigma. The reaction is followed by measuring the increase in absorbance at 340 nm. LD was assayed using the Cobas Bio. This assay has a day-to-day CV of 2.6% and was found to be linear to 1600 U/L using dilutions of brain homogenates.

MDH (EC 1.1.1.37) Assay: MDH was assayed by the method of Smith (51). The reaction is as follows:

$$\text{Malate} + \text{NAD} \text{ ----> } \text{Oxaloacetate} + \text{NADH} + H^+$$

The reaction mixture consists of 90 mmol/L diethanolamine buffer(Sigma), pH 9.2, 4.5 mmol/L $MgCl_2$, 2.9 mmol/L NAD, and 25 mmol/L malate. Activity is determined by following the increase in absorbance at 340 nm at 37 C. The assay was adapted to the Cobas Bio centrifugal analyzer and was found to have a within-run CV of 1.1% and to be linear to 300 U/L.

ENZYME ELECTROPHORESIS

CK isoenzyme electrophoresis was performed using the Corning (Palo Alto, CA.) Cardiotrac-CK kit. Electrophoretic separation was achieved using an agarose gel. The CK isoenzymes were visualized using the same reaction as for the CK enzyme assay which produces NADH. The fluorescent NADH was quantitated by using the Beckman Appraise scanning densitometer. LD isoenzyme electrophoresis was performed using the Beckman Paragon LD kit. Electrophoretic separation was achieved using an agarose gel. The LD isoenzyme bands were visualized using the same reaction as for the LD enzyme assay except that the resulting NADH further reacts in the presence of phenazine methosulfate to reduce nitroblue tetrazolium chloride, producing a blue formazan pigment. The LD isozyme bands were quantitated by using the Beckman Appraise scanning densitometer.

PROTEIN ASSAY

The method of Hartree was used (52). This is a modification of the Lowry method and it has the advantage of digesting particulate proteinaceous material such as that encountered in some of the experiments reported here. All assays were run in triplicate. An appropriate amount of sample, neat or diluted, was added to water to make up a total volume of 1 mL in a glass test tube. To the tube was added 0.9 mL of Solution A (2 g of potassium sodium tartrate and 100g of sodium carbonate dissolved in 500 mL of NaOH and then diluted to 1 L). Bovine serum albumin standards and a water blank were also prepared. The tubes were placed in a 55 C water bath for ten minutes, cooled to room temperature, and 0.1 mL of Solution B (2 g of sodium tartrate and 1 g of copper sulfate, hydrous, dissolved in 90 mL of water and 10 mL of 1N NaOH) was added. The tubes were incubated at room temperature for ten minutes. Three mLs of Solution C (Folin-Ciocalteu reagent diluted 1:40 with water) was added followed immediately by vortexing. The tubes were heated at 55 C for ten minutes and then cooled to room temperature. The absorbances were read at 650 nm. A standard curve was prepared using the blank and albumin standards readings and the sample protein concentrations were read from the standard curve.

FRESH TISSUE VS. IN VIVO FROZEN TISSUE EXPERIMENT

Male Sprague-Dawley rats weighing between 350-400 g were divided into two groups. One group was anesthesized with pentobarbital (54 mg/kg) and then decapitated. The skulls were opened and the brains removed as quickly as possible. The brains were then placed on analytical paper saturated with 0.9% saline which rested on a glass plate over a layer of ice. About 50 mg of frontal cortex was excised, weighed to the nearest 0.1 mg using a torsion balance to minimize time, and placed in a test tube containing five mL of

homogenization buffer, a 50 mmol/L Tris-HCL, 150 mmol/L NaCl, 10 mmol/L dithiothreitol (DTT) buffer, pH 7.4, sitting in ice. The tissue specimens were stored in the buffer on ice until further processing.

The other group of rats was also sedated with pentobarbital and then injected with lidocaine at the top of the skull. The brains were frozen in situ following the procedure of Ponten et al. (53). A skin incision was made down the midline of the skull. A funnel was placed on top of the skull after the skin had been pulled back and the interface between the funnel and the top of the skull was sealed with vaseline. Liquid nitrogen was then poured into the funnel and on to the top of the skull for a period of about three minutes, by which time the brain was frozen. The rat's head was then immersed in liquid nitrogen until the brain was removed. The brain was cut out from the skull by placing the rat in a cold box (-20 C) and shaving off the skull until the brain was exposed. About 50 mg of frontal cortex was excised, weighed on a torsion balance, and placed in five mL of the Tris buffer on ice until processed.

The fresh and frozen brain tissues were worked up at the same time using the standard homogenization and centrifugation procedure described here. The tissue (1:100 ratio of tissue to buffer) was homogenized in a Potter-Elvehjem homogenizer consisting of a glass tube and a motor-driven, tight-fitting (0.1 mm clearance) teflon-tipped pestle. Twenty up and down strokes were used and the homogenizer was kept in an ice filled beaker to minimize heating during homogenization. The homogenates were centrifuged at 20,000 x g for 30 minutes at 4 C in a Beckman L5-65 ultracentrifuge using a type 75 rotor. The clear supernatants were decanted and stored on ice until assayed for ACP, AST, CK, GDH, and LD. Enzyme concentrations were expressed as U/g wet tissue weight.

FRESH VS. IN VITRO FROZEN TISSUE EXPERIMENT

Another experiment was performed to confirm the effect on enzyme activity seen with freeze-thawing. In the first experiment, the frozen tissue was taken from rat brains that were frozen in vivo and in situ with liquid nitrogen, while the fresh tissue came from brains that had been removed from decapitated animals. In this experiment, fresh rat brain tissue was frozen in vitro and compared to fresh tissue not subjected to freeze-thawing. Male Sprague-Dawley rats weighing between 350-400 g were anesthesized with Metafane (methoxyflurane, Pitman-Moore, Washington Crossing, N.J.) and then decapitated. The brains were removed as quickly as possible and placed on analytical paper wetted with physiologic saline and placed on a glass plate sitting on ice. About 50 mg of tissue was excised from either the right or left side of the frontal cortex and placed in 5 mL of the Tris homogenization buffer, pH 7.4, on ice. About 50 mg of tissue was then excised from the opposite side of the frontal cortex, wrapped in aluminum foil, and dropped into a Dewar flask containing liquid nitrogen. After freezing for five minutes, the tissue was removed, rapidly thawed in a 37 C water bath with agitation for five minutes, and then placed in 5 mL of Tris buffer on ice. Fresh and frozen tissue was taken alternately from different sides of the frontal cortex with successive animals so that each group of specimens represented both left and right cortices. These specimens were homogenized and assayed as described for the previous experiment.

ISCHEMIA EXPERIMENT

Male Sprague-Dawley rats weighing between 300-350 g were anesthesized with pentobarbital (54 mg/kg) and decapitated. The brains were then removed at various times after decapitation (total ischemia) to test the effect on enzymatic activity of the time the brain tissue is subjected to ischemia

before enzymatic processes are essentially halted by freezing. All brains were immediately frozen in liquid nitrogen after removal from the skull. About 50 mg of frontal cortex was excised from each brain in a -20 C cold box. The tissue was processed via the standard homogenization and centrifugation procedure previously described.

Five groups consisting of three rats each were designated. Brains were removed from the skulls .1, .5. 1, 2, and 5 minutes after decapitation. It obviously was impossible to remove a brain instantaneously after decapitation but the brains from the .1 minute group were removed within 9-12 seconds after death.

The homogenate supernatants were assayed for ACP, AST, CK, LD, and GDH.

ENZYME SOLUBILIZATION EFFECT OF FREEZE-THAWING

The brains were removed from male Sprague-Dawley rats after Metafane anesthesia and decapitation. About 100 mg of tissue was excised from both right and left frontal cortex. One piece was designated fresh tissue (the fresh tissue was alternated between left and right cortex with successive animals) and placed in ten mL of Tris-HCl buffer, pH 7.4, the usual buffer for homogenization except that DTT was omitted as it was found to react as protein in the Hartree protein assay, and homogenized in a Potter-Elvehjem homogenizer using 20 strokes. This Tris homogenization buffer without DTT was used as the standard buffer throughout the rest of this work. The other piece of tissue from each brain, taken from the opposite side of the cortex, was designated as frozen tissue and was wrapped in aluminum foil and placed in liquid nitrogen for five minutes. The frozen tissue was then thawed for five minutes in a 37 C water bath. It was then homogenized in exactly the same way as the fresh tissue.

Two mLs of each homogenate were saved for protein and enzyme assay. The other eight milliliters were fractionated following the five fraction scheme of DeDuve (44,54). Five fractions were made from the cell homogenate: (1) N, nuclear, (2), H, heavy mitochondrial, (3) L, light mitochondrial, (4) P, microsomal, and (5) S, supernatant. The original homogenate was centrifuged at 600 x g for ten minutes in a Sorvall RC-3 refrigerated centrifuge set at 4 C to yield a pellet (the N fraction) and a supernatant. The N fraction was resuspended in ten mL of Tris-HCl homogenization buffer and the supernatant was centrifuged at 2450 x g for 15 minutes in the Sorvall RC-3. The resulting pellet (the H fraction) was resuspended in ten mL of homogenization buffer. The supernatant was centrifuged at 25,000 x g for ten minutes in a Beckman L5-65 refrigerated ultracentrifuge at 4 C using a type 75 rotor. The resulting pellet (the L fraction) was resuspended in ten mL of Tris buffer. The supernatant was centrifuged at 100,000 x g for 30 minutes in the Beckman L5. The final pellet (the P fraction) was resuspended in eight mL of Tris buffer and the final supernatant is the S fraction. All fractions were stored on ice or at 4 C in a refrigerator until assayed.

Electron photomicrographs were taken of the N, H, L, and P fractions to insure that the correct cellular components were contained in them. After centrifugation the pellets, while still in the tubes, were fixed in glutaraldehyde and then dislodged. The fixed pellets were cut into several blocks and each block was examined so that each layer of every pellet was sampled. Electron photomicrographs representative of each block were taken.

All fractions were assayed for protein and ACP, AST, CK, GDH, and LD.

All fractions were assayed for enzymatic activity immediately after preparation. The remains of all fractions were then stored frozen over night at -20 C and assayed again the next day after thawing by immersion in a 37 C water bath with agitation.

ACTIVATION ENERGY EXPERIMENT

A simple way of checking for an alteration of the specific catalytic activity of a molecule is to measure its activation energy, E_a, an intrinsic kinetic parameter. In this experiment, the E_a of enzymes in homogenates of fresh and frozen tissues was measured and compared. A significant change in E_a means that freeze-thawing changed the specific catalytic activity of the enzyme. If the E_a decreases, then activity increases as the enzyme's reaction is more favored. A decreased E_a can thus in part explain an increase in activity.

Two male Sprague-Dawley rats were decapitated after Metafane anesthesia. Their brains were removed and about 50 mg of tissue was excised from the left and right frontal cortex. A left frontal cortex piece of tissue from one brain and a right cortex piece from the other brain were placed in five mL of Tris-HCl homogenization buffer, pH 7.4, on ice. The other two pieces of brain tissue were wrapped in aluminum foil, frozen in liquid nitrogen for five minutes, thawed in a 37 C water bath for five minutes with agitation, and placed in five mL of homogenization buffer. All tissue samples were then processed via the standard homogenization and centrifugation procedure. The resulting supernatants were stored at 4 C until assayed. The supernatants were assayed immediately after preparation for AST, CK, GDH, and LDH using the methods described previously.

E_a is calculated using the following formula:

$$E_a = [RT_1T_2/(T_2 - T_1)] \ln V_2/V_1$$

where R is the universal gas constant, 8.314 Joules/deg-mol, T_1 is the lower temperature in degrees Kelvin (K), T_2 is the higher temperature, and V_1 and V_2 are the enzyme activities at the respective temperatures (55,56). All enzymes were assayed at 25 C (298 K) and 37 C (310 K). Hagelauer et al. made determinations of Ea by measuring activity at 25 C and 37 C in the same reaction mixture using a very accurately controlled, ultrasonically heated cuvette (55,56). Here, enzymes were assayed at the two temperatures in separate runs on the Cobas Bio centrifugal analyzer, which is undoubtedly not as desirable as the system used by Hagelauer et al. In order to average out the variation in enzymatic activity and hopefully obtain a more accurate estimate of Ea, every enzyme in each supernatant was assayed at both temperatures a total of five times or ten times and the Ea was calculated for each trial. The results were compared statistically using the two-tailed Student T test to determine if a significant change in Ea had occurred with freezing.

MITOCHONDRIAL ENZYMES EXPERIMENT

Rat brain mitochondria were prepared according to the method of Lai and Clark (57,58). Eight adult male Sprague-Dawley rats weighing 150-190 g were decapitated after Metafane anesthesia. The brain was rapidly removed from the skull of each animal and the rhombencephalon was separated by a transverse section. The part of the brain rostral to the transection (the forebrain) was used for the preparation of the homogenate. The eight rat forebrains were placed into a beaker of ice cold isolation medium containing 0.25 mol/L sucrose (Mallinckrodt), 0.5 mmol/L EDTA (J.T. Baker, Phillipsburg, N.J.), and 10 mmol/L Tris-HCl buffer (Sigma), pH 7.4.

The rat forebrains were finely chopped up with scissors while being frequently washed with isolation medium. The minced forebrains were suspended in 30 mL of isolation medium and homogenized manually using a Dounce homogenizer with a pestle having a total clearance of 0.1 mm (.002 inches) with 12 up-and-down strokes. The homogenate was then diluted with the isolation medium to a final volume of 60 mL.

The homogenate was centrifuged at 2,000 x g for three minutes in a Sorvall RC-3 preparative centrifuge at 4 C and the supernatant was carefully decanted. The supernatant was then recentrifuged at 2,000 x g for three minutes. The combined low speed pellets are Fraction A and the low speed supernatant is Fraction B. B was then centrifuged at 12,500 x g for eight minutes at 4 C in a Beckman L5-65 ultracentrifuge using a type 30 rotor to obtain a crude mitochondrial pellet, Fraction D, and a supernatant, Fraction C. D was resuspended in 12 mL of 3% Ficoll medium (1:1 dilution of a 6% Ficoll medium consisting of 6% w/w Ficoll (Sigma), 0.24 mol/L mannitol (Fisher, Fair Lawn, N.J.), 60 mmol/L sucrose, 50 micromolar EDTA, and 10 mmol/L Tris-HCl, pH 7.4). Six mL of this suspension was layered onto 25 mL of 6% Ficoll medium and centrifuged at 11,500 x g for 30 minutes at 4 C in the Beckman L5-65 using a type 30 rotor. The supernatant, Fraction E, was decanted along with the loose, fluffy, white top layer of the pellet, Fraction F. F was then resuspended in 5 mL of isolation medium and centrifuged at 11,500 x g for 10 minutes at 4 C in the Beckman L5-65 using a type 75 rotor. The resulting pellet, Fraction G, the final mitochondrial preparation, was resuspended in eight mL of isolation medium and divided into four equal fractions.

Electron photomicrographs were taken of the G fraction to ascertain the morphological quality of the mitochondrial preparation by looking for an

increase in mitochondria and a decrease in contaminating particles and structures.

Fractions A-G were assayed for protein and for the following enzymes: ACP, CK, GDH, LD, cytochrome c oxidase, fumarase, NAD- and NADP-linked ICDH, and MDH.

After the final G fraction was divided into four parts, one part was still designated the G fraction and was not subjected to any manipulation. Another part was designated the G-H fraction and it was homogenized with a Ten Broeck homogenizer using 12 up-and-down strokes. Another part was designated the G-F fraction and it was frozen for five minutes in liquid nitrogen and thawed for five minutes in a 37 C water bath with agitation. The last part was designated the G-F-H fraction and it was frozen and thawed as described for the G-F fraction and then homogenized as described for the G-H fraction. Each of these fractions was assayed for protein and for ACP, CK, fumarase, GDH, NAD- and NADP-linked ICDH, LD, and MDH. The activation energy, E_a, was also calculated for these same enzymes, with the exception of ACP and fumarase, as described previously using ten determinations of enzyme activity at both 25 C and 37 C. Statistically significant difference between average enzyme activities and E_a's for each enzyme in each fraction were determined using the two-tailed Student T test.

EFFECTS OF MULTIPLE FREEZE-THAWINGS

Male Sprague-Dawley rats weighing between 200-240 g were anesthesized with Metafane and decapitated. The brains were removed immediately and five pieces of frontal cortex, weighing about 50 mg each, were dissected from each brain. One piece was immediately placed in 5 mL of Tris homogenization buffer in a test tube and stored on ice until homogenized. Each piece was wrapped in aluminum foil and frozen in liquid nitrogen. All

pieces were thawed for five minutes in a 37 C water bath with agitation. One piece from each brain was subjected to this freeze-thawing process once, another piece twice, the third piece three times, and the final piece was frozen and thawed four times. After the designated number of freeze-thawings, each piece of tissue was placed in a test tube containing five mL of ice cold Tris homogenization buffer, pH 7.4. All tissue was then homogenized with a Potter-Elvehjem homogenizer using twelve up-and-down strokes (1:100 ratio of tissue to buffer). The homogenates were centrifuged at 20,000 x g for one half hour at 4 C in a Beckman L5-65 ultracentrifuge using a type 75 rotor. Each supernatant was then assayed for ACP, AST, CK, GDH, ICDH, LD, and MDH and enzyme concentrations were expressed as U/ g wet tissue weight.

ENZYME SOLUBILIZATION EFFECT OF DETERGENTS

Male Sprague-Dawley rats weighing between 200-240 g were anesthesized with Metafane and decapitated. The brains were removed immediately and eight pieces of frontal cortex, weighing between 20-30 mg a piece, were dissected from each brain. One piece was immediately placed in 5 mL of ice cold Tris homogenization buffer and this preparation served as a control as no enzyme solubilization treatment other than homogenization was used. Other pieces of brain tissue were placed in 5 mL of 1% detergent solutions made up in the standard Tris homogenization buffer. The following detergents were used: Triton X-100 (Technicon, Tarrytown, N.Y.); New Wetting Agent (NWA, Technicon); Clearing Solution (CS, Technicon); Brij 35 (Sigma); 3-12 Zwittergent (3-12 Z, Calbiochem, LaJolla, CA.); and 3-14 Zwittergent (3-14 Z, Calbiochem). The final tissue piece was wrapped in aluminum foil, frozen in liquid nitrogen, thawed at 37 C for 5 minutes with agitation, and placed in 5 mL of the homogenization buffer. Each piece of

tissue was then homogenized and centrifuged via the standard procedures. Each supernatant was kept at 4 C and assayed immediately for ACP, AST, CK, GDH, ICDH, LD, and MDH and enzyme concentrations were expressed as U/g wet tissue weight.

Adenylate kinase (AK) is known to interfer with the CK reaction. Therefore the CK assay was run without the addition of creatine phosphate, the triggering reagent, to determine what percentage of CK activity was due to AK which may also be solubilized by detergent treatment. Without creatine phosphate, the CK assay becomes an AK assay. The CK assay used includes AMP and diadenosine pentaphosphate to inhibit the AK reaction. If large amounts of AK are present the inhibitors may be overwhelmed.

ENZYME SOLUBILIZATION DOSE-RESPONSE CURVE OF NWA

Adult male Sprague-Dawley rats were anesthesized with Metafane and decapitated. The brains were removed immediately and six 20-30 mg pieces of cerebellum were dissected from them. One piece of brain tissue was placed in 5 mL of each of the following solutions of NWA made up in Tris homogenization buffer: .25%; .5%; 1.0%; .1.5%; and 2.0%. One piece of tissue was placed in 5 mL of the Tris homogenization buffer without NWA. Each piece of tissue was worked up via the standard homogenization and centrifugation procedure. The clear supernatants were stored at 4 C and assayed immediately for ACP, AST, CK, GDH, ICDH, LD, and MDH. The enzyme concentration were calculated as U/g wet tissue weight and enzyme concentrations were plotted against the NWA concentration to determine the optimal concentration of NWA for enzyme solubilization.

ENZYME ACTIVITY INTERFERENCE DUE TO DETERGENTS

Frozen brain homogenates were pooled in order to perform an interference study with detergents. The pool was centrifuged at 100,000 x g for one hour to remove membranes and other particulate matter from which enzymes might be released by detergent solubilization. To 1.8 mL of pooled brain homogenate was added 0.2 mL of physiologic saline. To six other aliquots of 1.8 mL of pooled brain homogenate were added 0.2 ml of 10% solutions of Triton X-100, NWA, CS, Brij 35, 3-12 Z, and 3-14 Z. The final concentration of detergents in these solutions was 1%, the same as in the detergent homogenization buffers used previously. All solutions were assayed in quadruplicate for AST, CK, GDH, ICDH, LD, and MDH to determine which if any of the detergents inhibited or enhanced enzyme activity and to what extent. Enzyme activities were reported as U/L.

The detergents uniformly enhanced CK activity. To determine whether the enhancement was due to solubilization of CK from small particles contained in the homogenate or due to a direct effect of detergent on the CK, a modified interference experiment was performed. Homogenate with and without 1% NWA was centrifuged for four hours at 100,000 x g to remove particulate matter. NWA, 1% in concentration, was added to an aliquot of the centrifuged homogenate. The homogenate without NWA, the homogenate to which NWA had been added prior to centrifugation, and the homogenate to which NWA had been added after centrifugation were all assayed for CK.

EFFECT OF DETERGENT SOLUBILIZATION AFTER FREEZE-THAWING

To determine the relative effectiveness of freeze-thawing and detergent treatment for enzyme solubilization, both treatments were applied to pieces

of rat frontal cortex. Pieces of cortex were dissected from rats which had been anesthesized with Metafane and decapitated. Pieces of cortex were frozen in liquid nitrogen and thawed rapidly at 37 C once, twice, six, or eight times. The tissue was then processed via the standard homogenization and centrifugation procedure. The supernatants were saved and the residual pellets were resuspended in 5 mL of a 1% solution of NWA made up in the Tris homogenization buffer. The pellets were then re-homogenized and centrifuged. The detergent supernatants were saved and assayed in parallel with the frozen-thawed homogenate supernatants for AST, CK, GDH, LD, and MDH. Enzyme concentrations for both sets of supernatants were reported as U/g wet tissue weight and the sum of a frozen-thawed supernatant and the corresponding detergent supernatant fluid was taken to represent the total enzyme concentration of the tissue.

ISOENZYME DISTRIBUTION AFTER ENZYME SOLUBILIZATION

Two male Sprague-Dawley rats were anesthesized with Metafane and decapitated. Three pieces of rat frontal cortex, weighing about 50 mg a piece, were dissected from each brain. One piece was placed in 5 mL of Tris homogenization buffer, one piece was placed in 5 mL of 1% NWA made up in Tris homogenization buffer, and the third piece was frozen in liquid nitrogen, rapidly thawed at 37 C, and then placed in 5 mL of Tris homogenization buffer. All tissue was then processed via the standard homogenization and centrifugation procedure. CK and LD isoenzyme electrophoresis was then performed on all of the resulting clear supernatants.

REGIONAL VARIABILITY EXPERIMENTS

RAT BRAIN

A total of 16 male Sprague-Dawley rats, weighing between 200-240 g, were anesthesized with Metafane and decapitated. The brains were removed immediately and dissected according to the method of Glowinski and Iversen (59). The rhombencephalon was separated by a transverse section from the rest of the brain and dissected into the cerebellum and the medulla oblongata and pons. A transverse section was then made at the level of the optic chiasma. The cerebrum posterior to this section was then divided into five regions: the hypothalamus, striatum, midbrain, cortex, and the hippocampus. Pieces of brain tissue from each of these regions, weighing about 50 mg, were prepared using a torsion balance. Each tissue specimen was wrapped in aluminum foil and placed in a Dewar flask containing liquid nitrogen and frozen. The frozen tissue specimens were removed from the liquid nitrogen and placed into test tubes containing 5 mL of the usual Tris homogenization buffer, pH 7.4, resulting in a 1:100 ratio of tissue to buffer, sitting in a 37 C water bath. The tissue specimens were thawed with agitation in the water bath.

After thawing, each tissue specimen was worked up via the standard homogenization and centrifugation procedure. The clear supernatants were decanted and stored at 4 C until assayed, which was always immediately after preparation.

Another five male Sprague-Dawley rats, weighing between 200-220 g, were anesthesized with Metafane and decapitated. The brains were removed immediately and dissected according to the method of Glowinski and Iversen as previously described (59). The fresh pieces of brain tissue were placed in test tubes containing 5 mL of the usual Tris homogenization buffer, pH 7.4,

resulting in a 1:100 ratio of tissue to buffer. Each specimen was worked up via the standard homogenization and centrifugation procedure. The clear supernatants were decanted and stored at 4 C until assayed, which was always immediately after preparation.

Another five male Sprague-Dawley rats, weighing between 220-270 g, were anesthesized with Metafane and decapitated. The brains were removed immediately and dissected according to the method of Glowinski and Iversen as previously described (59). The fresh pieces of brain tissue were placed in test tubes containing 5 mL of ice cold 1% NWA solutions made up in Tris homogenization buffer, pH 7.4, resulting in a 1:100 ratio of tissue to buffer. Each specimen was worked up via the standard homogenization and centrifugation procedure. The clear supernatants were decanted and stored at 4 C until assyed, which was always immediately after preparation.

Each homogenate was assayed for ACP, AST, CK, GDH, NADP-linked ICDH, LD, and MDH by the methods decscribed previously. Except for ACP, each enzyme was assayed in duplicate and the average value was used to calculate the enzyme content, expressed as U/g wet weight.

CAT BRAIN

Male cats weighing about five kg were anesthesized with Brevital (Lilly, Indianopolis, IN.) and sacrificed by an injection of saturated KCl solution. The brains were removed immediately and dissected. Two pieces of tissue, weighing about 50 mg each, were dissected from seven different brain regions: cerebellum, medulla, hypothalamus, striatum, midbrain, cortex, and hippocampus. Time elapsed from sacrifice of the animals to completion of the dissection ranged between 20-30 minutes. One piece of tissue from each region was placed in five mL of ice cold Tris homogenization buffer, pH 7.4. The other piece from each region was wrapped in aluminum foil and frozen

by immersion in liquid nitrogen. The frozen tissue was thawed in a 37 C water bath with agitation after being placed in five mL of the Tris homogenization buffer. All tissue was then worked up via the standard homogenization and centrifugation procedure. The supernatants were assayed for ACP, AST, CK, GDH, ICDH, LD and MDH by the methods described previously. Except for ACP, each enzyme was assayed in duplicate and the average value was used to calculate the enzyme content, expressed as U/ g wet weight. The protein content of all homogenates was also determined because it was felt that some of the water contributing to the wet tissue weight might have been lost during the time it took to remove the brain from the skull and dissect it, which was considerably longer than for the rat, and enzyme content was also calculated as U/ mg protein.

Cisternal lumbar punctures were performed on two cats immediately after death and about two mL of CSF was withdrawn and assayed for AST, CK, GDH, ICDH, LD, and MDH by the same methods used for the tissue homogenates.

RESULTS

FRESH TISSUE VS. IN VIVO FROZEN TISSUE EXPERIMENT

The results of this experiment are given in Table 1. Unexpectedly, the enzyme concentrations of the frozen-thawed homogenates were actually significantly higher in the frozen samples than in the fresh homogenates. The concentrations of the frozen homogenates range from 173% to 256% of those in the fresh homogenates. This elevation of activity appeared to be a general phenomenon associated with freeze thawing as all five enzymes assayed showed increases.

FRESH TISSUE VS. IN VITRO FROZEN TISSUE EXPERIMENT

The results of this experiment are given in Table 2 . Again, when tissue is frozen and thawed and then homogenized, the enzyme concentration in the resulting supernatant is increased when compared to the concentration in a supernatant layer from a fresh tissue homogenate. All five enzymes showed this elevation in activity with freeze-thawing with increases ranging from about 30% for CK and LD to 245% for GDH.

ISCHEMIA EXPERIMENT

The results of the ischemia experiment are summarized in Table 3. It is clear that there is no marked decrease in enzyme concentration with time after the initiation of total ischemia at least up to the five minutes observed.

ENZYME SOLUBILIZATION EXPERIMENT

Electron photomicrographs were taken of the N, H, L, and P fractions. The make-up of the fractions was as expected with the appropriate subcellular organelles present in each fraction. The N fraction contained nuclear membranes, the H and L fractions contained large and small mitochondria respectively, and the P fraction consisted of membrane

TABLE 1. Enzyme concentrations in homogenates prepared from fresh and in situ frozen rat brain tissue. The mean concentrations are expressed as U/g wet tissue weight with the standard deviation in parentheses. The mean concentrations were determined by averaging the results from seven different animals.

ENZYME	AVERAGE FRESH TISSUE CONC.	AVERAGE FROZEN TISSUE CONC.	RATIO OF FROZEN TO FRESH CONC.
ACP	.028 (.01)	.052 (.006)	1.85
AST	58 (8)	124 (11)	2.13
CK	430 (60)	743 (68)	1.73
GDH	2.5 (.3)	6.4 (1.9)	2.56
LD	48 (10)	107 (18)	2.21

TABLE 2. Enzyme concentrations in homogenates prepared from fresh and in vitro frozen rat brain tissue. The mean concentrations are expressed as U/g wet tissue weight with the standard deviation in parentheses. The mean concentrations were determined by averaging the results from four different animals.

ENZYME	AVERAGE FRESH TISSUE CONC.	AVERAGE FROZEN TISSUE CONC.	RATIO OF FROZEN TO FRESH CONC.
ACP	.058 (.02)	.111 (.01)	1.92
AST	81 (12)	155 (7)	1.91
CK	537 (63)	720 (46)	1.34
GDH	8.8 (2.0)	30.5 (1.4)	3.45
LD	86(7)	113 (9)	1.30

TABLE 3. Enzyme concentrations in homogenates prepared from rat brain tissue subjected to total ischemia for specified periods of time. The mean concentrations are expressed as U/g wet tissue weight with the standard deviation in parentheses. The mean concentrations were determined by averaging the results from three animals.

ENZYME	MINUTES SUBJECTED TO TOTAL ISCHEMIA	AVERAGE ENZYME CONCENTRATION
ACP	5	.047 (.005)
	2	.058 (.005)
	1	.052 (.006)
	.5	.043 (.006)
	.1	.048 (.001)
AST	5	176 (10)
	2	173 (4)
	1	171 (14)
	.5	157 (5)
	.1	170 (6)
CK	5	975 (64)
	2	955 (94)
	1	926 (100)
	.5	751 (80)
	.1	917 (78)
GDH	5	13.7 (.3)
	2	14.5 (2.3)
	1	13.1 (2.0)
	.5	16.7 (2.2)
	.1	13.9 (1.6)
LD	5	146 (19)
	2	158 (5)
	1	157 (13)
	.5	138 (10)
	.1	146 (7)

fragments, ribosomes, and vesicles. Some overlap or contamination one fraction by another fraction was noted but was considered to be unavoidable and within acceptable limits.

The data is organized in the form of a balance sheet, a method of presenting and tabulating data favored for cell fractionation work (60). Tables 4 and 5 show the results of a typical cell fractionation experiment. The wet weight of the fresh tissue was 98 mg and of the frozen tissue, 96 mg. Total protein in the fresh fractions amounted to 128 mg/g wet weight and total protein in the frozen fractions, 127 mg/g wet weight. Protein recovery for the fresh fractions was 92% and for the frozen fractions, 99%. In terms of protein, the significant differences between fresh and frozen tissue occur in the N and S fractions. The fresh N fraction amounts to 38% of the total protein whereas the frozen N fraction accounts for only 23% of the total protein. The fresh S fraction contains 25% of total protein while the frozen S fraction contains 40% of total protein. The amounts of protein in the other fresh and frozen fractions are essentially equivalent, within 4% of each other. Clearly, when freezing and thawing is used, protein is decreased in the N fraction and increased in the S fraction.

For ACP, the total concentration in the frozen homogenate was 130% of the concentration in the fresh homogenate. The combined concentrations of both sets of fractions were slightly greater than the concentrations of both the original homogenates, 113 and 108%, respectively. The concentration in the frozen S fraction was twice as great as that in the fresh S fraction.

For AST, the total concentration of the frozen homogenate was 171% of the concentration of the fresh homogenate. The combined concentrations of the frozen fractions represented 102% of the concentration of the frozen for homogenate but the fresh fractions accounted for only 80% of the total

TABLE 4. Balance sheet for cell fractionation experiment using fresh tissue.

	FRACTIONS							
	HOMO	N	H	L	P	S	TOTAL	%RECOV
PROTEIN CONTENT	140	49	28	13	6	33	130	92
% RECOV	-	38	22	10	5	25		
ACP CONC	.110	.035	.020	.038	.012	.020	.120	113
% RECOV	-	28	16	30	10	16		
ACP RSA	1.0	.74	.73	3.0	2.0	.64		
AST CONC	89	14	4	5	3	44	71	80
% RECOV	-	20	6	8	4	63		
AST RSA	1.0	.53	.27	.8	.8	2.5		
CK CONC	660	70	15	7	2	420	520	79
% RECOV	-	14	3	1	0	82		
CK RSA	1.0	.39	.14	.1	0	3.3		
GDH CONC	6.8	1.1	.19	.63	.04	3.8	5.7	84
% RECOV	-	19	3	11	1	66		
GDH RSA	1.0	.5	.14	1.1	.2	2.6		
LD CONC	108	17	4	3	0	62	86	79
% RECOV	-	20	5	3	0	72		
LD RSA	1.0	.53	.23	.3	0	2.9		

Legend: Homo=original homogenate; N=nuclear fraction; H=heavy mitochondrial fraction; L=light mitochondrial fraction; P=microsomal fraction; S=supernatant fraction. Protein content is given as mg/g wet tissue weight. Enzyme activity is given in U/g protein. Recoveries are calculated as the percent of protein content and enzymatic concentration in the original homogenate. Total protein content and total enzymatic concentration is calculated by summation of protein and enzymatic concentrations in all cell fractions except the original homogenate. Relative Specific Activity (RSA) is defined as the % enzyme concentration/% protein in a given fraction.

TABLE 5. Balance sheet for cell fractionation experiment using frozen-thawed tissue. For explanation of legend and definitions, see TABLE 4.

	FRACTIONS							
	HOMO	N	H	L	P	S	TOTAL	%RECOV
PROTEIN CONTENT	128	29	23	18	6	50	127	99
% RECOV	-	23	18	14	5	40		
ACP CONC	.15	.018	.031	.054	.008	.043	.16	108
% RECOV	-	13	21	38	6	31		
ACP RSA	1.0	.57	1.2	2.7	1.2	.77		
AST CONC	150	9	18	20	2	107	160	102
% RECOV	-	26	12	13	1	69		
AST RSA	1.0	.26	.67	.93	.2	1.7		
CK CONC	790	21	44	48	2	670	780	99
% RECOV	-	3	6	6	0	85		
CK RSA	1.0	.13	.33	.43	0	2.1		
GDH CONC	30	1.9	5.8	6.8	.07	16	31	104
% RECOV	-	7	19	22	0	53		
GDH RSA	1.0	3	1.0	1.6	0	1.3		
LD CONC	120	5	6	5	.3	98	115	93
% RECOV	-	5	5	4	0	86		
LD RSA	1.0	.22	.27	.29	0	2		

concentration of the fresh homogenate. The concentration of the frozen S fraction was 2.4 times as great as the concentration of the fresh S fraction.

For CK, the total concentration of the frozen homogenate was 120% of the concentration of the fresh homogenate. Recovery of the frozen fractions was 99% of the initial concentration of the frozen homogenate but the recovery of the fresh fractions accounted for only 79% of the fresh homogenate. The concentration of the frozen S fraction was 158% of that seen in the fresh S fraction.

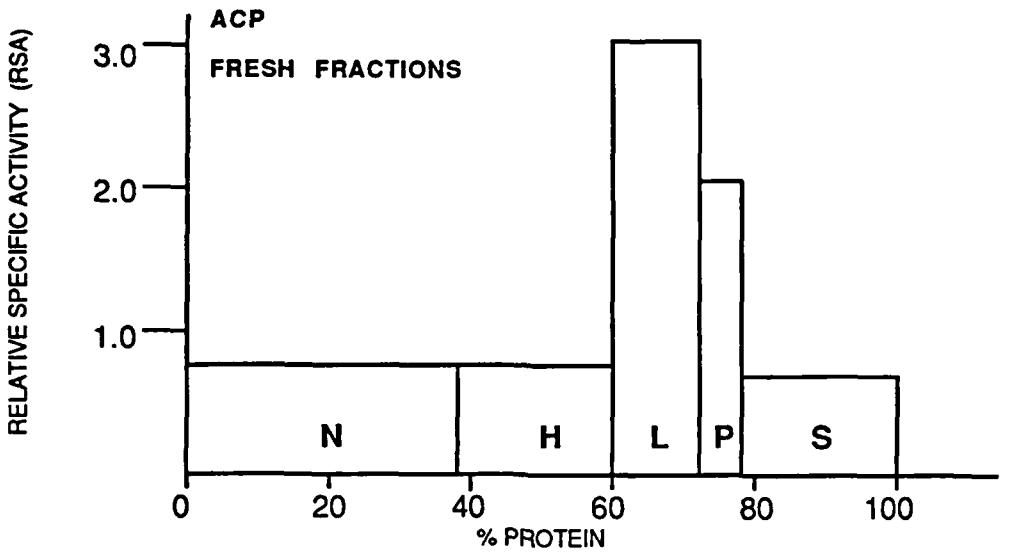
For GDH, the total concentration of the frozen homogenate was 433% of the concentration of the fresh homogenate. The recovery of the frozen fractions was 104% of the frozen homogenate but for the fresh fractions recovery was only 84% of the fresh homogenate's concentration. The concentration of the frozen S fraction was 434% of that seen in the fresh S fraction.

For LD, the total concentration of the frozen fraction was 114% of the concentration of the fresh homogenate. The recovery of the frozen fractions was 93% of the frozen homogenate but the recovery of the fresh fractions was only 79% of the fresh homogenate. The concentration of the frozen S fraction was 158% of that seen in the fresh S fraction.

The increases in enzyme concentration that occurred when freeze-thawing was used are statistically significnat, $p < 0.01$, when analyzed using the non-parametric sign test.

Figures 1 through 5 plot the Relative Specific Activity (RSA, % enzyme activity/% protein) of each fraction against the % protein in each fraction for every enzyme assayed. This type of graph was developed by DeDuve and is useful in showing where enzymatic activity is localized (54). It is clear that ACP is concentrated in the L fraction for both fresh and frozen homogenates

FIG. 1. ACP RSA in five subcellular fractions prepared from fresh and frozen-thawed rat brain tissue. The total ACP concentration for the fresh fractions is .127 U/g wet weight and for the frozen-thawed fractions .158 U/g wet weight.



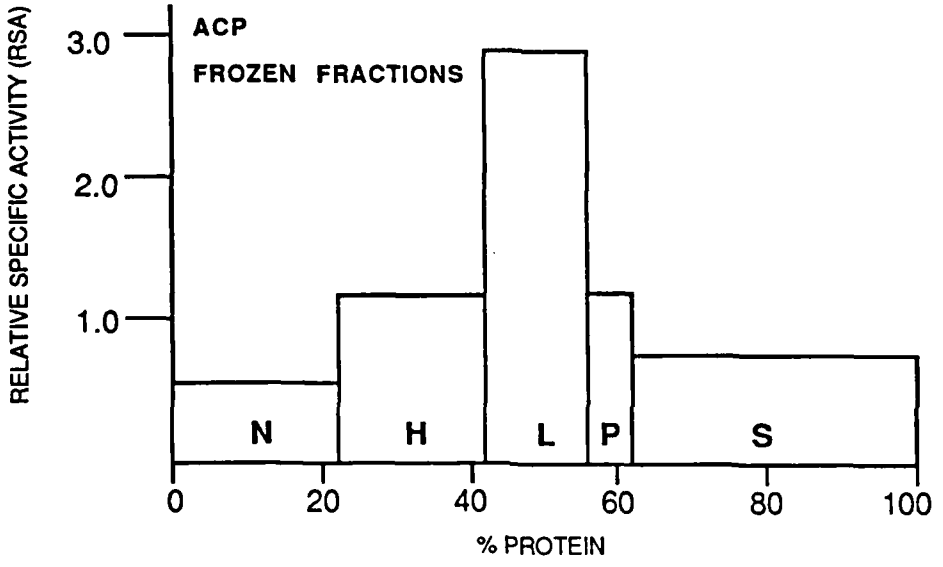


FIG. 2. AST RSA in five subcellular fractions prepared from fresh and frozen-thawed rat brain tissue. The total AST concentration for the fresh fractions is 71 U/g wet weight and for the frozen-thawed fractions 156 U/g wet weight.

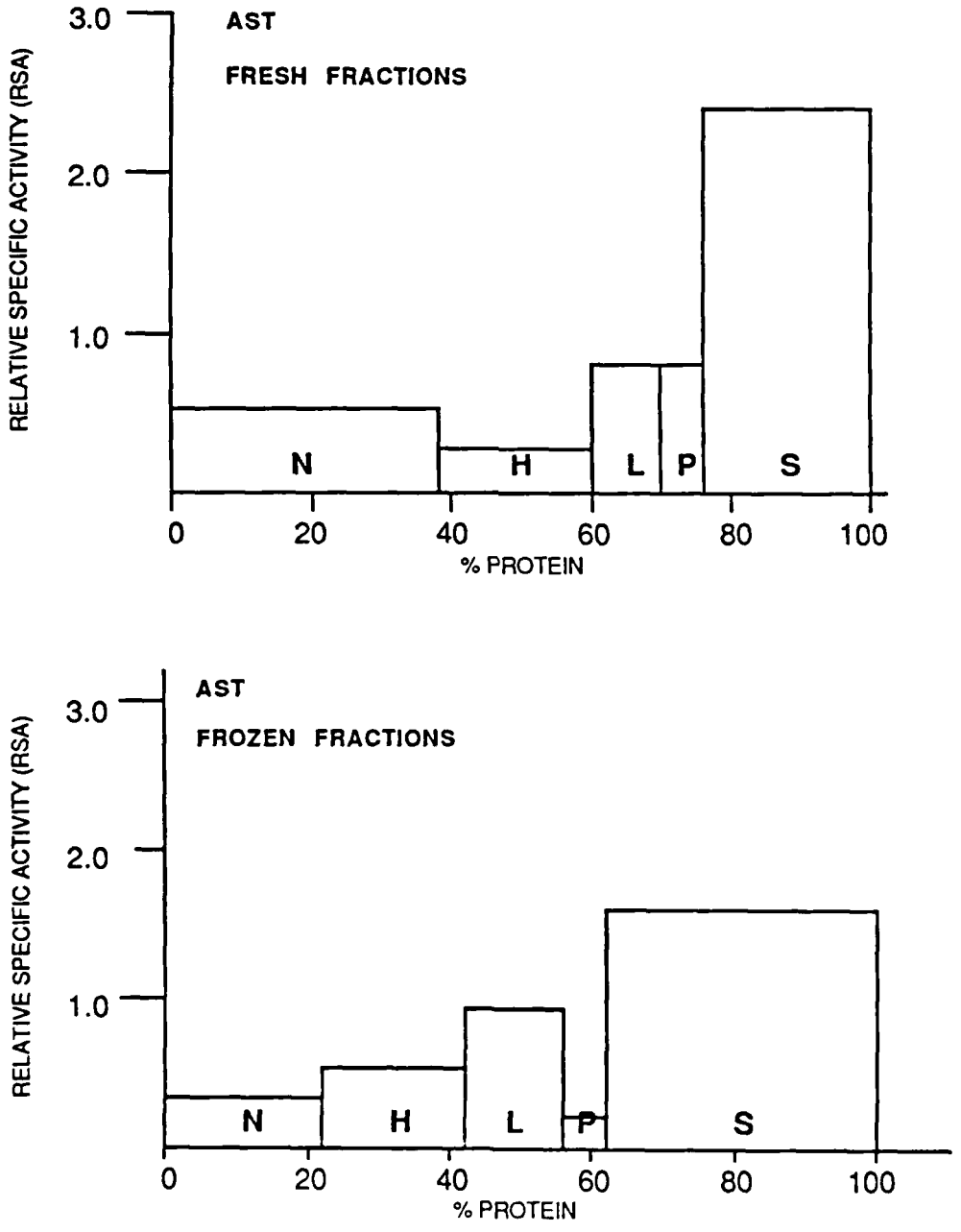
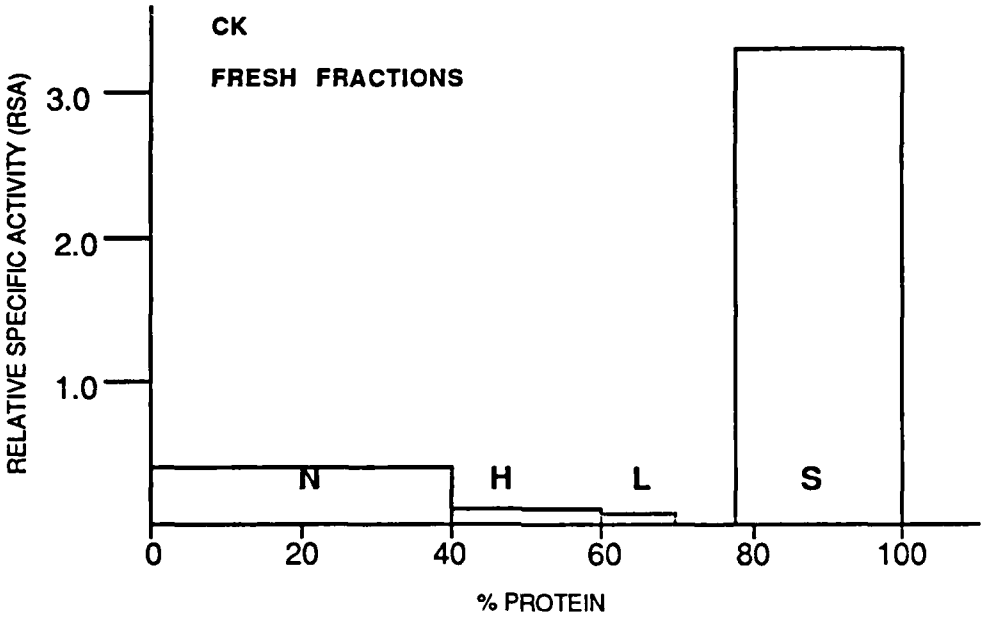


FIG. 3. CK RSA in five subcellular fractions prepared from fresh and frozen-thawed rat brain tissue. The total CK concentration for the fresh fractions is 517 U/g wet weight and for the frozen-thawed fractions 783 U/g wet weight.



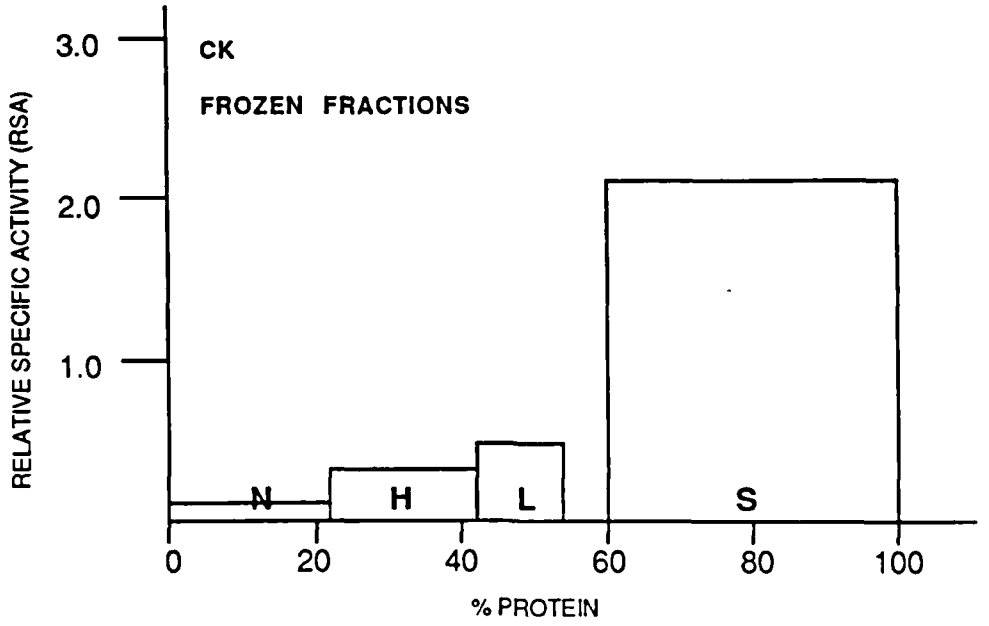


FIG. 4. GDH RSA in five subcellular fractions prepared from fresh and frozen-thawed rat brain tissue. The total GDH concentration for the fresh fractions is 5.7 U/g wet weight and for the frozen-thawed fractions 31 U/g wet weight.

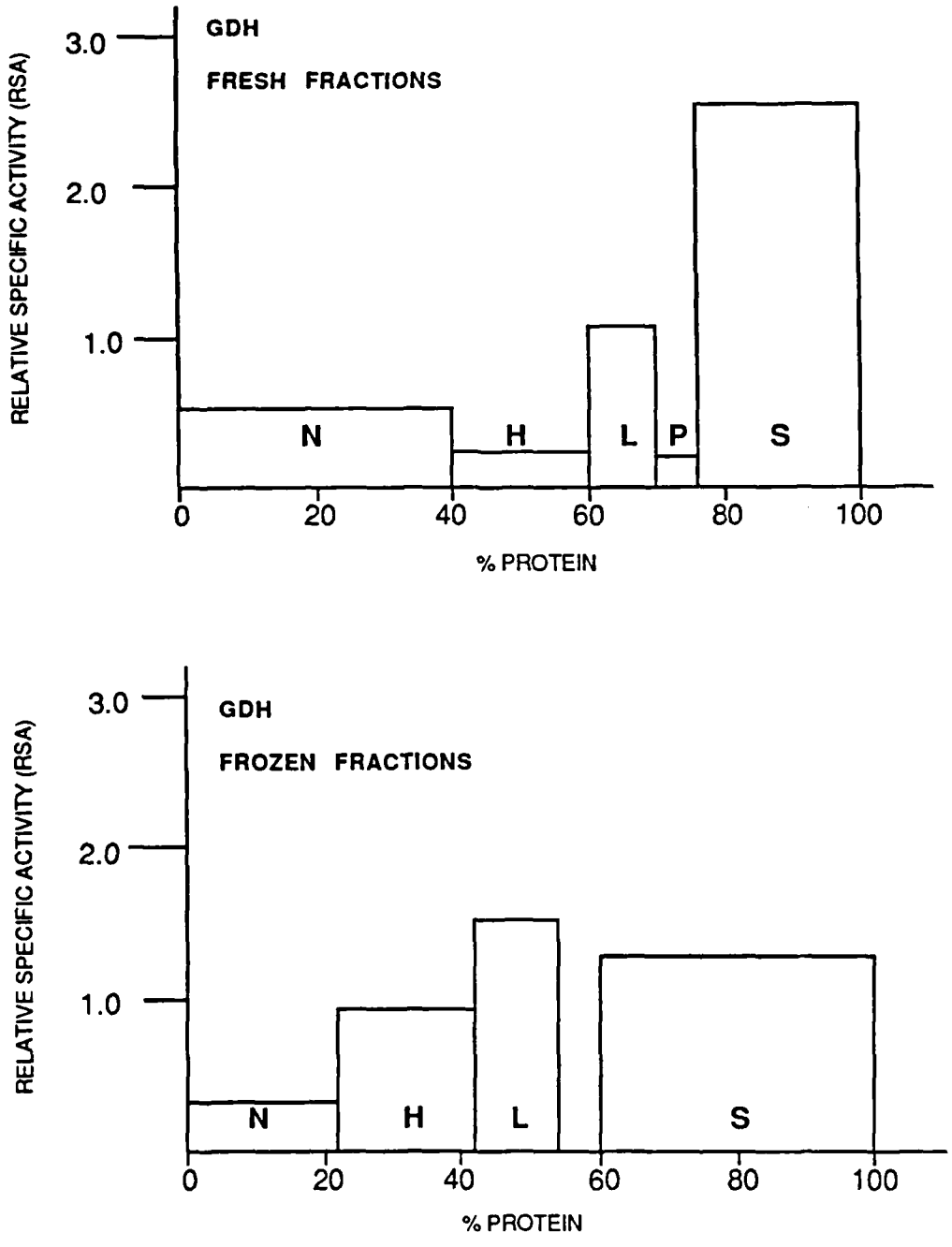
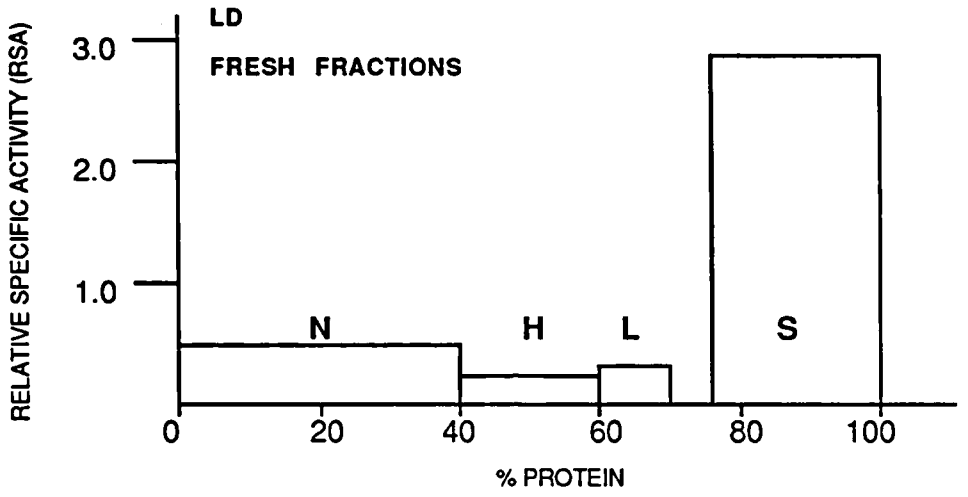
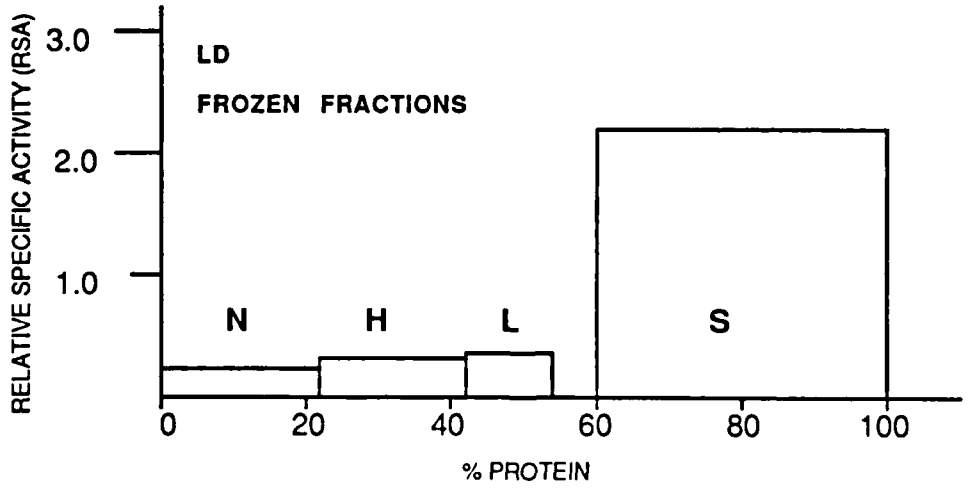


FIG. 5. LD RSA in five subcellular fractions prepared from fresh and frozen thawed rat brain tissue. The total LD concentration for the fresh fractions is 86 U/g wet weight and for the frozen-thawed fractions 115 U/g wet weight.





but the frozen S fraction contains more ACP activity than the fresh S fraction. ACP activity is expected to be rich in the L fraction as lysosomes overlap with mitochondria in cell fractionation schemes. More AST activity is seen to be found in the fresh particulate fractions in comparison to the analogous frozen fractions while the frozen S fraction clearly has more AST activity than the fresh S fraction. Both graphs show that AST is primarily a cytoplasmic enzyme, although a mitochondrial form does exist and makes a definite contribution to the total AST content. CK is also seen to be a primarily cytoplasmic enzyme as the bulk of activity is located in the supernatant fractions. The frozen S fraction contains more CK activity than the fresh S fraction. GDH activity is seen to be mainly in the mitochondrial and supernatant fractions. The frozen homogenate gives a clearer picture of GDH's mitochondrial location as high RSAs are found in both the H and L fractions while the fresh homogenate shows only an increased RSA in the L fraction. That LD is primarily a cytoplasmic enzyme is reflected by both graphs. It is of interest that considerably more LD activity is associated with the fresh N fraction than with the frozen N fraction.

Tables 6 and 7 are identical in format to Tables 4 and 5 but show the enzyme concentrations of fresh and frozen fractions after overnight storage at -20 C. The ACP concentration is seen to have decreased in both the fresh and frozen homogenates and fractions. While the AST concentraion in the frozen homogenate and fractions decreased, the AST concentration in the fresh homogenate and fractions increased after frozen storage. The increases in concentration occurred in the N, H, and L fractions. While the total CK concentration of the frozen fractions decreased, the total concentration of the fresh fractions increased, with the N, H, L, and P fractions accounting for the increase, the S fraction losing a little activity.

TABLE 6. Balance sheet for cell fractionation experiment for fresh tissue fractions which have been stored at -20 C overnight. For legend and definitions, see TABLE 4.

	FRACTIONS							
	HOMO	N	H	L	P	S	TOTAL	%RECOV
PROTEIN CONTENT	140	49	28	13	6	33	128	92
% RECOV	-	38	22	10	5	25		
ACP CONC	.030	.023	.010	.025	.012	.012	.087	271
% RECOV	-	28	12	31	15	15		
ACP RSA	1.0	.74	.55	3.1	3.0	.6		
AST CONC	107	23	23	11	0	43	99	92
% RECOV	-	23	23	11	0	43		
AST RSA	1.0	.61	1.0	1.1	0	1.7		
CK CONC	630	93	54	19	4	401	570	91
% RECOV	-	16	9	3	1	70		
CK RSA	1.0	.42	.41	.3	.2	2.8		
GDH CONC	17.5	6.2	6.1	3.4	0	3.8	19.7	112
% RECOV	-	32	32	17	0	20		
GDH RSA	1.0	.84	1.5	1.7	0	.8		
LD CONC	90	23	10	2	0	47	82	91
% RECOV	-	28	12	3	0	57		
LD RSA	1.0	.74	.55	.3	0	2.3		

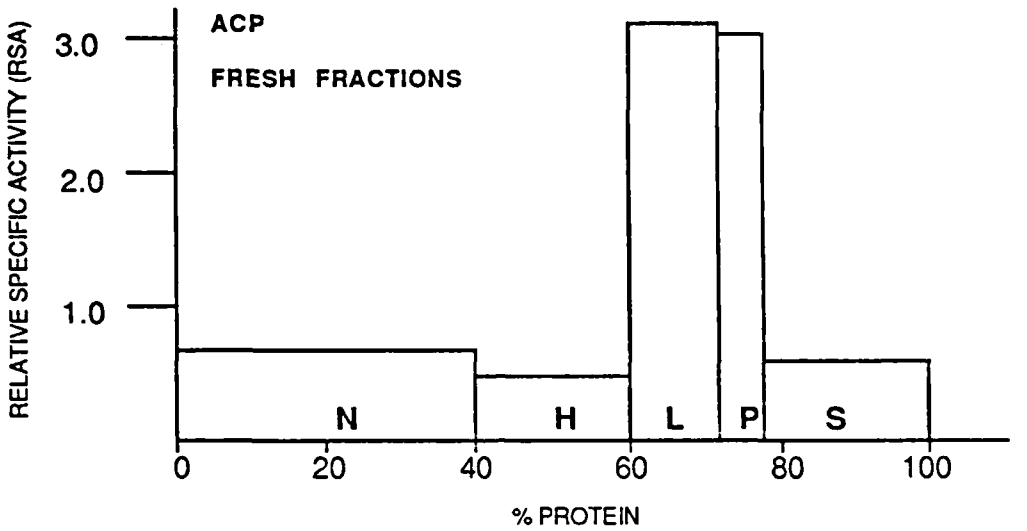
TABLE 7. Balance sheet for cell fractionation experiment for frozen-thawed tissue fractions which have been stored at -20 C overnight. For legend and definitions, see TABLE 4.

	FRACTIONS							
	HOMO	N	H	L	P	S	TOTAL	%RECOV
PROTEIN CONTENT	128	29	23	18	6	50	127	99
% RECOV	-	23	18	14	5	40		
ACP CONC	.093	.017	.022	.046	.014	.039	.142	152
% RECOV	-	13	16	33	10	28		
ACP RSA	1.0	.57	.88	2.4	2.0	.7		
AST CONC	118	.1	19	19	0	100	139	117
% RECOV	-	0	13	14	0	73		
AST RSA	1.0	0	.72	1.0	0	1.8		
CK CONC	504	11	39	46	4	644	745	147
% RECOV	-	1	5	6	1	87		
CK RSA	1.0	.04	.27	.43	.2	2.2		
GDH CONC	24	2.7	7.8	8.5	.5	17	36	149
% RECOV	-	8	21	24	2	46		
GDH RSA	1.0	.34	1.2	1.7	.4	1.2		
LD CONC	128	3	3	4	0	106	116	91
% RECOV	-	2	3	3	0	92		
LD RSA	1.0	.09	.16	.21	0	2.3		

For GDH, the total concentration of the frozen fractions increased 16% over the original concentration, but the total concentration of the fresh fractions increased 242% over their original concentration. While the frozen N, H, and L fractions showed modest increases, the same fresh fractions showed tremendous increases. GDH concentration in the S fractions remained constant. In the case of LD, total concentration of the fractions for both fresh and frozen homogenates remained virtually unchanged; but a noticeable increase occurred in the N and H fresh fractions while the fresh S fraction concentration decreased. In cases where the concentration of the enzyme decreased after overnight frozen storage, the loss of activity may simply represent the lability of the enzyme under these conditions or the lack of an optimal storage enviroment. AST, for example, will lose activity if stored without the cofactor 5-pyridoxal phosphate, which was not contained in the homogenization buffer.

Figures 6 through 10 are graphical expressions of enzyme concentration plotting RSA vs % protein and are analogous to Figures 1 through 5. For ACP, comparing Figs. 1 and 6, little change in the distribution of ACP RSA is noted after frozen storage. For AST, comparing Figs. 3 and 7 it can be seen that there has been little change for the frozen fractions except for the disappearance of activity in the N and P fractions. But for the fresh fractions, the amount of AST RSA accounted for by the N, H, and L fractions is seen to have increased. For CK, comparing Figs. 3 and 8, it can be seen that little has changed for the frozen fractions but the RSA in the fresh fractions has increased in the H, L, and P fractions. For GDH, comparing Figs. 4 and 9, it is seen that the same basic pattern exists for the frozen fractions while the RSA has significantly increased in the fresh N, H, and L fractions. Finally, for LD, comparing Figs. 5 and 10, the pattern for the

FIG. 6. ACP RSA in five subcellular fractions, prepared from fresh and frozen-thawed rat brain tissue, after overnight storage at -20 C. The total ACP concentration for the fresh fractions is .087 U/g wet weight and for the frozen-thawed fractions .142 U/g wet weight.



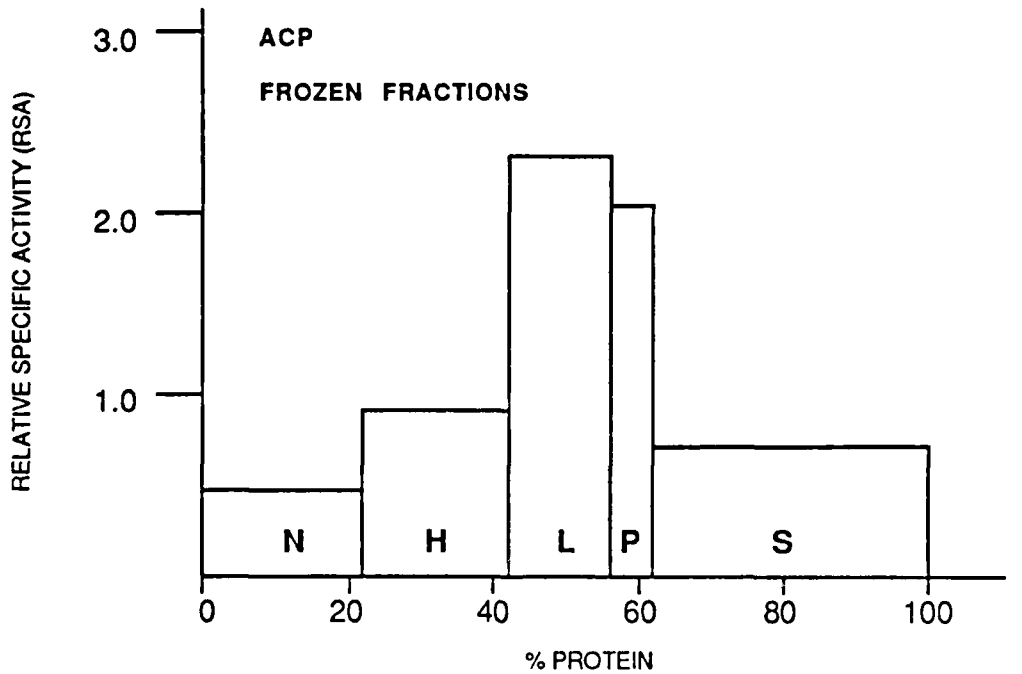
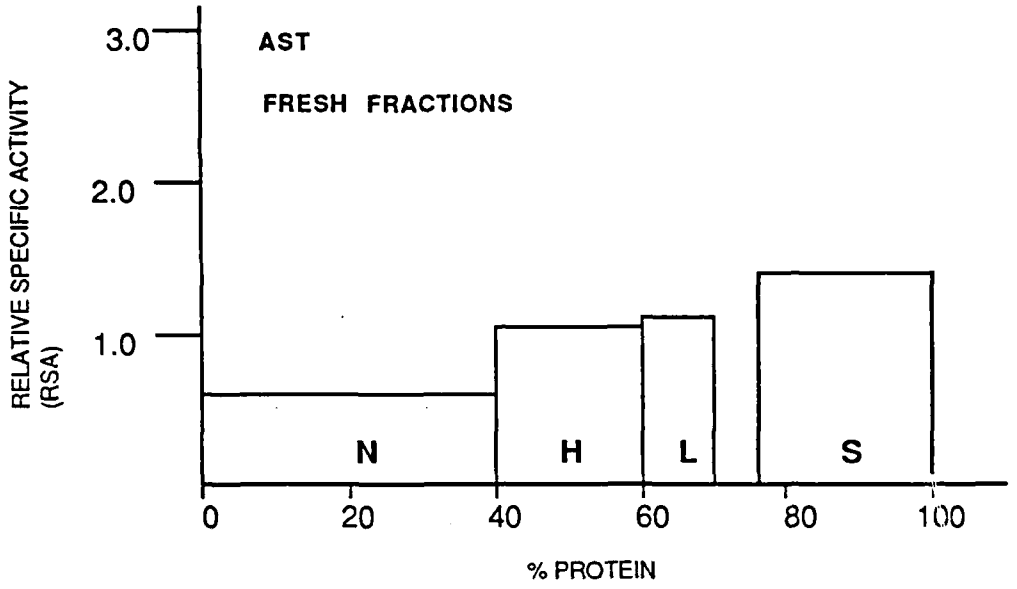


FIG. 7. AST RSA in five subcellular fractions, prepared from fresh and frozen-thawed rat brain tissue, after overnight storage at -20 C. The total AST concentration for the fresh fractions is 99 U/g wet weight and for the frozen-thawed fractions 139 U/g wet weight.



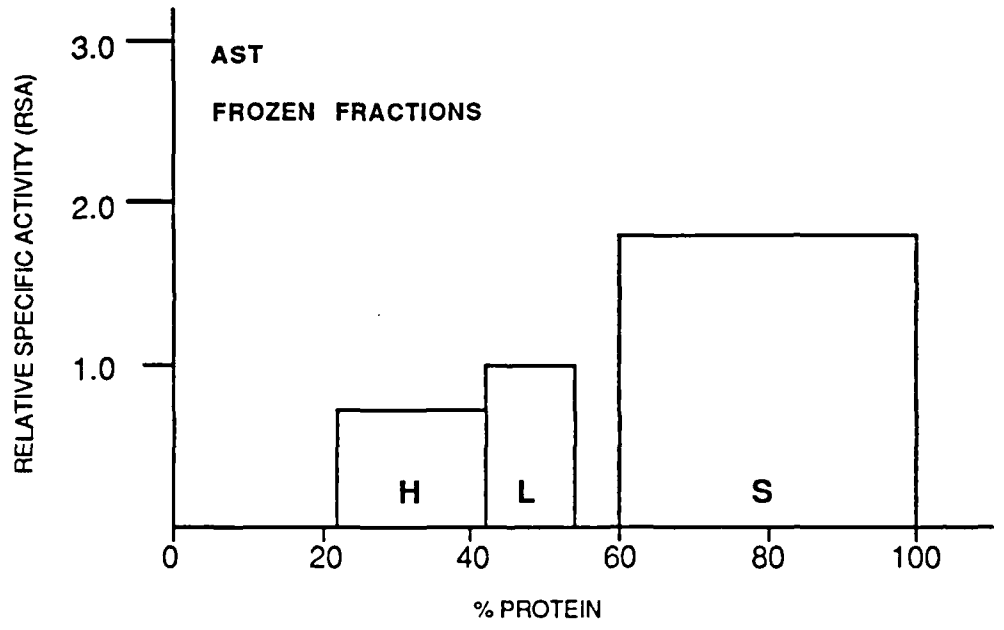


FIG. 8. CK RSA in five subcellular fractions, prepared from fresh and frozen-thawed rat brain tissue, after overnight storage at -20 C. The total CK concentration for the fresh fractions is 571 U/ g wet weight and for the frozen-thawed fractions 745 U/g wet weight.

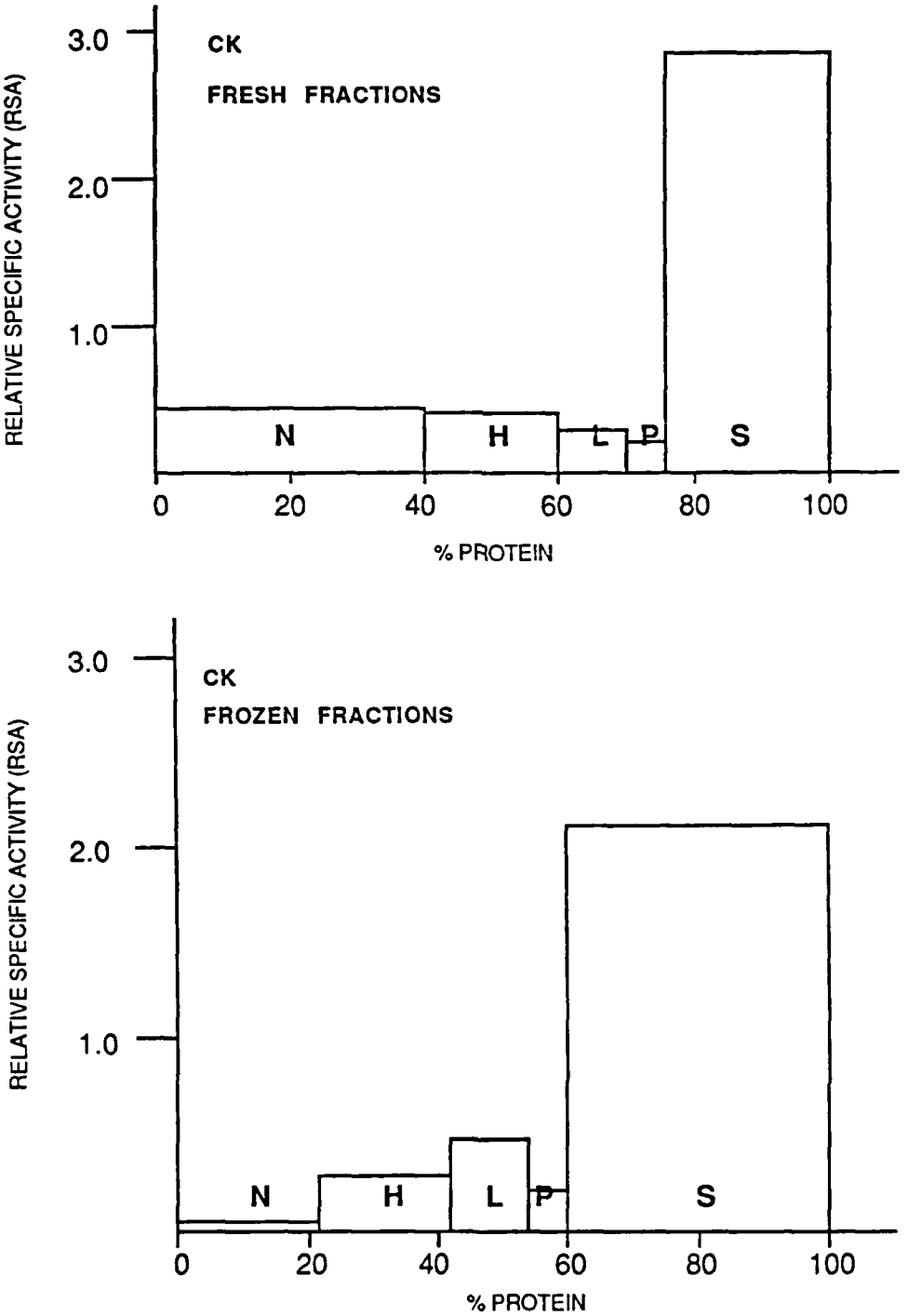
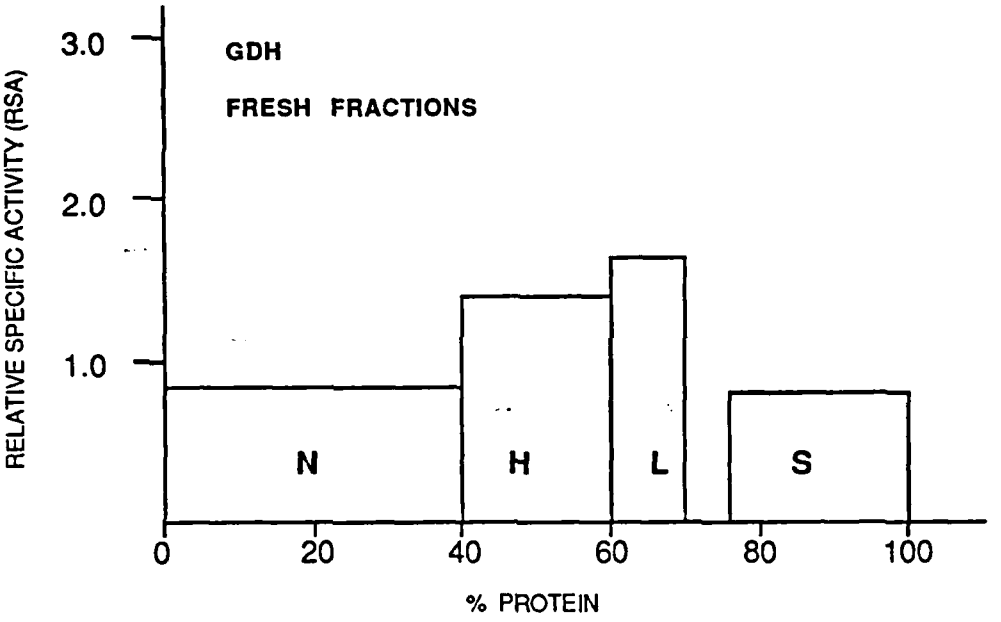


FIG. 9. GDH RSA in five subcellular fractions, prepared from fresh and frozen-thawed rat brain tissue, after overnight storage at -20 C. The total GDH concentration for the fresh fractions is 19.7 U/g wet weight and for the frozen-thawed fractions 36 U/g wet weight.



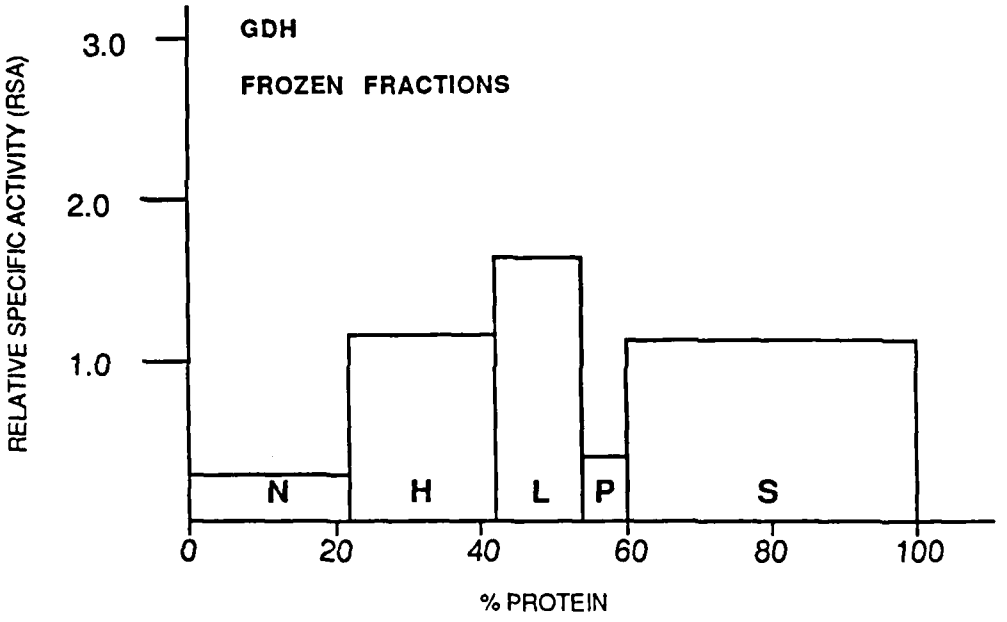
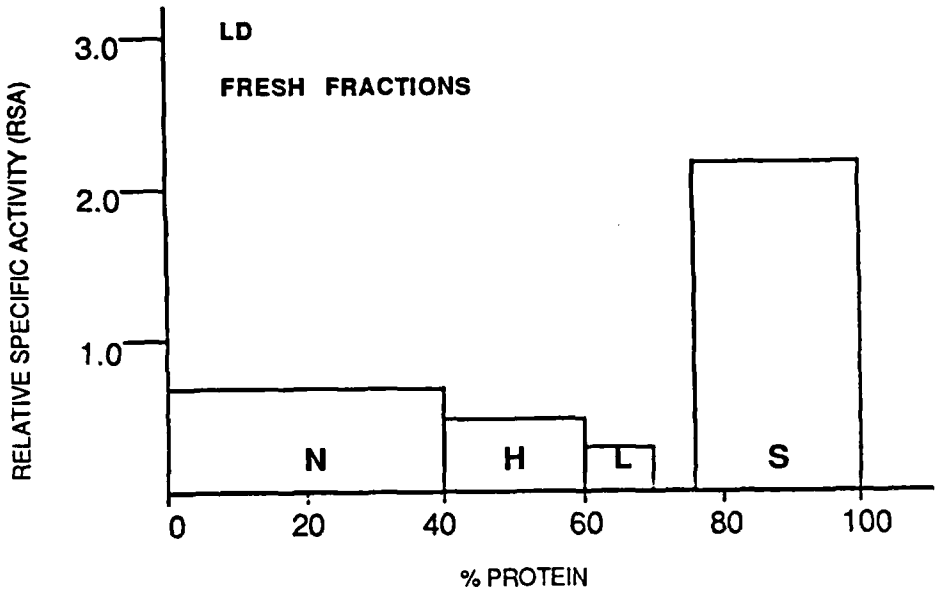
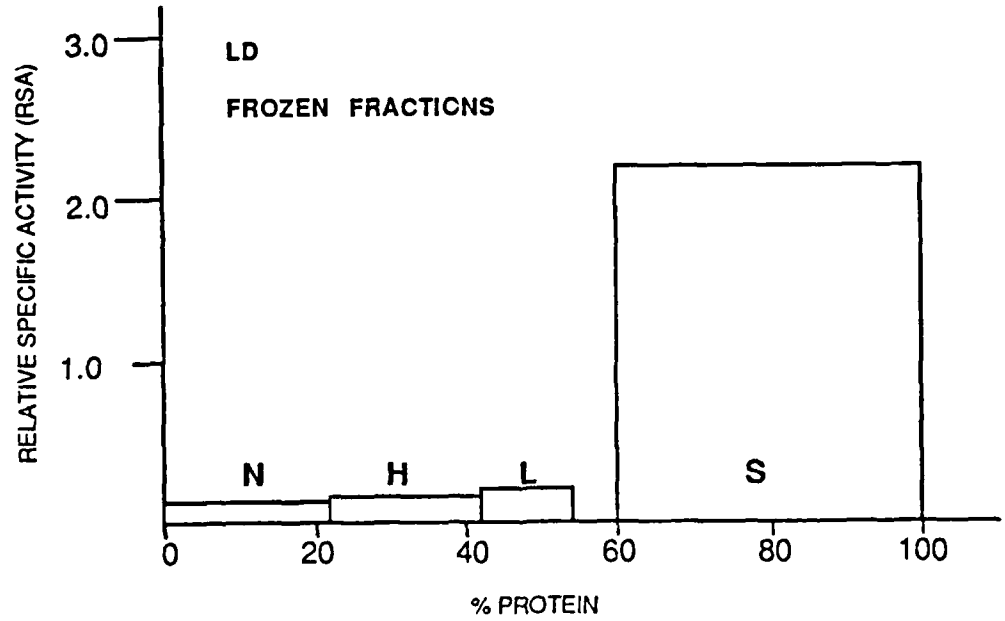


FIG. 10. LD RSA in five subcellular fractions, prepared from fresh and frozen-thawed rat brain tissue, after overnight storage at -20 C. The total LD concentration for the fresh fractions is 82 U/g wet weight and for the frozen-thawed fractions 116 U/g wet weight.





frozen fractions is little changed while for the fresh fractions the RSA for the N and H fractions has increased.

ACTIVATION ENERGY EXPERIMENT

The results are given in Table 8. For AST and CK, no statistically significant change in E_a was detected. For GDH and LD, the E_a did change significantly with freeze-thawing and did decrease. A decrease in E_a results in an increase in activity. How much the activity increases can not be readily calculated but Hagelauer and Faust have shown that for CK-MM catalytic activity increases exponentially with a decrease in E_a (55). What is important is that all four enzymes did not show a definite decrease in E_a which would result in increased activity.

MITOCHONDRIAL ENZYMES EXPERIMENT

Electron photomicrographs of the final mitochondrial pellet showed numerous mitochondria and a definite enrichment of this subcellular particle. Contaminating particles were, however, present and consisted of granules smaller than the mitochondria which were thought to be neurosecretory granules and lysosomes and fragments of nerves consisting of myelin which appeared as large concentric multi-lamellar arrays of membranes. It is not surprising that lysosomes should be found as a contaminant of the mitochondrial fraction considering the heterogeneity of both mitochondria and lysosomes and the resulting overlap between these two particles (60,61,62). Substantial amounts of myelin are also encountered in mitochondrial fractions (63). While the final pellet obtained did not represent a "pure" mitochondrial preparation, it was deemed to be acceptable for the purpose of this work as the biochemical data demonstrated a definite enrichment of the mitochondria and specific mitochondrial marker enzymes

TABLE 8. Activation energies (E_a) of homogenates prepared from fresh and in vitro frozen rat brain tissue.

ENZYME	SPECIMEN	AVERAGE Ea	RANGE
AST	FRESH 1	38	35-45
	FROZEN 1	41 (NS)	38-44
	FRESH 2	45	41-53
	FROZEN 2	43 (NS)	38-45
CK	FRESH 1	62	59-66
	FROZEN 1	58 (NS)	49-62
	FRESH 2	61	60-61
	FROZEN 2	62 (NS)	59-62
GDH	FRESH 1	30	28-31
	FROZEN 1	25 ($p<0.01$)	21-27
	FRESH 2	32	31-32
	FROZEN 2	29 ($p<0.01$)	28-30
LD	FRESH 1	62	60-63
	FROZEN 1	60 ($p<0.01$)	58-61
	FRESH 2	65	63-67
	FROZEN 2	63 ($p<0.01$)	61-65

E_a is expressed as kilojoules/mole (kJ/mol) with the S.D. in parentheses. The measured E_a represents the average of ten determinations. Statistical significance was determined using the two-tailed Student T test.

were ultimately to be measured so that enzyme activity originating from contaminants would be inconsequential.

Table 9 in the form of a balance sheet presents data from a typical mitochondrial preparation. Total protein is seen to decrease until in G only 2.5% of the original homogenate's protein is left. Only 3.2% of the original ACP activity, representing the lysosomes, is present in G while CK and LD activity, representing cytoplasmic contamination, have been reduced to 1.2% and 0.6% of their original activities. The initial specific activities (total units/protein) in the homogenate for ACP, CK, and LD are .00084, 7.37, and 1.39 respectively; the final specific activities in G for the same enzymes are .00109, 3.9, and 0.35 respectively. On the basis of these figures it is seen that an enrichment of lysosomes as indicated by the lysosomal marker enzyme ACP has occurred, which is not really unexpected given the overlap in density of mitochondria and lysosomes already mentioned. The specific activities of CK and LD, typically thought of as representing cytoplasmic enzymes, have been reduced to roughly 1/2 and 1/4 of their starting values indicating a decrease in cytoplasmic components. Beginning specific activities of the mitochondrial marker enzymes cytochrome c oxidase, GDH, and MDH are .228, .034, and 1.27 while the final specific activities of the same enzymes in G are .570, .232, and 2.32 respectively, resulting in enrichments of 2.5 x, 6.8 x, and 1.8 x respectively.

Another approach to judging the degree of enrichment of the mitochondrial fraction is to examine the initial and final ratios of lysosomal and cytoplasmic marker enzyme activities to mitochondrial marker enzyme activities. The initial cytochrome c oxidase/ACP value is 271 and the final value is 433, indicating a nearly two-fold enrichment of the mitochondrial enzyme relative to the lysosomal enzyme. The initial GDH/ACP value is 41

TABLE 9. Rat brain mitochondria balance sheet.

		FRACTIONS						
	HOMO	A	B	C	D	E	F	G
PROTEIN	912	540	376	167	160	137	47	23
% PROTEIN	100	59	41	18	18	15	5	3
CYTOCHROME c OXIDASE	208	108	35	0	31	3	23	13
% ACTIVITY	100	52	17	0	15	1	11	6
ACP	.77	.53	.27	.07	.09	.03	.04	.03
% ACTIVITY	100	69	35	10	12	10	5	3
LD	1270	723	588	443	100	20	28	8
% ACTIVITY	100	57	46	35	8	2	2	1
CK	6700	3200	3700	2600	730	280	210	89
% ACTIVITY	100	48	55	39	11	4	3	1
GDH	31	13	18	4	11	4	6	5
% ACTIVITY	100	42	56	12	36	14	19	17
MDH	1160	560	670	330	280	68	88	53
% ACTIVITY	100	48	58	28	24	6	8	5
NADP ICDH	18	4.9	10	7.4	2.4	.83	.73	.64
% ACTIVITY	100	28	58	42	14	5	4	4
NAD ICDH	2.4	1.3	1.4	0	2.3	1.1	1.3	.78
% ACTIVITY	100	55	57	0	97	44	54	32
FUMARASE	38	30	9	6	3	.6	1.9	1.5
% ACTIVITY	100	78	24	15	9	2	5	4

Enzyme activities are expressed as U/L except for cytochrome c oxidase which is expressed as nmol/min-L. Protein is expressed as mg. Percent (%) of protein or enzyme activity is in comparison to the amount in the homogenate, which is always 100%. Legend: Homo=original homogenate; A=low speed pellet; B=low speed supernatant; C=crude mitochondrial supernatant; D=crude mitochondrial pellet; E=Ficoll supernatant; F=Ficoll pellet; G=final washed mitochondrial pellet.

and the final value is 212, for a 5.2 fold enrichment. The initial cytochrome c oxidase/LD value is .164 and the final value is 1.63, for a 9.9 fold enrichment. The initial GDH/CK value is 0.25 and the final value is .663, for a 26.5 fold enrichment.

It may be concluded from the data in Table 9 that an enrichment of mitochondrial enzyme activity has been achieved relative to enzymatic activity attributable to contaminating fractions such as the cytosol and lysosomes.

EFFECTS OF FREEZE-THAWING AND HOMOGENIZATION

Table 10 demonstrates the effect on a variety of enzyme activities found in the mitochondrial preparation when it is subjected to homogenization, freeze-thawing, and freeze-thawing followed by homogenization. ACP activity emanating from the residual lysosomes increases by about the same amount, 12-18% above that of the G fraction, whether homogenization, freeze-thawing, or homogenization after freeze-thawing is employed. LD activity increases by 7-8% in the G-H and G-F fractions compared to the G fraction but these increases are not statistically significant. The 16% increase seen in the G-F-H fraction is significant and could be attributed to the release of mitochondrially bound LD which required the combined effects of freeze-thawing and homogenization. No statistically significant increases in CK activity were seen in the G-H, G-F, or G-F-H fractions when compared to the G fraction.

GDH activity was increased in the G-H and G-F fractions by 68% and 60% respectively and was doubled in the G-F-H fraction in comparison to the G fraction, indicating an additive effect of freezing and mechanical disruption. For MDH, a 3% increase in the G-H fraction is not statistically

TABLE 10. Enzyme solubilization effects of homogenization and freeze-thawing on mitochondrial preparations.

	FRACTIONS			
	G	G-H	G-F	G-F-H
PROTEIN	7.2	7.2	7.2	7.2
ACP CONC	.0104	.0123	.0116	.0120
%	100	118	112	116
LD CONC	1.71	1.85	1.83	1.98 (a)
%	100	108	107	116
CK CONC	21.2	21.8	22.3	22.5
%	100	102	105	106
GDH CONC	3.52	5.92 (a)	5.63 (a)	7.12 (a)
%	100	168	160	202
MDH CONC	20.9	21.6	27.8 (a)	27.4 (a)
%	100	103	133	131
NADP ICDH CONC	.184	.211	.312 (a)	.361 (a)
%	100	115	175	196
NAD ICDH CONC	.186	.178	.142 (a)	.112 (a)
%	100	96	76	60
FUMARASE	1.41	1.75	2.35	2.63
%	100	124	179	186

Each enzyme was assayed ten times in each fraction except for ACP and fumarase and the total concentrations were calculated from the average of these determinations. Enzyme concentration is expressed as U/g mitochondrial protein. Legend: G=original mitochondrial preparation; G-H=mitochondrial preparation after homogenization; G-F= mitochondrial preparation after freeze-thawing; G-F-H=mitochondrial preparation after freeze-thawing followed by homogenization. Each fraction contained an equivalent amount of protein. Statistical significance of the effects of freeze-thawing and homogenization was determined by comparison to the G fraction using the two-tailed Student T test.

a=$p<0.001$

significant but the 33% and 31% increases in the G-F and G-F-H fractions are. Similarly, the 15% increase in NADP-linked ICDH activity seen in the G-H fraction is not statistically significant but the 75% and 96% increases in the G-F and G-F-H fractions are. It is interesting that NAD-linked ICDH activity decreased with freezing and homogenization. This may be suggestive of the loss of a vital cofactor upon solubilization or of the importance of the microenviroment within the mitochondria to the activity of this enzyme. The 4% decrease in the G-H fraction is not meaningful statistically but the 34% and 40% decreases seen in the G-F and G-F-H fractions are. Fumarase activity increased 24% in the G-H fraction but was increased even more, 79% and 86% in the G-F and G-F-H fractions.

FREEZING AND HOMOGENIZATION EFFECTS ON ACTIVATION ENERGY

The activation energies are listed in Table 11. Activation energies for LD in all fractions covered a rather large range. This may be due to the fact that relatively little LD activity was present as LD is primarily a cytosolic enzyme. So great was the variation in Ea that a meaningful interpretation of the results is questionable. For CK, the Ea does not change significanctly with homogenization or freeze-thawing. For GDH, the Ea decreases with homogenization or freeze-thawing, and reaches its lowest value after combined freeze-thawing and homogenization, $p<0.001$. MDH does not show a significant change after homogenization but the Ea observed in the G-F and G-F-H fractions are lower and statistically significant. The results for NADP-linked ICDH are difficult to interpret for the same reason as the LD Ea data- there is tremendous variability in each fraction. The NAD-linked ICDH activation energies are really surprising as they are negative. NAD-

TABLE 11. Activation energies (E_a) of rat brain mitochondrial preparations.

	FRACTIONS			
Ea	G	G-H	G-F	G-F-H
LD Ea	73	124 (a)	57	63
RANGE	41-117	90-185	46-81	50-75
CK Ea	85.6	88.7	84.4	85.4
RANGE	83-87	85-90	79-86	83-89
GDH Ea	34.6	31.8 (a)	30.9 (a)	28.2 (a)
RANGE	32-36	30-32	29-32	27-28
MDH Ea	79	75	69 (a)	67 (a)
RANGE	65-86	64-83	62-74	61-69
NADP ICDH Ea	76	80	82	100
RANGE	34-111	35-123	69-94	65-125
NAD ICDH Ea	-22	-15	-8 (a)	-6 (a)
RANGE	(-30)-(-11)	(-43)-(-2)	(-19)-11	(-34)-11

The average E_a was calculated from the average of ten determinations at both 25 C and 37 C. E_a is expressed in kilojoules/mole (kJ/mol). Statistical significance was determined using the two-tailed Student T test comparing the G-H, G-F, and G-F-H fractions to the G fraction.
a=$p<0.01$

linked ICDH activity is actually increased at 25 C as compared to 37 C indicating an optimal reaction temperature that is different from most enzymes and which results in negative Ea values. Perhaps this enzyme is actually very temperature sensitive and may be undergoing denaturation in vitro at 37 C. Although there is considerable variability in the range of activation energies observed, it does appear that there is a statistically significant increase in Ea after freeze-thawing.

EFFECT OF MULTIPLE FREEZE-THAWINGS

The results of these experiments are shown in Figures 11-17. Maximal ACP activity is obtained after two to three freeze-thawings and appears to decrease with continued freeze-thawing. AST activity reaches a maximum after about three freeze-thawings and appears to decrease after a fourth freeze-thaw cycle. CK activity peaks after about two freeze-thaws and maintains at that level through four freeze-thaw cycles. GDH shows a peak of activity with three freeze-thawings and appears to decline in activity after one more cycle. ICDH activity seems to be maximal after about three freeze-thaw cycles and to be maintained at that level through a fourth cycle. LD activity also appears to peak after three freeze-thaws but appears to decrease after one more. MDH follows the same pattern as LD.

ENZYME SOLUBILIZATION EFFECT OF DETERGENTS

The results are given in Tables 12 and 13 and Figs 18-24. While freeze-thawing is considerably more effective than simple homogenization in releasing enzymatic activity, it is clear that homogenization of tissue in some detergent mediums is even a more potent approach to enzyme solubilization. How effective detergent enzyme solubilization treatment is depends on the particular enzyme and detergent. NWA is clearly the best choice for ACP. While two other non-ionic detergents cause noticeable increases in ACP

FIG. 11. ACP concentration in rat brain tissue homogenates after multiple freeze-thawings. Each line represents a separate experiment.

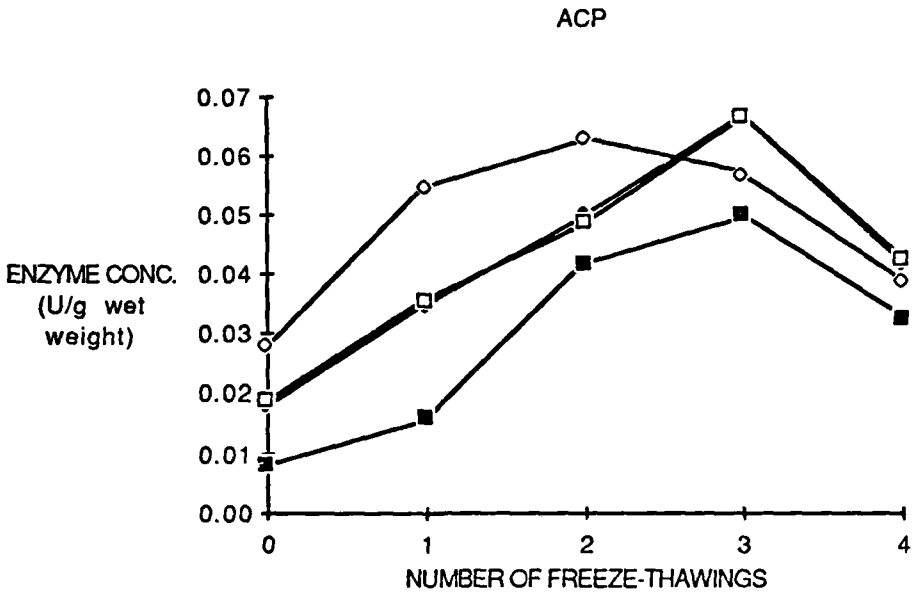


FIG. 12. AST concentration in rat brain tissue homogenates after multiple freeze-thawings. Each line represents a separate experiment.

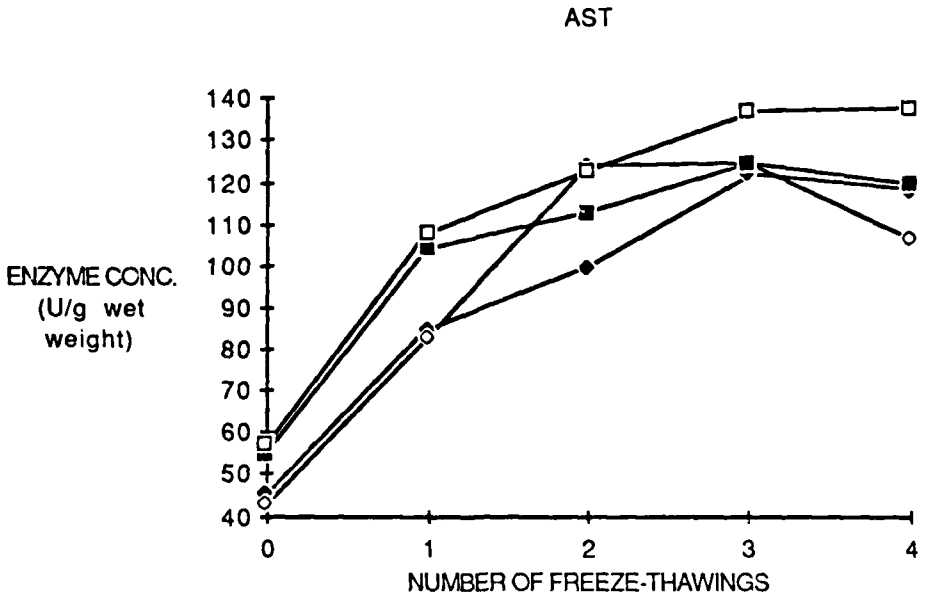


FIG. 13. CK concentration in rat brain tissue homogenates after multiple freeze-thawings. Each line represents a separate experiment.

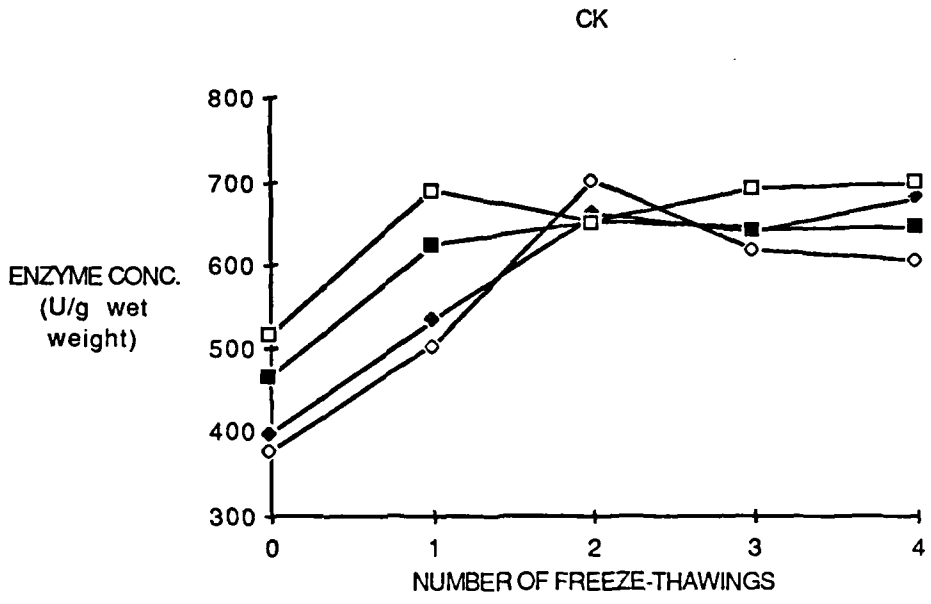


FIG. 14. GDH concentration in rat brain tissue homogenates after multiple freeze-thawings. Each line represents a separate experiment.

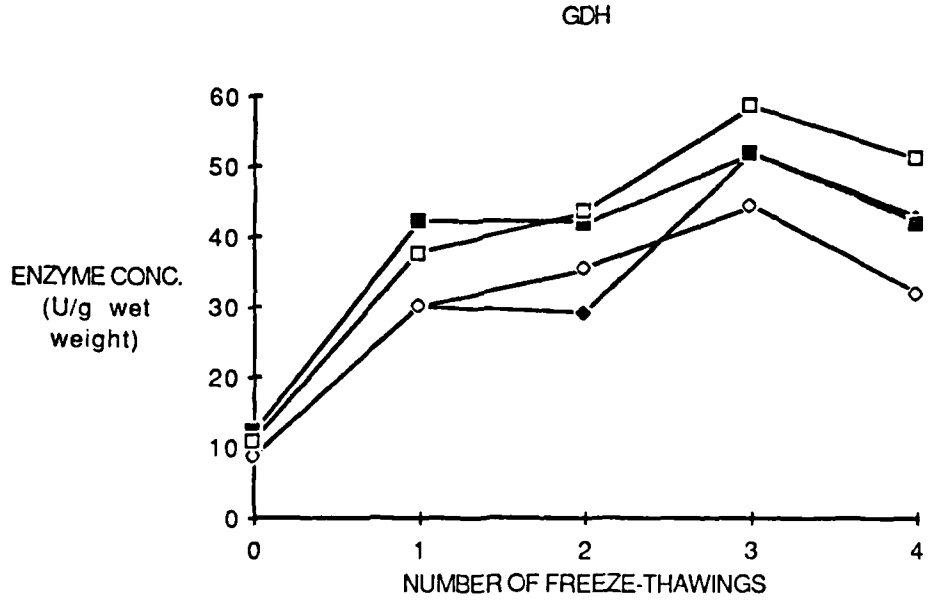


FIG. 15. ICDH concentration in rat brain tissue homogenates after multiple freeze-thawings. Each line represents a separate experiment.

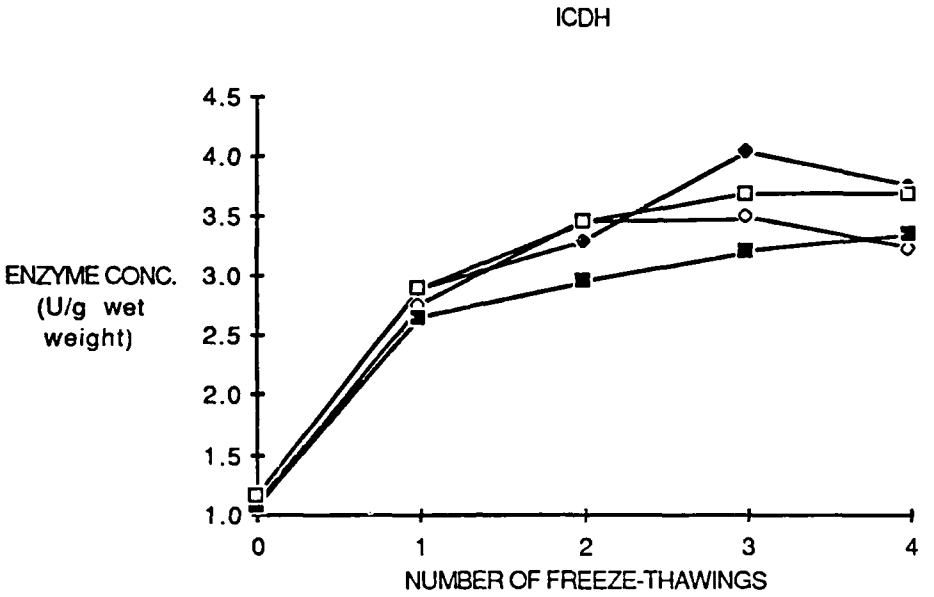


FIG. 16. LD concentration in rat brain tissue homogenates after multiple freeze-thawings. Each line represents a separate experiment.

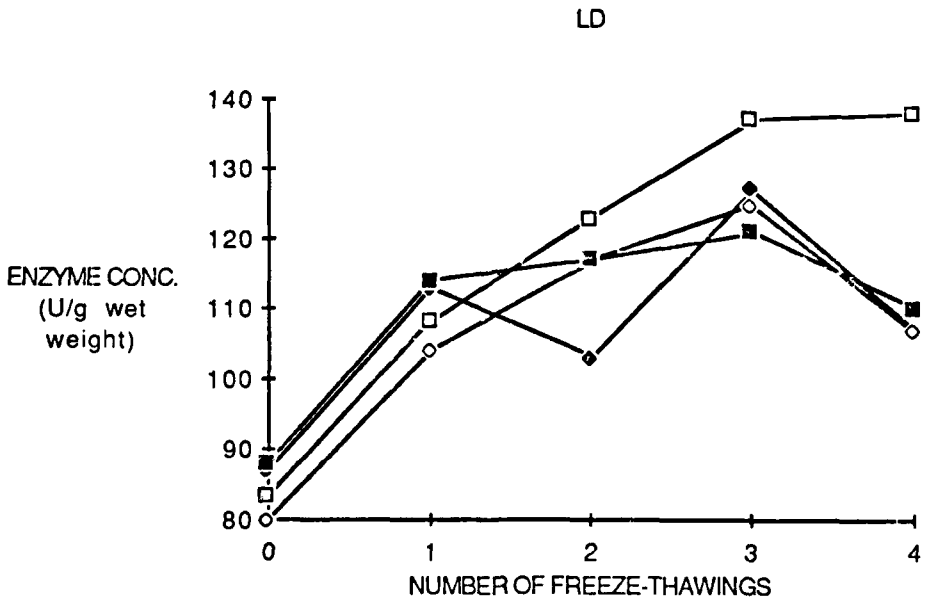


FIG. 17. MDH concentration in rat brain tissue homogenates after multiple freeze-thawings. Each line represents a separate experiment.

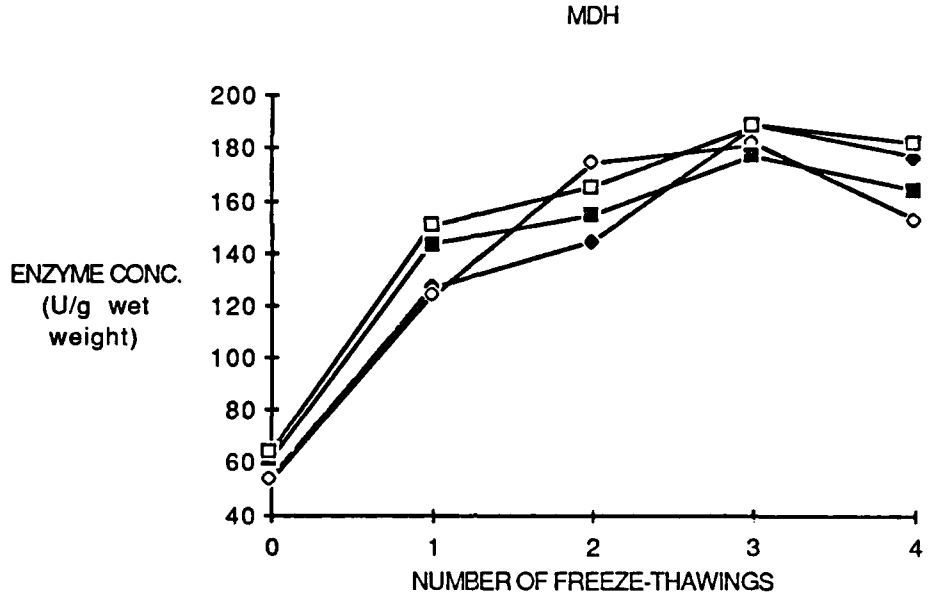


TABLE 12. Enzyme concentrations in rat brain homogenates after different enzyme solubilization treatments.

	ENZYME CONCENTRATIONS						
TREATMENT	ACP	AST	CK	GDH	ICDH	LD	MDH
NONE	.020 (.005)	33 (6)	345 (42)	3 (1)	4 (.5)	40 (5)	44 (7)
F-T	.079 (.01)	89 (7)	521 (51)	22 (4)	12 (2)	76 (5)	113 (11)
X-100	.091 (.01)	153 (20)	700 (50)	62 (11)	35 (3)	132 (24)	224 (14)
NWA	.143 (.02)	175 (7)	755 (52)	65 (16)	37 (7)	156 (25)	269 (21)
CS	.094 (.02)	171 (16)	826 (55)	61 (6)	12 (2)	143 (16)	284 (14)
BRIJ	.061 (.01)	97 (9)	605 (45)	42 (7)	14 (2)	89 (9)	160 (7)
3-12 Z	.035 (.007)	167 (22)	826 (57)	23 (2)	0	94 (14)	250 (25)
3-14 Z	.019 (.005)	185 (15)	804 (60)	28 (9)	11 (1)	145 (6)	266 (10)

Enzyme concentrations are given in U/g wet tissue weight and represent the average concentrations of four animals with S.D. in parentheses. All homogenates were made from 20-30 mg of rat brain frontal cortex tissue. All detergent concentrations were 1%, made up in Tris homogenization buffer, pH 7.4. Abbreviations: F-T= frozen-thawed; X-100= Triton X-100; NWA= New Wetting Agent; CS= Clearing Solution; Brij= Brij 35; 3-12 Z= 3-12 Zwittergent; 3-14 Z= 3-14 Zwittergent.

TABLE 13. Percent of enzyme concentration released from rat brain homogenates after different enzyme solubilization treatments.

	% OF ENZYME CONCENTRATION RELEASED						
TREATMENT	ACP	AST	CK	GDH	ICDH	LD	MDH
NONE	14	18	42	5	11	26	15
F-T	55	48	63	34	32	49	40
X-100	64	83	85	95	95	85	79
NWA	100	95	91	100	100	100	95
CS	66	92	100	94	32	92	100
BRIJ	43	52	73	65	38	57	56
3-12 Z	24	90	100	35	0	60	88
3-14 Z	13	100	97	43	30	93	94

The solubilization treatment yielding the highest concentration of enzyme was defined as the treatment that released 100% of enzyme activity and all other percentages were calculated in comparison to it.

FIGURE 18. Effect of 1% detergent solutions on the solubilization of ACP.

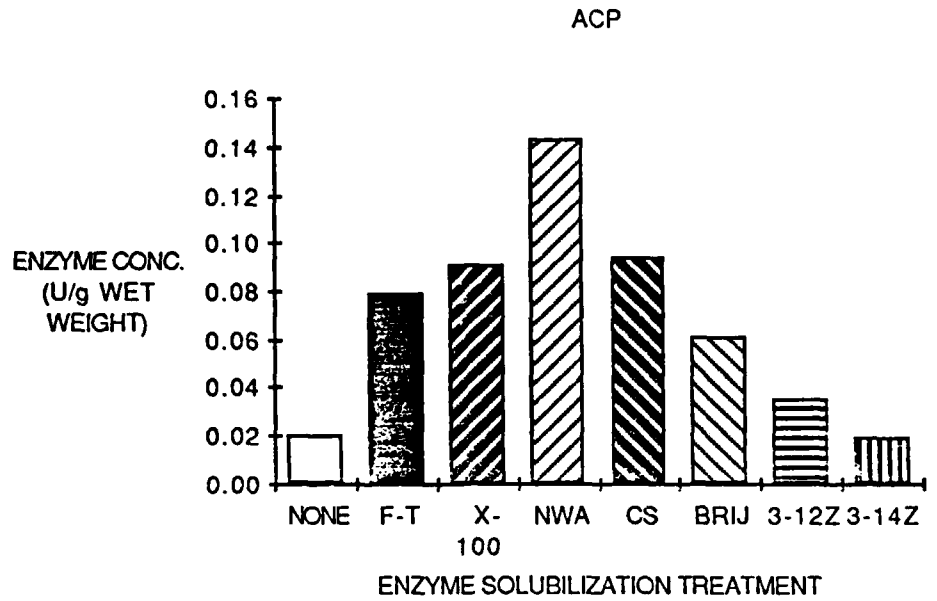


FIGURE 19. Effect of 1% detergent solutions on the solubilization of AST.

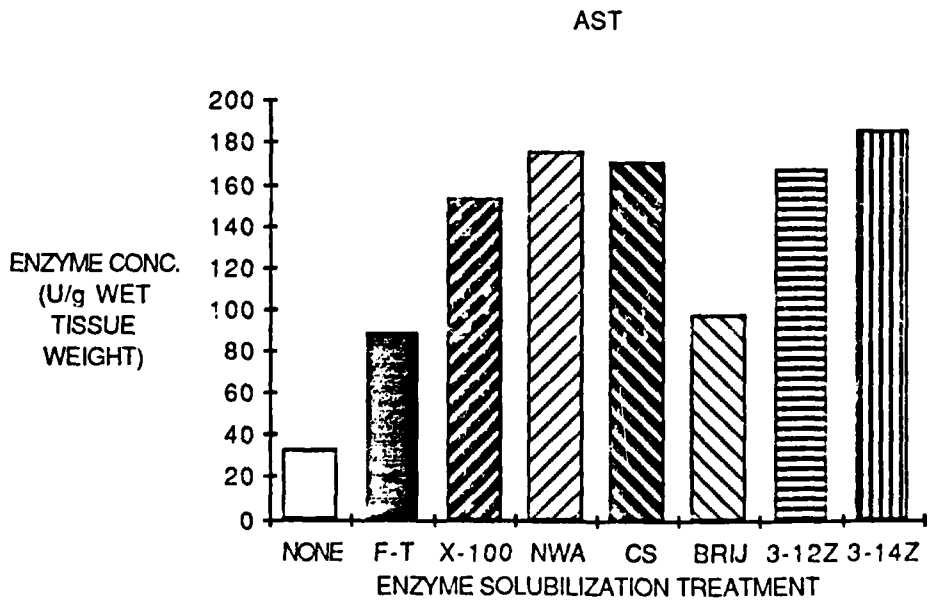


FIGURE 20. Effect of 1% detergent solutions on the solubilization of CK.

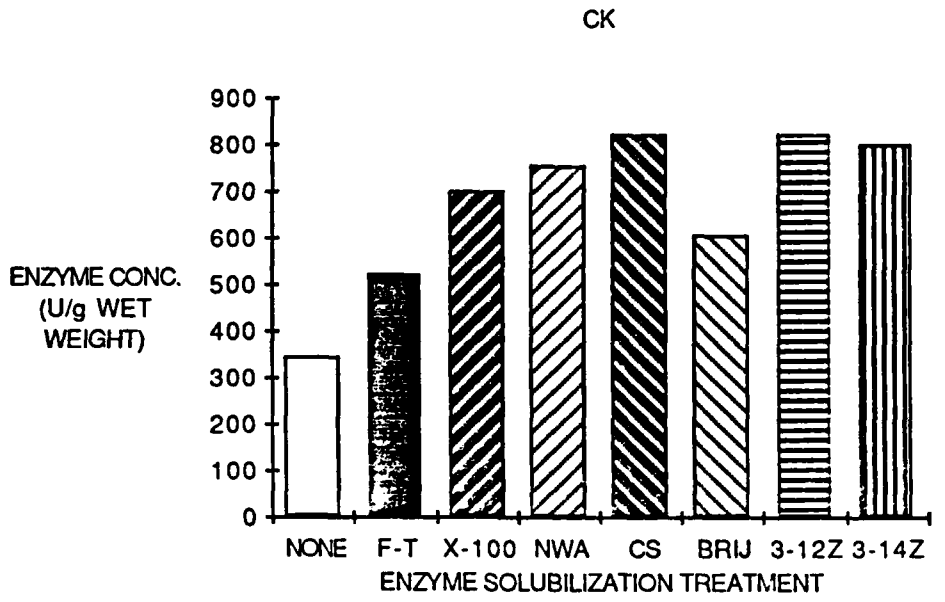


FIGURE 21. Effect of 1% detergent solutions on the solubilization of GDH.

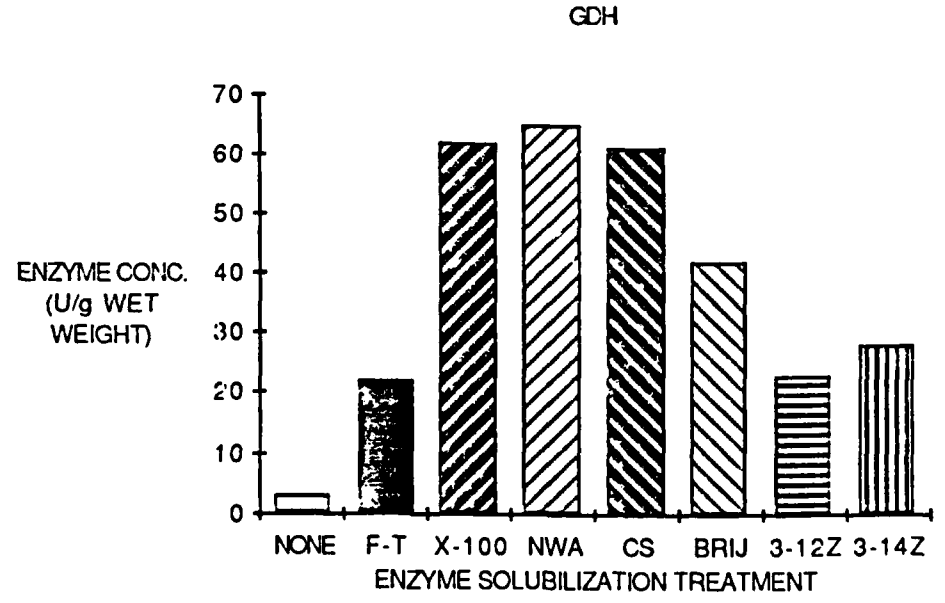


FIGURE 22. Effect of 1% detergent solutions on the solubilization of ICDH.

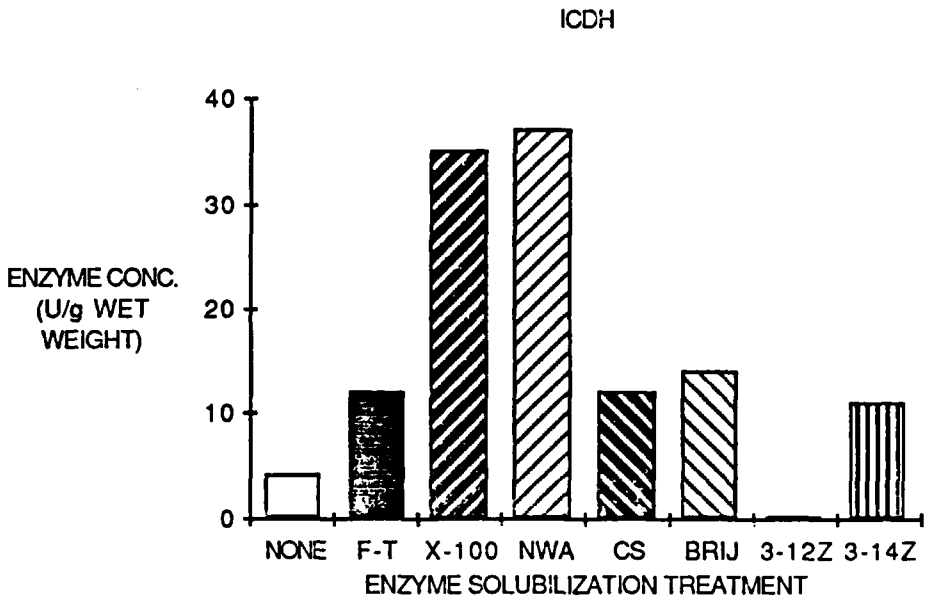


FIGURE 23. Effect of 1% detergent solutions on the solubilization of LD.

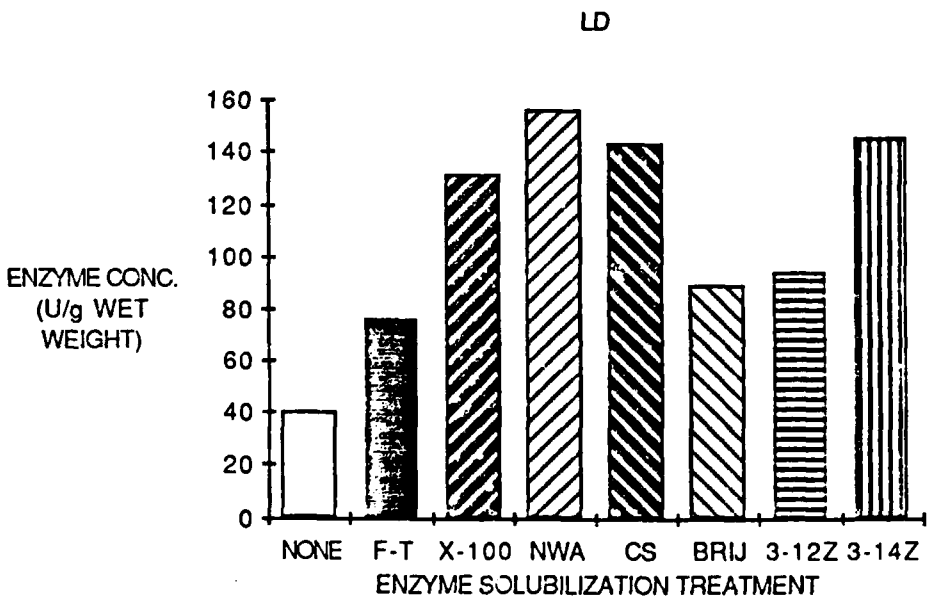
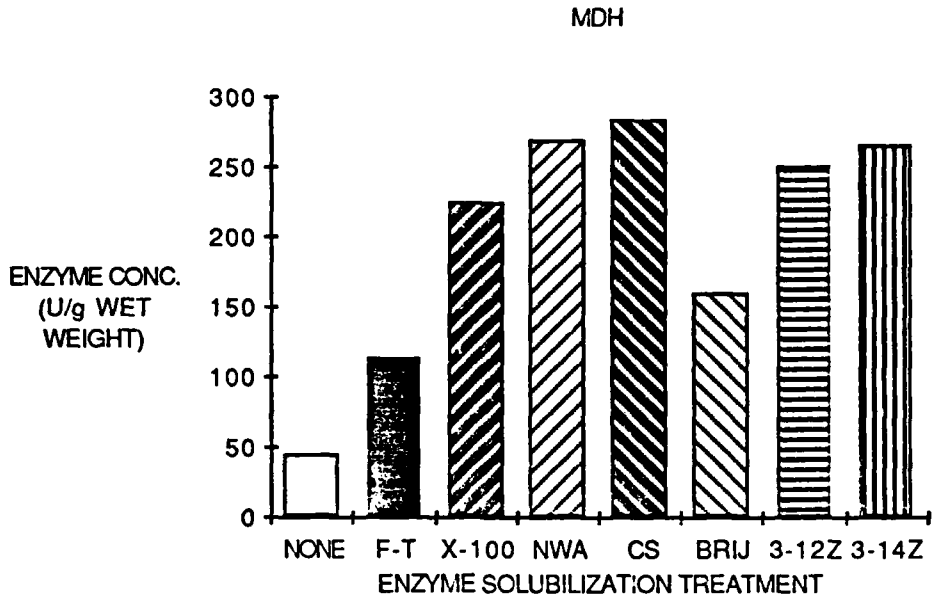


FIGURE 24. Effect of 1% detergent solutions on the solubilization of MDH.



activity, Brij and the two zwitterionic detergents are not as effective as free ›-thawing in promoting solubilization. For AST, Brij is roughly equivalent to freeze-thawing but all of the other detergents cause at least a 70% greater release of AST in comparison to freeze-thawing. In the case of CK, all of the detergents out-perform freeze-thawing with the CK concentrations ranging from 16-58% greater. For GDH, the zwitterionic detergents offer some improvement in enzyme solubilization over freeze-thawing, 5% and 27% for 3-12 Z and 3-14 Z, respectively. All of the non-ionic detergents are particularly effective in solubilizing GDH with the enzyme concentrations being 91-295% greater in comparison to freeze-thawing. A different pattern is seen with ICDH as 3-12 Z seems to totally inhibit ICDH activity and CS, Brij, and 3-14 Z perform no better than freeze-thawing. X-100 and NWA however cause the release of about three times the amount of ICDH as does freeze-thawing. For LD, Brij and 3-12 Z result in modest increases over freeze-thawing and the other detergents cause very substantial increases in LD release, varying from 73-200% greater than the concentrations seen with freeze-thawing. Finally, with MDH all the detergents substantially increase the amount of enzyme released in comparison to freeze-thawing.

AK activity was found to be present in the fresh, frozen-thawed, and detergent homogenates. While sufficient AK activity was present to overcome the AK inhibitors present in the CK reaction mixture, AK activity caused negligible interference. For the fresh, frozen-thawed, and NWA homogenates AK activity accounted for only 2-4% of the total CK activity.

NWA appears to be the most consistently effective detergent for solubilization of all seven of the enzymes studied and it was chosen for further evaluation as an enzyme solubilization reagent.

ENZYME SOLUBILIZATION DOSE-RESPONSE CURVE OF NWA

The results are given in Table 14. NWA at a concentration of 1% is the most effective for maximal enzyme solubilization. This concentration resulted in the highest enzyme concentrations for four of the seven enzymes assayed and was nearly equivalent to the optimal concentrations, and thus on the plateau of the dose-response curve, in the cases of the other three enzymes.

ENZYME ACTIVITY INTERFERENCE DUE TO DETERGENTS

The results are given in Tables 15 and 16. For AST, none of the six detergents studied had any appreciable effect on the amount of enzyme activity measured. For CK, all of the detergents actually enhanced CK activity with increases ranging from 7-21% over the control. Slight negative interferences were seen in the case of GDH with all of the non-ionic detergents, which ranged from 93-96% of the control value, but the zwitterionic detergents almost totally inhibited GDH activity, suppressing the measured activity to only 2-3% of the control. NWA, CS, and Brij have no appreciable effect on LD activity while X-100 and 3-14 Z result in the loss of about 8-9% of activity. The ionic detergent 3-12 Z decreases the LD activity to only 23% of the control. Six of the detergents have little effect on MDH activity with values ranging from 95-100% of the control activity; 3-12 Z results in 91% of the control value.

TABLE 14. Enzyme solubilization dose-response curve of NWA

	ENZYME CONCENTRATIONS						
% NWA	ACP	AST	CK	GDH	ICDH	LD	MDH
0	.031 (.007)	48 (11)	191 (6)	6 (1.3)	.72 (.1)	22 (2)	54 (10)
.25	.082 (.01)	121 (9)	858 (27)	41 (5)	3.5 (.2)	80 (9)	174 (10)
.5	.112 (.02)	190 (20)	1052 (53)	46 (1)	3.8 (.5)	85 (8)	249 (25)
1.0	.107 (.02)	207 (24)	958 (37)	52 (1.3)	4.2 (.1)	98 (5)	271 (33)
1.5	.099 (.02)	180 (18)	952 (43)	46 (2)	4.3 (.5)	88 (3)	245 (20)
2.0	.093 (.02)	184 (12)	913 (23)	59 (5)	4.2 (.3)	81 (5)	246 (18)

Enzyme concentrations are given in U/g wet tissue weight and represent the average of four experiments with the S.D. in parentheses.

TABLE 15. Enzyme activity interference due to detergents.

	ENZYME ACTIVITIES				
DETERGENT	AST	CK	GDH	LD	MDH
NONE	349 (3)	468 (35)	74 (4)	142 (3)	407 (4)
X-100	345 (12)	568 (17)	69 (1)	131 (7)	389 (9)
NWA	361 (4)	553 (44)	71 (3)	141 (2)	401 (2)
CS	355 (5)	501 (40)	70 (2)	138 (3)	395 (8)
BRIJ	354 (7)	542 (47)	71 (1)	143 (2)	402 (2)
3-12 Z	345 (1)	519 (22)	1.4 (.4)	32 (2)	363 (3)
3-14 Z	352 (5)	547 (21)	2.3 (.9)	129 (2)	381 (7)

Enzyme activities are expressed as U/L with the S.D. in parentheses. All specimens were run in quadruplicate. Detergents were present in 1% concentrations.

TABLE 16. Percent of enzyme activity in presence of detergents.

	PERCENT OF ENZYME ACTIVITY				
DETERGENT	AST	CK	GDH	LD	MDH
NONE	100	100	100	100	100
X-100	99	121	93	92	96
NWA	103	118	96	99	100
CS	102	107	95	97	99
BRIJ	101	116	96	101	100
3-12 Z	99	111	2	23	91
3-14 Z	101	117	3	91	95

The activity measured in the control specimen, to which only physiologic saline was added, is defined as 100%.

The enhancement of CK activity seen with the detergents appears to be due to a direct effect of the detergents on the enzyme molecule. Homogenate with and without NWA had equivalent CK activity after a hard centrifugation run designed to remove small particulate matter from which CK could be solubilized. A definite increase in CK activity was noted after NWA was added to the centrifuged homogenate.

EFFECTS OF DETERGENT SOLUBILIZATION AFTER FREEZE-THAWING

The results are given in Table 17. The data suggest that six freeze-thaw cyles or more has a negative effect on enzyme activities as the enzyme concentrations decreased for all four of the enzymes measured after six cycles in comparison to the concentrations after two cycles. In all cases, substantial amounts of enzyme were released when re-homogenization of the residual pellet in a 1% solution of NWA was performed after one or more freeze-thawings. For AST, 30-40% of the total enzyme concentration was released after NWA treatment following freeze-thawing. For Ck, 25-30% of the total enzyme concentration was released by the detergent treatment of the frozen-thawed pellets. For LD, 20-24% of the total enzyme concentration could be releaed from the residual pellets by re-homogenization in a NWA solution. Finally, with MDH 34-44% of the total enzyme concentration was released by the detergent treatment following freeze-thawing.

ISOENZYME DISTRIBUTION AFTER ENZYME SOLUBILIZATION

As seen in Fig. 25, when fresh tissue was homogenized or frozen-thawed and then homogenized, the resulting homogenate supernatant contained only the CK-BB isoenzyme, as anticipated (64). When a 1% solution of NWA

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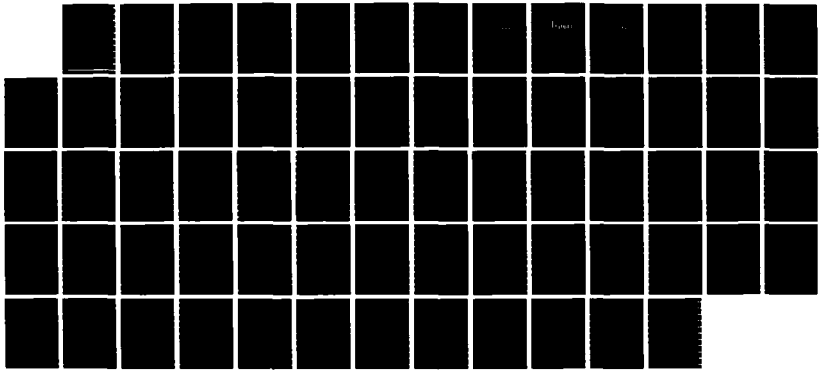

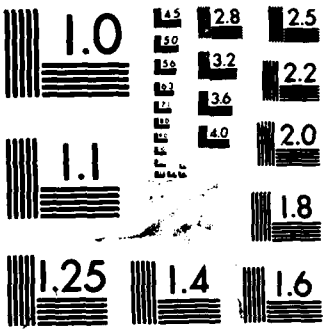

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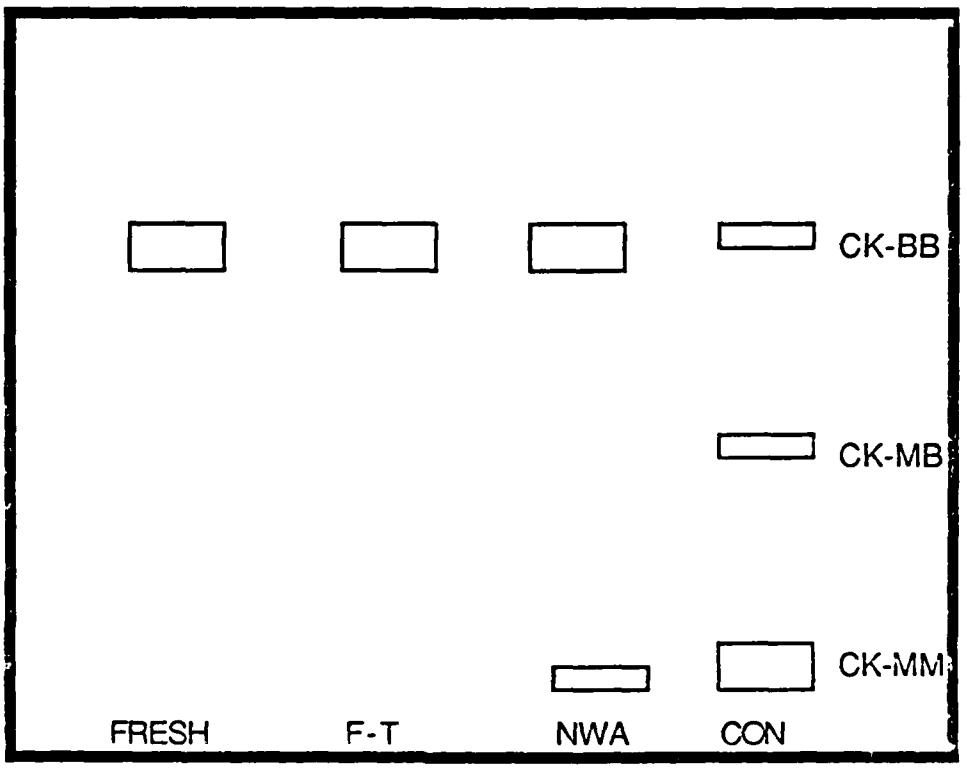
TABLE 17. Effects of detergent solubilization after freeze-thawing.

ENZYME	# FREEZE-THAWS	ENZYME CONC.	ENZYME CONC. AFTER NWA	TOTAL ENZYME CONC.
AST	1	94	68	162
	2	130	66	196
	6	103	42	145
	8	79	35	114
CK	1	595	206	801
	2	654	214	868
	6	483	178	661
	8	368	162	530
LD	1	76	21	97
	2	99	27	126
	6	70	23	93
	8	69	17	86
MDH	1	111	89	200
	2	145	85	230
	6	112	54	166
	8	109	56	165

Enzyme concentrations are in U/g wet tissue weight. Tissue specimens were frozen-thawed the specificed number of times, homogenized, centrifuged, and the supernatants were decanted. The residual pellets were re-homogenized in 1% NWA Tris homogenization buffer, pH 7.4.

FIGURE 25. CK isoenzyme distribution after enzyme solubilization.



FRESH= SIMPLE HOMOGENIZATION OF FRESH TISSUE
F-T= FREEZE-THAWING FOLLOWED BY HOMOGENIZATION
NWA= NWA DETERGENT HOMOGENIZATION
CON= SERUM CONTROL

Electrophoresis was performed on agarose gel and bands were visualized using a scanning fluorescence densitometer.

made up in Tris homogenization buffer was used in the preparation of the homogenate, two peaks were observed on the electrophoresis scan. One peak, CK-BB, accounted for about 92% of the total CK, and the other peak represented about 8%. The second, smaller peak appears at about the same position as the CK-MM peak of the control serum. It is quite possible that it represents CK-MM derived from blood in the brain. However, CK-MM from blood would also be expected to be found in the fresh and frozen-thawed homogenates, but it is not. It is also possible that this peak represent mitochondrial CK. Mitochondrial CK has been found in two forms: a large (350,000 M.W.) type that migrates cathodal to the MM band, and a dimer (80,000 M.W.) type that migrates at the MM band position, or so close to it as to be virtually indistinguishable from it (65,66,67,68). Thus the small peak seen on the NWA homogenate electrophoresis scans may be the low molecular weight form of mitochondrial CK.

Whether simple homogenization, freeze-thawing, or detergent treatment is used, the LD isoenzyme pattern is essentially the same. The total LD content breaks down as follows: LD-1, 19-21%; LD-2, 18-23%; LD-3, 21-25%; LD-4, 25-30%; and LD-5, 5-9%. No atypical bands were detected.

REGIONAL VARIABILITY EXPERIMENTS

RAT BRAIN

The results are given in Tables 18,19, and 20 and Figs. 26-32 for brain tissue that was homogenized without any special enzyme solubilization treatment, for tissue that was frozen and thawed before homogenization, and for tissue that was homogenized in a 1% solution of NWA made up in homogenization buffer. Thus, there are three different sets of tissue homogenates for all seven enzymes and brain regions. The enzyme

TABLE 18. Regional enzyme concentrations in the rat brain using fresh tissue.

BRAIN REGIONS	ENZYMES CONCENTRATIONS						
	ACP	AST	CK	GDH	ICDH	LD	MDH
CEREBELLUM	.023 (.008)	57 (6)	792 (57)	6.0 (1.4)	1.44 (.2)	59 (5)	69 (3)
MEDULLA OBLONGATA AND PONS	.025 (.01)	57 (7)	444a (33)	8.2a (.8)	1.66 (.3)	48a (6)	61 (8)
HYPOTHALA-MUS	.032 (.008)	39a (4)	292a (14)	6.7 (.5)	1.52 (.2)	41a (4)	45a (4)
STRIATUM	.024 (.008)	48 (4)	487a (63)	6.1 (1.5)	1.16 (.2)	52 (4)	58a (5)
MIDBRAIN	.027 (.01)	52 (8)	388a (39)	6.7 (2.1)	1.41 (.3)	47a (7)	57 (10)
CORTEX	.028 (.008)	40a (5)	455a (42)	5.3 (1.4)	1.02a (.2)	58 (5)	57a (2)
HIPPOCAMPUS	.026 (.016)	39a (8)	417a (35)	5.7 (1.7)	.92a (.3)	53 (7)	51a (7)

The enzyme concentrations are expressed as U/g wet tissue weight with the S.D. in parentheses and represent the average concentration of five animals. All brain tissue was homogenized without freeze-thawing. The mean enzyme concentrations of the other six brain regions were compared to that of the cerebellum and statistical significance was determined using the two-tailed Student T test.
a = $p<0.01$

TABLE 19. Regional enzyme concentrations in the rat brain using frozen-thawed tissue.

BRAIN REGIONS	ENZYME CONCENTRATIONS						
	ACP	AST	CK	GDH	ICDH	LD	MDH
CEREBELLUM	.044 (.008)	96 (7)	900 (57)	22.3 (6)	3.2 (.35)	81 (5)	116 (9)
MEDULLA OBLONGATA AND PONS	.048 (.007)	100 (14)	514a (37)	38.5a (10)	3.13 (.41)	65a (5)	110 (13)
HYPOTHALAMUS	.057 (.011)	91 (11)	398a (45)	41.4a (8)	3.56 (.51)	81 (10)	110 (14)
STRIATUM	.043 (.007)	95 (11)	609a (58)	22.0 (7)	2.44a (.37)	92a (9)	112 (12)
MIDBRAIN	.049 (.007)	114a (15)	501a (36)	35.7a (8)	3.07 (.24)	94a (8)	126 (15)
CORTEX	.049 (.006)	101 (7)	586a (40)	24.5 (7)	2.58a (.28)	107a (5)	134a (11)
HIPPOCAMPUS	.050 (.007)	90 (9)	485a (58)	21.3 (4)	2.58a (.29)	89a (9)	113 (12)

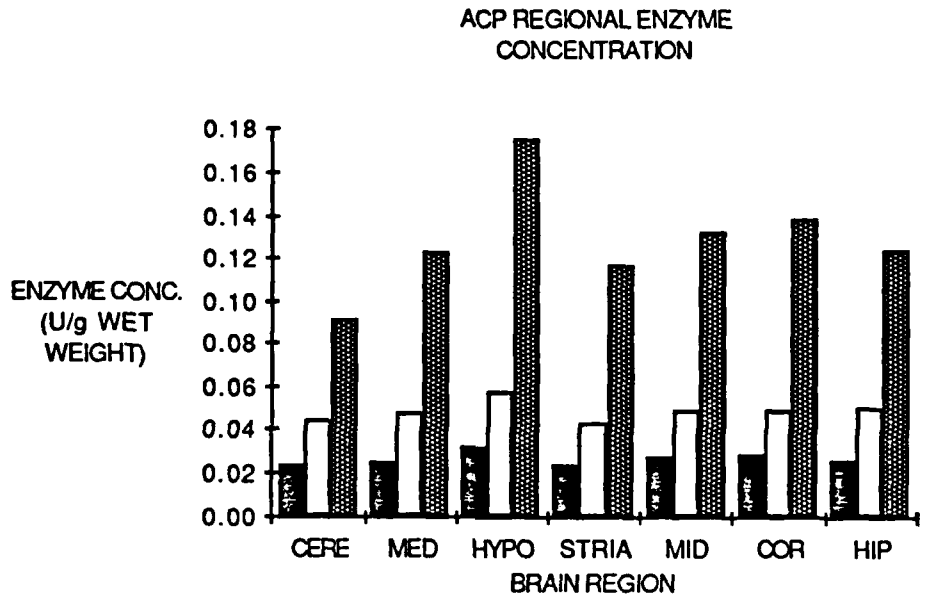
The enzyme concentrations are expressed as U/g wet tissue weight with the S.D. in parentheses and represent the average concentration of sixteen animals. All brain tissue was frozen-thawed before homogenization. The mean enzyme concentrations of the other six brain regions were compared to that of the cerebellum and statistical significance was determined using the two-tailed Student T test.
a = $p < 0.01$

TABLE 20. Regional enzyme concentrations in the rat brain using NWA detergent solubilized tissue.

BRAIN REGIONS	ENZYME CONCENTRATIONS ACP	AST	CK	GDH	ICDH	LD	MDH
CEREBELLUM	.091 (.019)	207 (17)	1286 (86)	63 (5)	4.33 (.5)	112 (8)	227 (17)
MEDULLA OBLONGATA AND PONS	.122 (.019)	202 (26)	769a (81)	117a (17)	4.72 (.7)	100 (11)	215 (35)
HYPOTHALA-MUS	.175a (.021)	204 (24)	709a (55)	121a (14)	5.68 (1.1)	124 (24)	222 (11)
STRIATUM	.117 (.01)	207 (9)	998a (59)	77 (6)	4.04 (.4)	148a (10)	227 (29)
MIDBRAIN	.132a (.018)	209 (14)	795a (23)	91a (8)	5.25 (.6)	157a (13)	236 (33)
CORTEX	.138a (.012)	200 (16)	965a (76)	59 (10)	3.76 (.6)	144a (14)	242 (23)
HIPPOCAMPUS	.124 (.011)	197 (10)	772a (88)	66 (10)	3.34 (.8)	143a (10)	261 (29)

The enzyme concentrations are expressed as U/g wet tissue weight with the S.D. in parentheses and represent the average concentrations of five animals. All brain tissue was homogenized in a 1% solution of NWA. The mean enzyme concentrations of the other six brain regions were compared to that of the cerebellum and statistical significance was determined using the two-tailed Student T test.
a= $p<0.01$

FIGURE 26. ACP regional enzyme concentration in the brain.



Solid black bars= homogenization only
Open bars= freeze-thawing and homogenization
Mottled bars= homogenization in an NWA detergent solution

FIGURE 27. AST regional enzyme concentration in the brain.

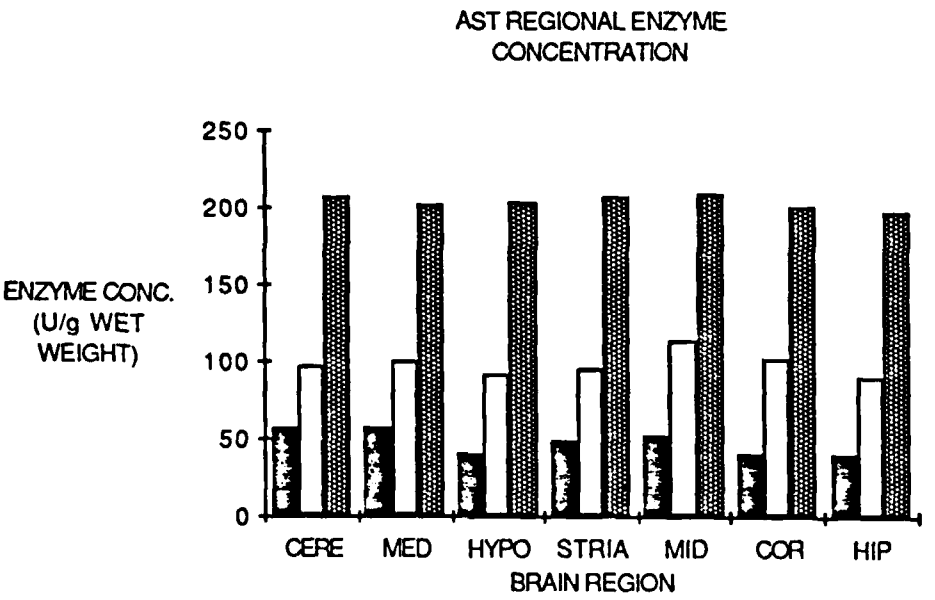


FIGURE 28. CK regional enzyme concentration in the brain.

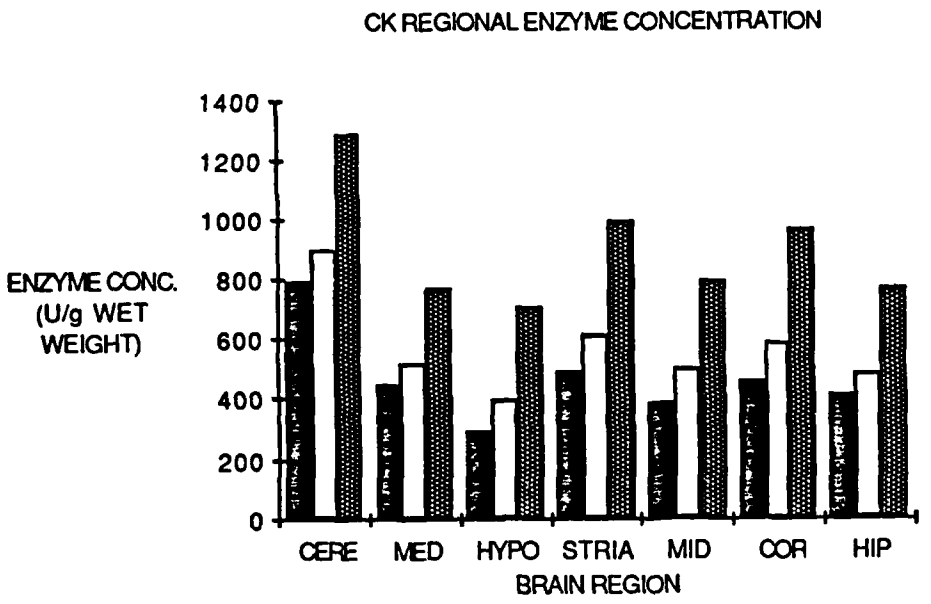


FIGURE 29. GDH regional enzyme concentration in the brain.

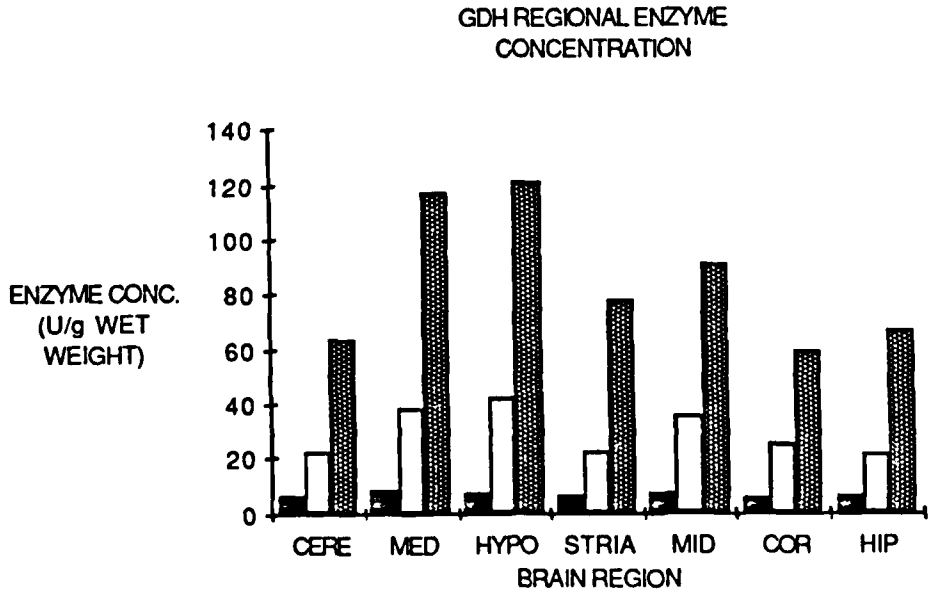


FIGURE 30. ICDH regional enzyme concentration in the brain.

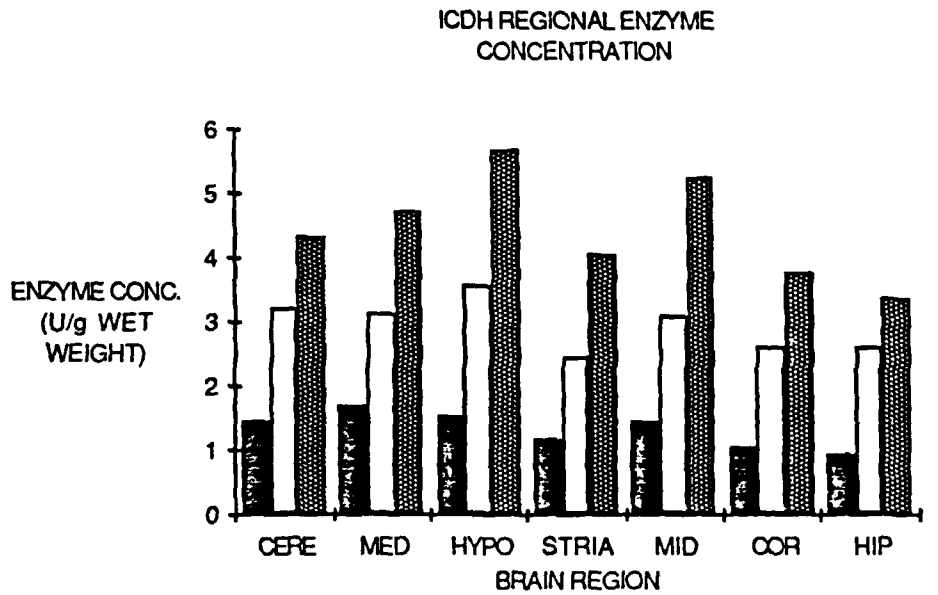


FIGURE 31. LD regional enzyme concentration in the brain.

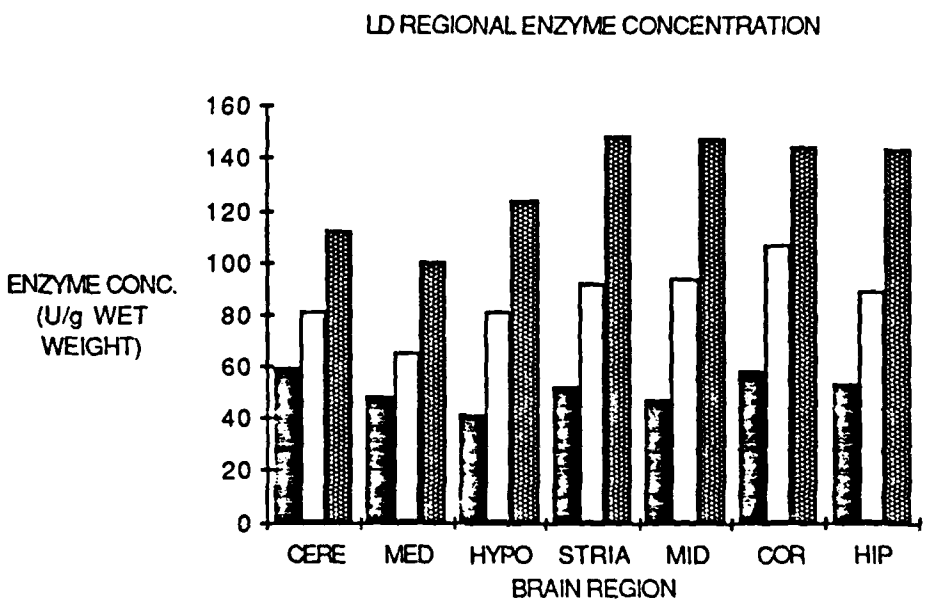
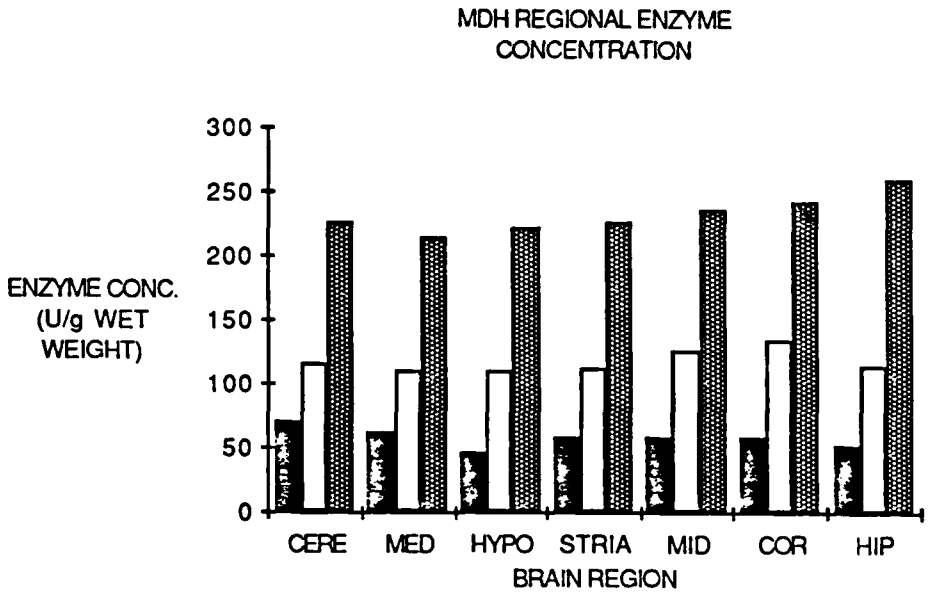


FIGURE 32. MDH regional enzyme concentration in the brain.



concentration in the cerebellum was used as a basis for comparison to the other six regions. Any statistically significant differences in concentration are relative to the value for the cerebellum.

For ACP the fresh and frozen-thawed tissue homogentates show little regional variability. The fresh homogenate's concentrations range from .023-.032 U/g wet weight and the frozen-thawed homogenate's from .043-.057. When NWA is used to solubilize ACP from the lysosomes, there is a suggestion of variability. The hypothalamus, for example, has about twice as much ACP as does the cerebellum. Five of the seven regions examined, however, are very close in ACP content, ranging from .117-.138. Three of the regions showed a statistically significant difference when compared to the cerebellum.

AST shows remarkably little variability regardless of the tissue treatment. The concentrations of all regions are tightly grouped: fresh, 39-57 U/g wet weight; frozen-thawed, 90-114; and NWA treated, 197-207. The detergent treated homogenates show the smallest differences in concentrations. While two regions among the fresh homogenates showed a significant difference when compared to the cerebellum and one region among the frozen-thawed homogenates, none of the NWA homogenates showed a statistically notable difference.

CK has the greatest reginal variability of all of the enzymes assayed. Furthermore, the variability pattern seen with CK holds consistently with all three tissue treatments. The cerebellum clearly has the greatest concentration of CK and it is nearly twice as great as that found in the hypothalamus. The striatum and cortex have the next highest CK concentration and are about equivalent to each other but both contain about

30% less CK than the cerebellum. The medulla oblongata and pons, the midbrain, and the hippocampus have basically equal CK concentrations and have less than the striatum and cortex. The hypothalamus has the least CK of the seven regions studied. Again, the same relative pattern of regional variability is seen in all three sets of homogenates.

Six of the fresh homogenates have very similar GDH concentrations, ranging from 5.3-6.7 U/g wet weight. The medulla has a value of 8.2 which is higher and statistically significant. When freeze-thawing is used, four of the regions show roughly equivalent values, 21.3-24.5, and three of the regions, the medulla, hypothalamus, and midbrain, are distinctly higher in concentration, having at least 45% more GDH. When NWA homogenates are examined, it is seen that the same four regions as with the frozen-thawed homogenates fall in an equivalent range, 63-77, while again the medulla, hypothalamus, and midbrain are significantly higher.

For ICDH fresh homogenates, four regions are close in concentration, ranging from 1.41-1.66 U/g wet weight. Three regions, the striatum, cortex, and hippocampus are noticeably lower and the values for the cortex and hippocampus are significantly different. This same pattern holds for the frozen-thawed homogenates and the values of the striatum, midbrain, and hippocampus are all significantly lower than those of the other four regions. The same pattern is seen once again with the NWA treated homogenates but although the concentrations of the striatum, cortex, and hippocampus are less than those of the other regions, the difference is not statistically significant.

For LD, the fresh homogenate concentrations fall in a fairly tight group, ranging from 41-59 U/g wet weight, although the values of the medulla, hypothalamlus, and midbrain are significantly lower than that of the cerebellum. The concentrations of LD in the frozen-thawed homogenates

are also close for six regions, ranging from 81-107, and only the medulla is significantly low at 65. For the NWA detergent homogenates, four regions may be grouped together, 143-157, while three regions, the cerebellum. medulla, and hypothalamus, are considerably lower, the cerebellum and medulla significantly so.

Finally, MDH shows no marked regional variability. The fresh homogenates all fall in the range 45-69 U/g wet weight, with four regions being significantly lower in concentration in comparison to the cerebellum at 69. For the frozen-thawed homogenates, the range is 110-134 and only the cortex at 134 is significantly higher than the cerebellum, 116. The NWA treated homogentes range from 215-261 and none differ significantly from the cerebellum, 227.

When the protein content is determined for the homogenates of the regions and enzyme concentration is calculated in terms of U/mg protein,the same relative patterns of regional variability, or the lack thereof, are seen.

CAT BRAIN

The results are given in Tables 21 and 22 for brain tissue that was homogenized without freeze-thawing and brain tissue that was frozen and thawed before homogenization, respectively. One piece of tissue from each animal from each brain region was frozen-thawed and a complementary piece of tissue was not and both were processed identically afterwards.

ACP concentrations ranged from .009-.016 U/g wet weight in Table 21 and from .021-.026 in Table 22, showing little regional variability.

AST concentrations ranged from 22-37 U/g wet weight in Table 21. In Table 22, AST values ranged from 47-72, with only the concentration of the striatum showing a statistically significant difference from that of the cerebellum. Both sets of data suggest little regional variability for AST.

TABLE 21. Regional enzyme concentrations in the cat brain using fresh tissue.

BRAIN REGIONS	ENZYME CONCENTRATIONS ACP	AST	CK	GDH	ICDH	LD	MDH
CEREBELLUM	.011 (.007)	34 (8)	537 (89)	6.9 (1.7)	1.02 (.35)	55 (10)	51 (12)
MEDULLA	.015 (.007)	33 (7)	277 (21)	14.4a (2.7)	1.25 (.34)	26 (16)	45 (15)
HYPOTHALAMUS	.013 (.005)	22 (5)	195 (13)	3.9a (0.9)	0.89 (.31)	32 (4)	33 (7)
STRIATUM	.009 (.003)	37 (10)	513 (37)	6.1 (1.4)	1.15 (.25)	50 (6)	53 (10)
MIDBRAIN	.016 (.008)	36 (5)	305 (55)	4.3 (1.2)	0.92 (.29)	37 (3)	48 (7)
CORTEX	.015 (.007)	27 (10)	350 (89)	4.4 (1.6)	0.85 (.19)	48 (10)	39 (8)
HIPPOCAMPUS	.010 (.004)	22 (5)	357 (30)	3.9a (.7)	0.88 (.29)	39 (3)	33 (2)

The enzyme concentrations are expressed as U/g wet tissue weight with the S.D.in parentheses and represent the average concentration of four animals. All brain tissue was homogenized without freeze-thawing. The mean enzyme concentrations of the other six brain regions were compared to that of the cerebellum and statistical significance was determined using the two-tailed Student T test.

a= $p < o.o1$

TABLE 22. Regional enzyme concentration in the cat brain using frozen-thawed tissue.

BRAIN REGIONS	ENZYME CONCENTRATIONS ACP	AST	CK	GDH	ICDH	LD	MDH
CEREBELLUM	.021 (.013)	51 (6)	764 (26)	13.7 (3.2)	1.69 (.32)	64 (12)	72 (12)
MEDULLA	.025 (.014)	47 (24)	388a (25)	24.4 (6.6)	1.67 (.36)	35 (2)	67 (20)
HYPOTHALAMUS	.026 (.014)	52 (7)	300a (16)	9.8 (2.8)	1.68 (.29)	57 (3)	69 (7)
STRIATUM	.022 (.005)	72a (11)	760 (60)	10.6 (4.6)	1.86 (.08)	66 (15)	97 (5)
MIDBRAIN	.026 (.014)	63 (5)	368a (55)	10.9 (3.5)	1.75 (.35)	65 (13)	74 (12)
CORTEX	.023 (.011)	54 (8)	490 (60)	11.9 (2.8)	1.59 (.43)	58 (2)	81 (14)
HIPPOCAMPUS	.021 (.017)	48 (3)	500 (72)	12.5 (4.1)	2.04 (.11)	66 (2)	81 (5)

The enzyme concentrations are expressed as U/g wet tissue weight with the S.D. in parentheses and represent the average concentration of four animals. All brain tissue was frozen-thawed before homogenization. The mean enzyme concentrations of the six other brain regions were compared to that of the cerebellum and statistical significance was determined using the two-tailed Student T test.
a = $p<0.01$

Table 21 shows considerable regional variability for CK. The pattern is: cerebellum = striatum (537 and 513 U/g wet weight) > cortex = hippocampus (350 and 357) > midbrain = medullla (305 and 277) > hypothalamus (195). Table 22 also indicates appreciable regional variability for CK. The pattern is: cerebellum = striatum (764 and 760) > cortex = hippocampus (490 and 500) > medulla = midbrain (388 and 368) > hypothalamus (300). The concentration of CK in the cerebellum and striatum is nearly twice that of the medulla and midbrain and over 2.5 times greater than that of the hypothalamus.

Table 21 shows that the medulla has, with a concentration of 14.4, at least twice as much GDH as the other six regions, which range from 3.9-6.9. Table 22 shows that the GDH concentration in the medulla, 24.4 U/g wet weight, is also nearly twice as great as that in the other six regions, 9.8-13.7.

ICDH shows little regional variability in Table 21, with values ranging between 0.88-1.25 U/g wet weight, or in Table 22, with values ranging from 1.59-2.04.

LD concentrations ranged from 26-55 U/g wet weight in Table 21 and from 36-66 in Table 22. In both cases, the medulla had the lowest LD concentration and was nearly half as great as that in some of the other regions. The LD concentration in the other six regions were considerably closer to one another.

The MDH concentrations in Table 21 ranged fro 33-53 U/g wet weight and from 67-97 in Table 22, indicating no marked regional variability for MDH.

The differences in the values reported in Tables 21 and 22 reflect the increased enzyme solubilization effect of freeze-thawing that has previously been noted.

When enzyme concentrations are expressed as U/mg protein, after the actual protein content of each homogenate was determined, the same relative patterns of regional variability, or the lack thereof, are seen.

CSF enzyme values in the two cats on which cisternal lumbar punctures were performed shortly after death are:

AST, 9-11 U/L

CK, 10-12 U/L

GDH, 2 U/L

ICDH, undetectable

LD, 10-13 U/L

MDH, 5-6 U/L.

DISCUSSION

FRESH TISSUE VS. FROZEN TISSUE EXPERIMENTS

Fresh tissue is the preferred specimen for brain enzyme studies. Yasmineh had suggested that about 70% of CK activtity was lost in brain tissue taken 12-24 hours after death and Urdal et al. reported a 50% decrease in brain CK in autopsy tissue abtained at about 20 hours after death (36,69). The optimal tissue samples would probably be fresh surgical brain specimens. But in practical terms, it is difficult to obtain normal brain tissue from human subjects. Even if a reasonable quantity of such tissue must be excised along with a brain tumor or other abnormal brain tissue, it would be difficult to obtain tissue from preselected brain regions. The impracticality and unpredictability of obtaining fresh human brain tissue means that an animal model must be used. The rat was chosen as these animals are readily available, relatively cheap, and the best and most recent work on brain enzyme regional variability has been based upon rat studies.

Intuitively, it seemed that removing and processing the rat brain as quickly after sacrifice as possible would result in the highest enzymatic activities and values that would most closely reflect the actual enzyme concentration as a minimal amount of activity should be lost. But some previous studies of tissue enzyme content have used tissue that was frozen after collection and stored frozen until processed and assayed (36,69). The in situ freezing of rat brains has been shown to be superior to decapitation in preserving labile brain metabolites such as ATP and phosphocreatine which are subject to rapid destruction via autolytic processes (53). Freezing is a generally accepted means of maintaining enzyme activity at near original levels until the time chosen for measurement. Clearly there are advantages to freezing

and storing tissue specimens until it is convenient to assay them. It was decided to first examine the enzyme activity found in fresh tissue removed immediately from the rat and processed and fresh tissue that had been subjected to freezing and then subsequently thawed and processed to determine if freezing would adversely affect the measured enzyme activity by causing it to decrease.

It is apparent from these experiments that homogenates made from fresh tissue that has been frozen and thawed will result in estimates of enzymatic concentration that differ considerably from those obtained from homogenates prepared from fresh tissue that has not been subjected to freezing and thawing. The effect of freeze-thawing appears to be a consistent, generalized phenomenon as all five enzymes assayed, which represent molecules with different types of functional activities and different intracellular distributions. These enzymes respond in the same way, namely, by showing increased activity and hence higher concentrations. This effect has been reported previously.

Strominger and Lowry noted that the activities of MDH and LD increased 5-10% in rabbit brain homogenates that had been frozen (30). They attributed the increases to incomplete homogenization and better disruption of the tissue by freezing. Stahl and Swanson in 1975 reported that freezing of guinea pig and human brain prior to homogenization resulted in increases in succinate dehydrogenase, monoamine oxidase, and LD activity (70). Neither of these reports addresses what effect freezing and thawing might have on the estimation of total enzyme activity in a tissue specimen.

Some reports of the concentration of CK in the brain may reflect the impact of tissue processing procedures, specifically freezing and thawing, on the final results. Roberts in 1975 measured the CK activity in fresh human

brain tissue obtained from surgery and found a value of 200 U/g wet weight of tissue (71). Tsung also used brain tissue removed during surgery and worked up immediately and found a CK concentration of 157 U/g wet weight (72). Urdal also processed fresh surgical brain tissue but froze it at -20 C and stored it at -70 C until assay (69). CK concentrations of 494 and 555 U/g wet weight were obtained, more than double the values reported from the not frozen brain tissue samples. All authors used fresh human tissue and CK activity was measured using modern, optimized assays. The values obtained for the fresh, unfrozen tissue, 157 and 200 U/g wet weight, are close to each other but clearly considerably less than the 494 and 555 U/g wet weight values obtained using fresh, frozen tissue. It must be pointed out that freeze-thawing is not necessarily responsible for this difference; the papers cited simply speak of homogenizing brain tissue and do not specify what region of the brain the tissue came from. If considerable regional variability of CK exists, it might account for these discrepant findings.

THE FREEZE-THAW PHENOMENON

A basic question raised by the experimental results reported here is: Why does freeze-thawing of tissue increase the enzyme activity of homogenates in comparison to the activity of fresh tissue homogenates?

Four possible explanations have been considered: (1) phosphorylation or dephosphorylation of enzymes can cause changes in enzymatic activity and these phenomena can be triggered in the brain by decapitation/depolarization (2) ischemia caused by decapitation can bring about alterations of enzymatic activity so that the fresh brain homogenates may show decreased activity in comparison to the brain tissue frozen in situ, (3) homogenization of frozen-thawed tissue may result in more thorough disruption of the cells and increased solubilization of enzymes, and (4) the freeze-thawing process

might change the specific catalytic activity of the enzyme molecules, via an alteration of the structure of the molecule or a change in its enviroment, resulting in increased activity.

The first possible explanation deserves consideration because it is known that various enzymes' activities are modified through a phosphorylation-dephosphorylation mechanism (73). Phosphorylation can change the maximum velocity, Vmax, of the reaction catalyzed by an enzyme. A well known example is pyruvate dehydrogenase (PDH) in the brain. It has been shown that decapitation, resulting in a calcium influx, activates PDH phosphatase in the brain which dephosphorylates brain PDH resulting in activation of the enzyme and increased PDH activity (74,75). This increase in PDH activity is not seen in brains which are frozen in situ or frozen immediately after decapitation. The phenomenon described is just the opposite of what is observed in the experimental data presented here as the enzyme activities are higher in the frozen tissue as compared to the unfrozen tissue from decapitated animals. The phosphorylation-dephosphorylation explanation of course is only applicable to enzymes whose activity is regulated in this way, such as PDH. ACP, AST, CK, and LD are not regulated by a phosphorylation-dephosphorylation mechanism. GDH is reported to be regulated in this way, in the same fashion as PDH (76). Nevertheless, GDH does not appear to show the expected activation in fresh tissue from decapitated animals vis-a-vis tissue frozen in situ that PDH demonstrates. The phosphorylation-dephosphorylation explanation is further disproved in the second set of experiments described in which all animals were decapitated and one portion of brain was used fresh and an analogous portion was used after freeze-thawing. GDH may undergo activation in fresh tissue after decapitation as opposed to when the brain is

quick frozen in situ but in the case of this enzyme at least any such effect is overshadowed by freeze-thawing which causes an increase in GDH activity in the frozen-thawed tissue.

The other alternative hypotheses, 2, 3, and 4, which have been considered have been tested experimentally.

ISCHEMIA EXPERIMENT

Ischemia, a decrease in blood flow, was considered to be a possible cause of decreased enzymatic activity in fresh tissue homogenates in comparison to in situ frozen tissue homogenates. When decapitation occurs, complete, abrupt ischemia results. Ischemia may cause physiological changes in the tissue that are deleterious to enzymes. For example, proteolytic enzymes might be released from lysosomes and destroy other enzymes (77). When brain tissue is frozen very quickly in situ these adverse effects on enzymes would not be seen.

The experimental results presented in Table 3 suggest that there is no rapid degradation of enzymatic activity in brain tissue for up to five minutes after the imposition of total ischemia. The average enzymatic concentration of brain tissue subjected to five minutes of ischemia is essentially equivalent to that of brain tissue fixed by freezing immediately after death. Therefore the differences in concentration between fresh and frozen tissue are not due to any rapid and dramatic changes brought about by ischemia.

It is interesting to compare the enzyme concentrations found in this ischemia experiment to those found in the original experiment comparing fresh tissue values to frozen in situ values. The enzyme concentrations from the ischemia experiment more closely resemble those of the in situ frozen tissue than those of the fresh tissue. The common link between the ischemia results and the in situ frozen results is the fact that in both cases the tissue was

subjected to freezing and thawing. The indication is that the freeze-thawing process is critical and primarily responsible for the increase in enzyme concentration seen in frozen-thawed tissue homogenates.

ENZYME SOLUBILIZATION EFFECT OF FREEZE-THAWING

It is possible that the increased enzyme concentration seen in homogenates of previously frozen tissue in comparison to homogenates of fresh tissue is a result of simple solubilization. That is, the mechanical stress of freezing and thawing serves to break up and disrupt the tissue cells, ripping open membranes and subcellular organelles such as lysosomes and mitochondria, allowing enzymes attached to or contained in these structures to escape and wind up in the supernatant fraction of the homogenate. The fresh tissue is only exposed to the disruptive forces of homogenization alone, and since a Teflon pestle is used rather than a ground glass pestle which would provide for more harsh homogenization, more of the cell's structural elements may remain intact and the enzymes associated with them will never be measured as they will be sedimented out via centrifugation of the homogenate and remain in the pellet.

That freeze-thawing could have a tremendous effect on enzymatic activity by causing the release of enzymes from structures that they were attached to or contained in was probably best described by DeDuve et al. (78,79,80). It was found that acid phosphatase could be "activated" in mitochondrial preparations by freeze-thawing. This "activation" actually was a liberation of acid phosphatase from lysosomes. The enzyme was inactive when contained within the intact lysosomes but when freeze-thawing disrupted and broke apart the lysosomal membrane, acid phosphatase spilled out of the ruptured membranes, was liberated, and was free to react with substrate. DeDuve's work showed that up to 80% of ACP could be contained in the

lysosomes, where it was inaccessible for assay, and could be released by freeze-thawing (78). He concluded that:*"Repeated freezing and thawing probably acts in several ways, splitting or denaturing architectural components, creating local increases in osmotic pressure and causing tearing effects due to ice crystals"* (79).

The same results with regards to ACP are seen in this investigation. ACP activity increases in homogenates after tissue is frozen and thawed or after homogenates are frozen and thawed. A simple explanation of the findings would be that lysosomes are more extensively disrupted in the frozen-thawed tissue than in the fresh tissue and more ACP molecules appear in the supernatant layer and result in increased ACP activity. Intuitively, it would seem quite possible that all enzyme activities are to a certain extent latent and increased by the same basic mechanism, namely, increased solubilization. CK and LD are typically thought of as cytoplasmic enzymes but their activities are not confined totally to the cytosol. It has recently been noted that certain enzymes may not have a fixed intracellular distribution, as previously thought, but may exist in soluble and, to some extent, membrane bound forms. These enzymes are termed "ambiquitous" meaning that they are found both in the cytosol in a free form and bound to membranes (81,82). Some portion of the LDH activity contained in a cell has been shown to be associated with the mitochondria, found on the outer membrane and intramembrane space, and the microsomes (83-86). Theoretically, then, any treatment such as freeze-thawing which more effectively rips up the internal structure of a cell, such as the mitochondrial membranes, than simple homogenization, could result in the increase of enzymatic activity of LD.

The total CK activity of a cell is definitely known to consist in part of a mitochondrial fraction (66,67,87,88,89). In the brain it is generally accepted

that only the CK-BB and the mitochondrial isozymes are found (32,33,35). Booth and Clark have reported that about 5% of the CK activity of the adult rat brain is mitochondrial CK (90). Again, a portion of enzymatic activity is not freely accessible in the cytoplasm but sequestered within the cell so that any treatment that enhances solubilization of enzymes can be expected to increase measured enzymatic activity.

AST exists mainly as a cytoplasmic enzyme but a mitochondrial form also exists and contributes to the AST activity in serum (91-94). In the case of this enzyme as well solubilization by freeze-thawing may result in increased enzymatic activity.

Finally, GDH was chosen for assay specifically in this study because it is a mitochondrial matrix marker enzyme (95-97). One expects to see GDH activity only if the enzyme has been released from the mitochondrial matrix compartment or if substrates and products can pass through the intact mitochondrial membranes. Increased GDH activity would be anticipated if the mitochondrial membranes are disrupted and increased amounts of GDH are released or made more accessible to the substrates.

To test whether solubilization could explain the increased activity seen in homogenates from frozen tissue, cell fractionation experiments were performed. Brain tissue homogenates from fresh and frozen tissue were broken down into portions representing various particulate and supernatant fractions. Enzyme activity and protein content of each fraction was measured. In this way a better understanding of what happens to the subcellular organelles when freeze-thawing is used could be gained by looking at differences in the enzymatic activities in each fraction

The results show that freeze-thawing increases enzymatic activity via an enzyme solubilization process that brings out latent enzyme activity. Latent

enzymatic activity may be defined as activity that is not measurable because enzymes are not accessible to react with substrate. Latent activity may be unmasked by making the enzymes available to react through some type of solubilization process. When tissue is frozen and thawed, more protein appears in the supernatant fraction than in the particulate fractions, indicating that more protein is released from cellular structures than when fresh tissue is homogenized without benefit of freeze-thawing before hand. Some of the increased protein in the frozen supernatant is clearly accounted for by enzymes as the enzymatic activities for all five enzymes assayed are markedly higher in the frozen supernatants than in the fresh supernatants. This is plainly shown by the RSA graphs in addition to the balance sheet data. These graphs show not only that latent enzyme activity has been made accessible but that a redistribution of enzymatic activity has occurred as a result of solubilization. Activity has been decreased in the subcellular organelle fractions and increased in the supernatant fraction.

The data resulting from the overnight frozen storage are particularly illuminating. It shows that in general activity in the original frozen fractions decreased slightly or remained about the same, except for GDH which increased. But activity in the original fresh particulate fractions in many cases increased. These increases in the activity of the fresh particulate fractions can be explained by postulating that frozen storage served to more completely disrupt and tear apart the particles and membrane structures in these fractions. The enzymes were solubilized and made accessible to the substrate molecules and now could be assayed. The shifts in the RSA patterns for the fresh fractions perhaps most vividly reflect this solubilization process and of course the previously noted redistribution of activity among the fractions. While activity in the supernatant fractions sometimes decreased,

the particulate fractions, particularly the nuclear fraction, showed small, moderate, or marked increases. Perhaps the enzymes trapped within subcellular organelles or associated with membranes or other structures were protected while the enzymes that were free in solution in the supernatants were subject to denaturation.

Supporting the biochemical data are the electron photomicrographs which show that when freeze-thawing was not used, more intact, complete mitochondria are visible in the pellet whereas with freeze-thawing there are fewer whole mitochondria and more mitochondrial fragments in the pellet.

These observations echo the caveat of Visser et al. who noted that erroneously high variability of tissue enzyme content estimations may in part be attributed to the intracellular compartmentization of enzymes (29). They specifically noted that AST estimates in tissue can be erroneous if the mitochondria are not sufficiently disrupted to release the AST which they contain.

In conclusion, freeze-thawing acts to increase enzyme activity most likely by simple solubilization of enzyme molecules which would remain locked in particulate structures where they are not free to react with substrate molecules or in which their activity is restricted and diminished. This process causes a considerable redistribution of activity from particulate fractions to the supernatant of a homogenate. Enzymatic activity still associated with subcellular organelles, membranes, or any other structure of notable size and density will be sedimented out during the centrifugation of the homogenate and essentially be lost as it will be contained in the pellet and not available for assay in the supernatant.

ACTIVATION ENERGY EXPERIMENT

It is possible that the increased enzymatic activity found in homogenates of frozen-thawed tissue may be due in part to an increase in the specific catalytic activity of the enzymes. If freeze-thawing results in either a change in the molecular configuration of an enzyme or in the enviroment in which it finds itself, the observed specific catalytic activity can be increased. Both situations have been shown to occur experimentally.

The M chain of the CK-MM and CK-MB isoenzymes is known to be modified by a serum protein, a carboxypeptidase, resulting in a change in the isoenzyme's activation energy, Ea, and Michaelis-Menten constant, Km (55,56). Cytochrome c oxidase when released from its normal site, bound to the intact inner mitochondrial membrane, demonstrates a change in Km and a large increase in its maximal activity, Vmax (98). In contrast, succinate dehydrogenase, another enzyme attached to the inner mitochondrial membrane, shows a decreased Km and Vmax when solubilized from the intact membrane (99). Solubilization of an enzyme does not necessarily affect its specific catalytic activity, however. The ambiquitous enzyme hexokinase is found in the brain in two subcellular fractions. Approximately 20% of the total hexokinase activity is cytosolic and the other 80% is bound to the outer mitochondrial membrane. When solubilized mitochondrial hexokinase and cytosolic hexokinase were compared, it was found that there were no differences in their kinetic parameters (100).

Thus it is of interest to see if freeze-thawing affects the kinetic characteristics of enzymes so as to boost their specific catalytic activity, either through a change in the structure of the molecule as in the case of CK-MM, or via solubilization as in the case of cytochrome c oxidase.

Hagelauer and Faust reported that the E_a's of CK-BB prepared from the uterus and from serum are 63 and 61 kJ/mol respectively (55). The values reported in this study, ranging from 58-62 kJ/mol, for the CK-BB found in brain tissue are certainly comparable and in good agreement. Rej and Vanderlinde reported that the E_a for AST is in the range of 51-52 kJ/mol based upon work with purified cytoplasmic and mitochondrial AST from man and the pig (101). Hafkenscheid and Kohler reported that the E_a for AST is about 50-52 kJ/mol for the enzyme found in commercial control sera (102). The values reported in this study, ranging from 38-45 kJ/mol, are all lower. No drastic differences exist in the assays used, all being essentially based upon that recommended by the Scandinavian Society for Clinical Chemistry (103). The lower results obtained in this study may be due to the fact that AST in crude homogenates was measured rather than the purified enzyme assayed by Rej and Vanderlinde or the AST found in the control sera, most likely purified animal extracts, assayed by Hafkenschied and Kohler. Buhl et al. report an E_a of 65 kJ/mol for LD 1 and an E_a of 46.5 for LD 5 (50). The E_a's reported in this study for LD, ranging from 60-65 kJ/mol, are certainly in the same range and the fact that they are much closer to the E_a of LD 1 is not surprising as most of the LD activity in the brain is composed of the anodal isozymes (34).

For two enzymes, AST and CK, it appears that the increased activity observed with freeze-thawing is due to increased solubilization of these enzymes and not to any change in their specific catalytic activity as their E_a's were not observed to change with freeze-thawing. The activation energies of both GDH and LD did change with freeze-thawing and did decrease indicating that the increased activity of these enzymes seen in frozen tissue

homogenates may in part be due to a decrease in E_a resulting in increased specific catalytic activity.

Even for GDH and LDH, although the changes in E_a are statistically significant, they are small. It is likely that freeze-thawing exerts most of its effect on increasing enzymatic activity through solubilization rather than through alteration of enzyme kinetic parameters.

The results of the activation energy experiment should be interpretted cautiously as AST activity consists of two isoenzymes, a cytosolic and a mitochondrial form. CK in brain is essentially all CK-BB but mitochondrial CK is also present. LD activity is made up of five different isoenzymes and recombination of the tetramers may occur. There is some evidence that GDH, although a specific mitochondrial matrix enzyme, may have three different isoenzymes (33). Therefore the activation energies measured may reflect not one molecule but two or more different molecules with the same enzymatic activity but possibly with different activation energies and specific catalytic activities. Freeze-thawing may cause a detectable change in E_a for a mitochondrial isoenzyme through solubilization but have no effect on the cytosolic form of the same enzyme. Or the structure of one isoenzyme, and its activity, may be altered by freeze-thawing, but no change may occur with the other isoenzyme(s). It is therefore difficult to accurately know exactly why E_a may be changed.

MITOCHONDRIAL ENZYMES EXPERIMENT

It has been determined that homogenates prepared from frozen-thawed tissue possess greater enzymatic activity than homogenates prepared from fresh tissue. In an effort to understand this phenomenon, various possible explanations have been investigated. It has been concluded that enzyme solubilization, that is, the release of enzymes from intracellular organelles

and membranes or the increased accesssibility of these enzymes to substrates, caused by freeze-thawing, is primarily responsible for the observed increase in activity and that an increase in the specific catalytic activity of some enzymes may also in part account for the elevation of activity. The effects of freeze-thawing appear to represent a general phenomenon based upon the results obtained with the five different enzymes assayed.

So far, this research has dealt with homogenates prepared from whole cells. From the enzymatic viewpoint, a whole cell is a rather complicated system. A given enzymatic activity, as mentioned previously, can result from several different isoenzymes, distinguishable from one another by some means but all catalyzing the same specific reaction. A given enzymatic activity can also be derived from more than one source within the cell. It was decided to further investigate the freeze-thawing phenomenon at the subcellular level to gain further insights and better understand what is happening inside of the cell when freeze-thawing is employed. The approach taken here is similar to that of Leong et al. who turned to the examination of the nonsynaptic (free) and the synaptic mitochondria to determine if the heterogeneous distribution of energy-metabolising enzymes seen at the brain regional level was also reflected at the subcellular level (8).

In these studies, rat brain mitochondrial preparations were made and various mitochondrial marker enzymes were assayed. The mitochondrial preparations were divided into four portions: (1) the original mitochondrial preparation, (2) the preparation after homogenization, (3) the preparation after freeze-thawing, and (4) the preparation after freeze-thawing followed by homogenization. The same marker enzymes were assayed in each fraction so that the effects of the various manipulations on enzyme activity could be assessed in comparison to the enzyme activity found in the original

preparation. It was expected that freeze-thawing would, in general, result in increased enzyme activity in comparison to the original preparation and that freeze-thawing coupled with homogenization would result in increased enzyme activity in comparison to the homogenized preparation.

The mitochondrial enzymatic activities for NAD- and NADP-linked ICDH, fumarase, MDH, and CK are within the same range as those observed by Lai et al. and Leong et al. when expressed in terms of nmol/min/mg of mitochondrial protein (6,8). This finding is not surprising since the same basic scheme for preparation of brain mitochondria, namely that of Lai and Clark, was used.

No dramatic changes in the activities of ACP, CK, and LD were seen after freeze-thawing and/or homogenization of the mitochondrial preparations. The increase in activity for all of these enzymes was no greater than 16% in the frozen-thawed and homogenized fraction as compared to the original preparation. This result may be expected since these enzymes represent contaminants in the mitochondrial preparation, derived from lysosomes and the cytoplasm. On the other hand, it is not surprising that increases were seen as lysosomes are inadvertently enriched in the mitochondrial preparation. As discussed previously, and CK and LD both have mitochondrially associated activities that can be released by manipulation of the mitochondria.

The enzymes of interest for diagnostic purposes are the mitochondrial marker enzymes. Homogenization alone does not increase MDH activity, but causes only moderate increases in NADP-linked ICDH and fumarase activity, and results in a large increase in GDH activity. Freeze-thawing alone caused large increases in the activities of all of these enzymes, ranging from 30% for MDH up to 80% for fumarase. While freeze-thawing coupled with homogenization did not further increase MDH activity above that

observed with freeze-thawing alone, for GDH, NADP-linked ICDH, and fumarase, activities were boosted another 40%, 21%, and 7% respectively. As expected, the disruptive force of freeze-thawing can greatly increase the solubilization, and thus the measurable enzymatic activity, of the mitochondrial matrix enzymes by tearing up the mitochondrial membranes and releasing or making accessible the enzymes contained within them. While the degree of activity elevation may vary with a given enzyme, it is clear that a combination of freeze-thawing and homogenization results in markedly greater activity than homogenization alone of mitochondria and mirrors the results seen previously with whole tissue.

The case of NAD-linked ICDH deserves special mention as it is an anomaly. It alone of the mitochondrial enzymes shows a decrease in activity with freeze-thawing and a further decrease with freeze-thawing followed by homogenization. As will shortly be discussed further , NAD-linked ICDH may possess peculiar properties. The decreases observed after freezing and homogenization have been shown to be real statistically. The effect of freeze-thawing increasing enzymatic activity appears to be a widespread phenomenon applying to many enzymes but not to all as the case of NAD-linked ICDH points out.

The data presented here supports the conclusion that freeze-thawing leads to increased enzymatic activity through enzyme solubilization brought about by the mechanical stress that it exerts on whole cells and subcellular organelles such as mitochondria. Part of the increase in activity may be attributable to a change in the specific catalytic activity of enzymes caused by freezing as well. The activation energy of CK was not found to be altered after freezing, which is in agreement with results previously reported. The E_a's of GDH and MDH were both decreased after freezing. The E_a of GDH

had previously shown this same behavior. This lowering of the E_a may be due to the release of these enzymes from their native enviroment within the mitochondrion or due to a change in conformation or a combination of both of these possibilities. While NADP-linked ICDH activity increased with freezing, no statistically significant change in its E_a occurred so that release from a subcellular organelle enviroment or modification of the enzyme's structure by freezing does not necessarily decrease E_a.

NAD-linked ICDH again does not follow the pattern of the other enzymes. Its E_a changes but it has a negative value to begin with and it increases to a less negative value after freezing. This enzyme again seems to be atypical as its activity is higher at 25 C than at 37 C. The fact that its E_a increases after freezing and homogenization suggests that its activity should decrease. This is exactly what is observed. Its behavior is consistent in terms of the activity measured after freeze-thawing and homogenization and the change in E_a observed after these manipulations. It may be speculated that when deprived of its native enviroment inside of the mitochondrial matrix, perhaps losing a crucial cofactor, a conformational change occurs resulting in lower activity and/or freezing may be detrimental to the enzyme's activity. Or this enzyme may simply undergo denaturation at 37 C.

It would have been interesting to have taken electron photomicrographs of the mitochondrial pellets before and after the various manipulations designed to increase enzyme solubilization. Increased enzymatic activity could then have been correlated with increased mitochondrial disruption as witnessed but fewer intact mitochondria and more mitochondrial fragments in the EM pictures.

In conclusion, for most enzymes freeze-thawing followed by homogenization will result in a greater release of measurable enzyme activity

via a solubilization process than homogenization alone. Freeze-thawing coupled with homogenization is therefore a preferable means if one desires to assess the total enzymatic content of a tissue specimen as long as E_a remains unchanged.

EFFECTS OF MULTIPLE FREEZE-THAWINGS

Brain tissue was frozen and thawed up to four times before being homogenized to determine how much the enzyme concentration increases in the homogenate supenatant fluid with successive freeze-thawings. For all seven enzymes studied, the greatest increase in enzyme concentration was seen after the first freeze-thaw cycle. The maximal enzyme concentration was usually seen after two or three freeze-thawings. After four freeze-thaw cycles, the enzyme concentrations typically leveled off and remained at about the same value as after three freeze-thaws or the concentration decreased. These observations are generally in agreement with those of Gianetto and DeDuve for ACP, beta-glucuronidase, and cathepsin (80). These investigators found the activity of these three enzymes to be maximal after 4-6 freeze-thawings but they did not specify the conditions of freeze-thawing and they may well have used an approach other than freezing in liquid nitrogen followed by rapid thawing at 37 C and agitations. It is quite likely that the exact freeze-thaw processs used will affect the amount of enzyme released and the rapidity of release. Presumably two to three cycles releases the maximal amount of enzymes from subcellular organelles and other cellular structures in which the enzymes may be sequestered or to which they may be attached. Depending upon the nature of the specific enzyme molecules, four freeze-thawings may cause denaturation, as witnessed by an apparent drop in the enzyme concentration, or have little effect, as seen when the enzyme concentration remains essentially the same. Of course the

process of solubilization and denaturation may be taking place concurrently for some enzymes. While each successive freeze-thaw cycle may release more of the total enzyme complement of a tissue, enzyme molecules that had already been solubilized may undergo structural changes and be deactivated.

SUMMARY OF FREEZE-THAW EXPERIMENTS

Freezing and thawing tissue followed by homogenization will allow a better estimate of the total enzyme concentration in the tissue than simple homogenization. Whether the extent of cell disruption and enzyme release effected by freeze-thawing is similar to that seen in clinical situations, for example, a traumatic head injury, is not certain. It seems likely though that cell death and cellular metabolic perturbations caused by injury and pathologic processes do result in the break up of not only of cell membranes but subcellular particles and the internal structures of the cell as well, especially as necrosis progresses. It is probably not an unreasonable approach to measure the enzyme content of tissue that has only been homogenized and of tissue that has undergone the more destructive process of freeze-thawing and homogenization when assessing the regional variability of enzymes in the brain. Since there is bound to be difficulty in precisely reproducing the same amount of cellular disruption with each freeze-thaw and for the sake of simplicity, it was decided to subject tissue specimens to a single freeze-thaw cycle.

ENZYME SOLUBILIZATION EFFECTS OF DETERGENTS

In addition to freeze-thawing, it was decided to explore another approach to enzyme solubilization, namely, detergent solubilization, which could possibly allow even a better estimate of the total enzyme content of rat brain.

Detergents are known to solubilize membranes and thus release enzymatic activity (104). Nonionic detergents are particularly useful for this

purpose as they do not usually denature proteins. It is of interest to compare the effects of detergents with that of freeze-thawing, both of which lead to increases in the amount of measurable enzymatic activity.

Zuretti et al. used Triton X-100 to assess the residual latent activity of lysosomal preparations that had previously received extensive hypoosmotic shock treatment (105). Salminen et al. coupled Triton X-100 with hypotonic shock and freeze-thawing to measure the releasable activity of several lysosomal enzymes including beta-glucoronidase, cathepsin C, arylsulphatase, and acid ribonuclease (106,107).

Elduque et al. have shown that treatment of a mitochondrial preparation by successive extraction with a buffer or successive sonications releases only about 50% of the MDH activity that can be extracted by just one treatment with a 1% solution of Triton X-100 (108).z Skorkowski et al. found a 2% solution of Triton X-100 to be effective in solubilizing NAD- and NADP-specific forms of MDH from mitochondria (109). Robinson and Srere used 1% Triton X-100 to measure the total activity of the mitochondrial matrix enzyme citrate synthase (110).

Nucleotidase, a membrane bound enzyme, has been effectively solubilized by Lutoslawska et al. using new zwitterionic detergents which are more effective than nonionic detergents such as Triton X-100 for this purpose (111).

Triton X-100 and Brij 35 were chosen for investigation as they are common non-ionic detergents, Triton in particular having been much used in research. New Wetting Agent (NWA) and Clearing Solution (CS) are two non-ionic detergents used in the clinical chemistry laboratory. Their composition is not known as they are proprietary reagents of Technicon. The zwittergent detergents, 3-12 and 3-14, were chosen as they are relatively

new reagents and have been shown to be more effective than non-ionic detergents in solubilizing 5'-nucleotidase (111).

The data indicate that various detergents are extremely effective in comparison to simple homogenization and a single freeze-thawing followed by homogenization in solubilizing enzymes and bringing out latent activity. It is clear, though, that different detergents have different effects with a given enzyme. The ionic detergents, zwittergents 3-12 and 3-14 offered no advantage in terms of enzyme solubilization for ACP, GDH, and ICDH. Ionic detergents have not traditionally been favored for enzyme solubilization as they are more likely to cause denaturation than non-ionic detergents and that is likely what happened with these three enzymes (GDH activity is inhibited by the zwittergents as is discussed later) (104). Both of these detergents were very effective with AST, CK, and MDH, though. The non-ionic detergents in general worked quite well for enzyme solubilization. Triton X-100, NWA, and CS in particular were effective across the board with all seven enzymes studied except for NADP-linked ICDH in the case of CS.

Adenylate kinase (AK) is an ubiquitous enzyme of metabolic importance as it can generate high energy ATP from two molecules of ADP. It is also a potential interferent in the CK assay. While sufficient AK was present in the fresh, frozen-thawed, and detergent supernatants to overwhelm the AK inhibitors used in the CK reaction mixture, it accounted for no more than 4% of the total CK activity. Thus the CK concentrations reported reflect a true solubilization of CK rather than a cumulative effect due to the release of substantial quantities of AK as well as CK.

NWA was singled out for further study as it was the one detergent that showed considerable enhancement of enzyme solubilization for all seven enzymes and thus was the best prospect for use in a regional variability study.

Since the purpose of using detergents is to bring out latent enzyme activity hidden away in organelles or buried in membranes, it is not surprising that the most dramatic increases in enzyme concentration after detergent treatment are seen for enzymes such as AST, GDH, and MDH, found partly or wholly in the mitochondrial matrix, as opposed to the primarily cytosolic enzymes CK and LD. Still, the increases seen for the cytosolic enzymes are quite impressive and belie the simplistic notion that a cell, or a subcellular organelle, is a simple sack of enzymes and that enzymes will readily spill out of the interior if the membrane is disrupted. The extent and importance of enzyme attachment within the cell has recently been reviewed by Werner et al., who also point out the misconceptions associated with the traditional idea of "soluble" cytosolic enzymes (112).

ENZYME SOLUBILIZATION DOSE RESPONSE CURVE OF NWA

In order to guage the relative effectiveness of six different detergents, 1% solutions of each made up in Tris homogenization buffer were used. NWA emerged from this trial as the best all around detergent for enzyme solubilization but whether a 1% solution was optimal was not known. The data indicate that a 1% solution, although not always producing the highest enzyme concentration for all seven enzymes, was the best compromise concentration to use in regional variability studies.

ENZYME ACTIVITY INTERFERENCE DUE TO DETERGENTS

Although all of the six detergents studied in most cases seemed to be at least as useful for solubilizing enzymes as freeze-thawing and better than

simple homogenization, an interference study was necessary to determine if the detergents caused either an enhancement or loss of activity. If a detergent increased the measured activity, an overestimation of enzyme concentration might result suggesting that the detergent treatment was more effective for enzyme solubilization than is actually the case. Conversely, if a detergent caused a loss of activity, this effect would work contrary to the detergents enzyme releasing properties and would have to be taken into account.

For the five enzymes listed, one or both of the zwitterionic detergents were responsible for noteworthy loss of GDH and LD activity. This effect has already been mentioned in the discussion of GDH enzyme solubilization by detergents and is presumably due to denaturation of GDH and LD by the ionic detergents.

For four of the five enzymes, all four of the non-ionic detergents had no consequential adverse effect on activity. Interestingly enough, all four of the non-ionic detergents, and both of the zwitterionic detergents, had an enhancing effect on CK activity. CK values were 7-21% higher in the prescence of the detergents in comparison to the control. NWA, for example, shows and 18% enhancement. Correcting the CK concentration value for NWA in Table 12 for this artificial increase in activity, NWA still released about 100 U/g wet weight more CK than freeze-thawing and about 270 U/g wet weight more than simple homogenization. So it would appear that two phenomenon, enzyme solubilization and an enhancement of activity, take place when an NWA homogenization buffer is used. While this enhancement phenomenon is not ideal, as long as it can be assumed to be constant it can be tolerated in a regional variability study where the purpose is to detect relative difference in CK concentration from region to region.

EFFECT OF DETERGENT SOLUBILIZATION AFTER FREEZE-THAWING

The important conclusion to be drawn here is that even though repeated freeze-thawing may release successively greater amounts of enzyme, there is always some portion of the total enzyme content that is not solubilized by freeze-thawing but remains latent. This portion of enzyme content can be released by detergent treatment.

Not surprisingly, the amount of detergent solubilizable enzyme is less for the primarily cytosolic enzymes CK and LD, ranging from 25-30% and 20-24%, respectively, than for AST, 30-40%, which has a substantial mitochondrial component, and MDH, 34-44%, which is basically a mitochondrial matrix enzyme. If attempting to measure the absolute total amount of enzyme present in a tissue, cell, or organelle, a combination of solubilization techniques may be called for, as tried by Salminen et al. for lysosomal enzymes (106,107).

ISOENZYME DISTRIBUTION AFTER ENZYME SOLUBILIZATION

An interesting difference between the CK isoenzyme pattern seen with NWA homogenates and those seen with the fresh and frozen-thawed homogenates is the existence of a small peak representing about 8% of the total CK and located about on the point of application of the gel in addition to the large CK-BB peak which is expected. As explained previously, the extra peak could be explained by either the presence of CK-MM originating from blood in the brain tissue or the low molecular weight form of mitochondrial CK. The fact that the fresh and frozen-thawed homogenates, made at the same time from adjacent pieces of rat cortex, had no similar band is circumstantial evidence that the extra band is not CK-MM but rather

mitochondrial CK. The easiest way to conclusively prove the identity of the small band might be to treat it with anti-CK-MM antibody before adding substrate to the gel, which would block the CK-MM active site and cause the band to disappear if it was CK-MM , but not if it was the mitochondrial isoenzyme. Conversely, treating the band with anti-mitochondrial CK antibody would result in just the opposite results but also allow the band to be identified. Unfortunately, neither of these antibodies was available. Considering the enhanced enzyme solubilization effect of NWA, it would not be surprising if the extra band was the mitochondrial form of the enzyme, but this can not be conclusively stated. Booth and Clark had found mitochondrial CK to represent about 5% of the total CK content of rat brain, intriguingly close to the roughly 8% reported here (90).

No band representing adenylate kinase was found in any of the homogenates. This supports the previous conclusion that AK does not contribute substantially to the CK activity of the brain homogenates.

There was no remarkable changes seen in the LD isoenzyme pattern regardless of which tissue processing treatment was used. While LD isoenzymes may bind to the mitochondrial membrane, no distinct mitochondrial isozyme is known. It is likely that a more potent enzyme solubilization technique only serves to release more LD belonging to the five known isoenzyme bands. While some shift in the relative amounts of LD found in the five bands may occur, no band appears to change radically with a more vigorous solubilization technique.

REGIONAL VARIABILITY OF ENZYMES IN THE BRAIN

The regional variability of enzymes in the rat brain has been investigated. The rat was chosen for study first since this animal is readily available and relatively inexpensive, thus allowing a number of animals to be used to

generate a significant data base. Values for the concentrations of some enzymes in various regions of the rat brain have previously been reported in the literature so that a basis for comparison with the values found in this work exists. Adult rats weighing between about 200-400 g, about 60 days old, were used (113). Seven different enzymes were chosen for study. Three are clinically useful enzymes commonly assayed in hospital laboratories and previously measured in the CSF as potential prognostic markers in cases of central nervous system damage. They are AST, CK, and LD. The other four enzymes, ACP, GDH, ICDH, and MDH, have been assayed previously in the course of this study and all have some definite potential clinical usefulness. By assaying seven enzymes it is more likely that any noteworthy regional variability, if its exists for any enzyme, will be detected and any generalized pattern of regional variability for more than one enzyme will also be discerned. Seven different brain regions, representing major and well-defined substructures of the brain and regions which are likely to be affected by brain damage, were chosen to enhance the chances of detecting regional variability, as well as three different approachs to tissue processing.

It appears that ACP is distributed fairly evenly through the brain. As ACP is a lysosomal marker enzyme, by inference lysosomes are presumably found in equal amounts in all brain regions. Lysosomes, with their complement of lytic enzymes, are necessary for routine maintenance of cells and for use in extraordinary situations, such as disease processes. But the relatively homogeneous distribution of ACP suggests that there is no one region which has a greater or lesser need for lysosomes and their enzymes than any other region. A standard complement of lysosomes per cell may be sufficient in all regions.

AST does not show any remarkable regional variability. Wakim and Fleisher in 1956 assayed AST in the cerebral cortex, internal capsule, medulla oblongata, and cerebellar cortex of the dog brain and found concentrations ranging from 1.8-6.6 micromoles/hour/mg of fresh tissue (114). It was concluded that AST activity in cortical grey tissue is definitely higher than in white matter. Using tissue from a cancer patient who died eight hours before dissection of the brain, Miyazaki in 1958 measured AST in seven brain regions (31). He found AST to range from a low of 0.52 micromoles/hour/ mg of tissue in the hypothalamus to a high of 0.83 micromoles/hour/mg in the cerebellum and noted that the observation that AST was higher in the cortex than in white matter was not confirmed by his findings. The present results are roughly in agreement with those of Miyazaki showing essentially equivalent concentrations of AST in various brain regions. The two- to three-fold differences among regions reported by Wakim and Fleisher were not observed.

CK is the enzyme which showed the greatest regional variability. Presumably the differences in concentration of CK in the various regions are related to the metabolism and energy needs of those regions. Phosphocreatine is a molecule which can serve as an energy reservoir and CK can act to either increase the amount of stored energy or to release it when necessary.

Booth and Clark reported a value of 207 U/g wet weight in rat cerebral cortex (90). This is only about a third of the value obtained in the present work, 455 U/g wet weight, for the fresh homogenate. Booth and Clark used fresh tissue and an optimized assay and their lower results are probably attributable to differences in tissue handling and processing. The value observed in this study increases greatly when liquid nitrogen freezing and

subsequent rapid thawing or NWA detergent treatment is used as in this study to specifically maximize enzyme activity through solubilization.

The CK concentrations reported here are also much higher than those of Chandler et al. which range from about 20-120 U/g wet weight, using an optimized assay and the Cobas Bio centrifugal analyzer and a reaction temperature of 30 C (39,40). The Cobas Bio analyzer was also used in this study but the assay temperature was 37 C. Assuming a Q_{10} of 2 for CK, Chandler's CK concentrations might have been doubled if the assay were run at 37 C instead of 30 C, but the values would still have been well below those reported here. Chandler et al. used human autopsy brain tissue which was not subjected to a freeze-thaw process. These authors did detect regional variability of CK and the pattern seen in the present study, i.e., greater CK activity in the cerebral cortex in comparison to the medulla, is in agreement with Chandler's findings although the activities reported here are much higher and the differences are likewise much greater than in Chandler's work.

The values obtained for GDH in the frozen-thawed homogenates are in excellent agreement with those of Leong and Clark: cerebellum, 22.7 vs. 22.3; medulla oblongata, 38.1 vs. 38.5; hypothalamus, 33.1 vs. 41.4; striatum, 25.6 vs. 22.0; midbrain, 34.3 vs. 35.7; and cortex, 24.6 vs. 24.5 (U/g wet weight, first values those of Leong and Clark, second values from the present work) (4). These authors used fresh tissue and esentially the same assay used in the current work except that the reaction temperature was 25 C rather than 37 C. They did not use freeze-thawing but seemingly were successful in obtaining an equivalent release of GDH from the mitochondria by using a glass homogenizer with a very small clearance, 0.0075 inches. They ranked the regions on the basis of GDH content in the order: medulla

oblongata and pons > midbrain=hypothalamus > cerebellum=striatum=cortex. In this study, the concentrations in the medulla and hypothalamus are equivalent and the midbrain ranks slightly below them, but otherwise the same pattern was obtained. The hippocampus, not measured by Leong and Clark, is also equivalent in GDH content to the cerebellum, cortex, and striatum.

The values for ICDH found in the frozen-thawed homogenates are very similar to those of Leong et al. (38). These authors report concentrations ranging from 1.79 to 2.34 U/g wet weight in comparison to values of 2.44 to 3.56 U/g wet weight reported here. That the values of Leong et al. are consistently lower may be attributable to running the assay at 25 C instead of 37 C. The present results agree with those of these authors as no remarkable regional variability is noted for ICDH.

The values for LD in the frozen-thawed homogenates agree well with those of Leong et al., ranging from 65-107 U/g wet weight in the present work in comparison to 75-118 U/g wet weight (38). The values of Leong et al. were consistently higher, but only slightly, than those reported here. This is a little surprising as these authors used 25 C as the assay temperature instead of 37 C as used in this study. Leong et al. did not use freeze-thawing but they did incorporate Triton X-100 into the assay mixture to allow measurement of the maximal activity. The effect of the Triton detergent may account for their higher values, especiallly if the supernatants contained fragments of membranes and/or organelles from which the LD could be released. They ranked the regions on the basis of LD content in the order: striatum > cortex > midbrain = hypothalamus > cerebellum > medulla oblongata (2,38). The findings of the present work for the NWA homogenates result in ranking the regions as follows : midbrain = cortex =

striatum = hippocampus > cerebellum = hypothalamus = medulla oblongata. The differences in the patterns are likely due to the enhanced enzyme solubilization technique used in this study. Some regional variability is observed in both sets of data.

The concentrations of MDH found in this study, ranging from 215-261 U/g wet weight in the NWA homogenates, whose values were much higher than those of the fresh and frozen-thawed homogenates, differ considerably from those reported by Leong et al., which ranged from 512 to 668 U/g wet weight (38). Why the MDH values of these authors are so much higher is not obvious and is surprising in light of the very reasonable comparability of values for other enzymes. The assay systems used are not identical. Lai and Clark used a potassium phosphate buffer, pH 7.4, and a 25 C reaction temperature whereas the present work employed a diethanolamine buffer, pH 9.2, and a 37 C reaction temperature (115). These were the major differences and perhaps are sufficient to account for the variance seen. Leong found the least MDH activity in the medulla oblongata and the hypothalamus which agree with the results reported here. They found the highest MDH activity in the cerebellum with slightly less MDH in the cerebral cortex. The present study found the cerebral cortex and the hippocampus, using the NWA values, to be richest in MDH and the midbrain to have the next greatest concentration of MDH. While Leong et al. found greater regional differences in MDH than the present work, it may be concluded that basically equivalent concentrations of MDH are seen in all of the regions studied.

In conclusion, ACP, AST, ICDH, and MDH do not exhibit marked regional variability in the rat in the seven brain regions examined. CK,

GDH, and LD, on the other hand, do vary considerably in concentration in one or more regions.

The regional variability of enzymes in the cat brain has also been examined. Values for enzyme concentrations in the cat brain are not available in the literature but they are generally in the same range as those in the rat brain, although the rat concentrations tend to be higher than those of the cat.

ACP, AST, ICDH, and MDH show no marked regional variability. LD concentrations are also very equivalent but the medulla shows the lowest values of all seven regions in both the frozen-thawed and fresh homogenates. The GDH concentration in the medulla is about twice as high as in the other six regions, which are basically equivalent.

CK is the enzyme which exhibits the greatest regional variability in the cat brain, as it did in the rat brain. The CK concentrations in the cerebellum and striatum are roughly twice as great as in the medulla, hypothalamus, and midbrain. CK concentrations in the cortex and hippocampus are equivalent and fall in between the other two groups. This pattern of regional variability for CK varies from that seen in the rat brain but in both animals the cerebellum contains the most CK and the hypothalamus the least.

It would have been interesting to have used NWA detergent treatment for enzyme solubilization of cat brain tissue. Values from NWA homogenates presumably would have generally corroborated the findings in the fresh and frozen-thawed homogenates, as they did in the rat brain.

In conclusion, ACP, AST, ICDH, LD, and MDH are not subject of regional variability in the cat in the seven brain regions examined. GDH is distinctly higher in one region than in the other six and the CK concentration shows considerable heterogeneity for the regions studied.

Based upon assay of two cat CSF specimens obtained by cisternal puncture. it is seen that AST, CK, GDH, LD, and MDH are present in cat CSF. ICDH was not detectable. The cat CSF enzyme values for AST, CK, and LD are in agreement with those reported by Maas (16,17).

COMPARISON OF CAT AND RAT ENZYME CONCENTRATIONS

By comparing the values in Tables 19 and 22, both for frozen-thawed homogenates, it can be seen that the concentrations of all seven enzymes in the rat brain are in general higher than in the cat brain. ACP, AST, ICDH, and MDH show no great regional variability in either animal. LD concentrations are reasonably equivalent from region to region in both animals except for the medulla in which the LD is lowest in both. The rat brain has higher concentrations of GDH in three regions, the medulla, hypothalamus, and midbrain, than in the other four, while only one region, the medulla, in the cat brain is much higher than the others. CK shows the most marked variability in both animals but the pattern is different. While the cerebellum in the rat is richest in CK, both the cerebellum and striatum in the cat have the highest concentrations. The hypothalamus has the lowest concentration of CK in both animals.

It is not surprising that there are some broad similarities between the two animals and some specific differences. Presumably the similarities in regional enzyme concentrations reflect like metabolic needs in the brains of the cat and rat and the differences in regional enzyme patterns reflect the unique metabolic requirements of the two species.

CRITICAL CONCERNS

The original purpose of this study was to determine if there were differences in regional enzyme concentrations in the brain. Enzyme activity

in homogenates was measured and quantitated per unit of tissue weight. The answers that have been obtained are confounded by several factors. First, activity is a somewhat fickle entity to measure. Unlike assaying a molecule such as glucose, in which case a concentration value is reported which ultimately translates to a specific number of molecules of glucose, activity does not necessarily translate into a specific number of enzyme molecules. As is evident from a review of the literature, the reported concentration of a given enzyme in tissue will depend upon how the activity is measured, e.g., substrate concentrations, assay temperature, pH, etc. These factors can affect the results of assay for simpler molecules as well but differences in methodology have a major impact on enzyme activity values and thus on enzyme concentrations. Standardization and optimization does much to improve the situation, at least for measurement in a patient serum specimen. But when tissue is being processed, other factors can influence the enzyme activity measured. Witness the drastic effects of enzyme solubilization seen in this study.

An assay methodology that would not measure a secondary expression of an enzyme such as activity but would get to the heart of the matter and detect directly the number of enzyme molecules present would be useful. An immunological assay, such as an RIA test, that would produce an enzyme concentration in terms of mass or number of molecules would likely be preferable to measuring activity. Such assays do exist for some enzymes but they are not at all prevalent or commonly used.

Even if an assay which measures the number of enzyme molecules directly were readily available, in a regional variability study the problems associated with tissue processing would have to be dealt with. How does one know that all existing enzyme molecules have been liberated from the tissue and

measured? The problem of enzyme latency is still present even if the complications associated with measuring activity were no longer an issue.

Yet another complication can be mentioned. Perhaps the enzyme assays should be optimized for each brain region examined as the catalytic properties of an enzyme or the distribution of isoenzymes may differ from region to region.

It is likely that the results presented here are not definitive in terms of describing with absolute accuracy the amount of a given enzyme contained in a particular brain region. But hopefully they do reflect the relative amounts of enzymes in the regions and do show meaningful differences in regional variability.

CLINICAL IMPLICATIONS

The question of regional variability of enzymes in the brain has been addressed by this research primarily for the purpose of determining which enzymes, if any, in the CSF are likely to be useful as prognostic indicators in cases of brain injury. Maas has demonstrated that brain injury will result in a release of enzymes from the brain into the CSF and that in general, the more severe the brain damage, the higher the elevation of CSF enzymes (16,17). The problem that has arisen in clinical studies is that elevated CSF enzyme levels do not always correlate with patient outcome and therefore the prognostic value of CSF enzymes is uncertain. This study has established that in the rat and cat some enzymes exhibit significant regional variability which can make it difficult to equate CSF enzyme levels with the extent of brain damage and the likelihood of recovery. CK specifically is the one enzyme that is probably not a good candidate for a prognosticon in cases of brain injury as it exhibits marked regional variability. Ironically, it is the enzyme that has been most studied in the CSF just for this purpose. While it is likely

that increased CSF CK levels correlate with brain tissue damage, it is also likely that there will be too many instances where the CSF CK elevation will not accurately reflect the amount of tissue damage to make it a completely reliable prognostic indicator. AST or LD, having more homogeneous concentrations throughout the brain, would probably be more suitable.

GDH and MDH, being basically mitochondrial enzymes, might be very interesting to examine in this regards. If severe tissue damage has occurred, including necrosis, it is likely that mitochondria will be destroyed and release their enzyme complement. Elevated CSF levels of mitochondrial enzymes may better reflect the severity of cell damage than typically cytoplasmic enzymes, even though GDH is more concentrated in one region, the medulla, than in others. Both prospective and retrospective clinical studies are called for to prove the utility of CSF enzyme measurements. The results of this study may prove useful in interpretting the findings of clinical investigations, e.g., explaining why one enzyme correlates better with prognosis than another, and may suggest which enzymes are more likely to offer a good prognostic correlation but it can not prove that CSF enzyme measurements are truly useful prognosticons.

AST, CK, and LD are all routinely assayed in the clinical laboratory, making them attractive enzymes to measure in the CSF. GDH and MDH are not commonly assyed but could be with little difficulty if the value of CSF determinations for these enzymes were proven. But enzymes are in general fairly ubiquitous molecules. Molecules more specific for the nervous system might be a better bet as prognostic markers. O'Callaghan and Miller have suggested that quantification of nervous system specific proteins in the CSF would be more useful than enzymes in gauging not only the extent of cell death but even the specific types of neuronal cells affected (116). Greengard

et al. have ennumerated a large group of neuronal phosphoproteins of possible clinical significance (117). These phosphoproteins fall into three categories: (1) those widely and fairly evenly distributed throughout the nervous system, (2) those widely but unevenly distributed, being enriched in certain nerve cells, and (3) those restricted to one brain region only, presumably localized in a single neuronal cell type. Ubiquitous neuronal phosphoproteins include synapsin Ia and Ib and proteins IIIa and IIIb. The widely but unevenly distributed phosphoproteins include the enzyme tyrosine hydroxylase. Cell specific phosphoproteins are the substrates for a GMP-dependent protein kinase and a cyclic AMP-dependent protein kinase. Protein III has been found in the CSF of normal human subjects and it has been suggested that neuronal damage in the brain can be detected and quantitated by measureing CSF protein III.

As promising as the various types of phosphoproteins may be in clinical settings as prognosticons, their assays are cumbersome and slow. If phosphoproteins can be shown to be of value to the neurosurgeon, it would certainly be worthwhile to develop assays for them that are suitable for the clinical laboratory. The advantage of measuring CSF enzymes is that these tests can be performed readily and easily at present by nearly any clinical laboratory. It is probably prudent to pursue both approachs to discover valuable prognostic markers for as many pathological conditions affecting the central nervous system as possible.

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