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THE SUBCELLULAR LOCALIZATION OF SULFUR AMINO ACID METABOLISM IN RAT BRAIN

by

DAVID KEITH RASSIN

A dissertation submitted to the Graduate
Faculty in Biomedical Sciences in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy, The City
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1974

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

THE SUBCELLULAR LOCALIZATION OF SULFUR AMINO ACID METABOLISM IN RAT BRAIN

by

DAVID KEITH RASSIN

Advisor: Professor Gerald E. Gaull

Evidence for the participation of some amino acids in synaptic function has become convincing, especially for glycine and gamma-aminobutyric acid. A number of the sulfur containing amino acids have also become implicated as neurotransmitter substances: cystathionine, cysteine, cysteine sulfinic acid, cysteic acid, hypotaurine and taurine. These amino acids are the substrates and products of the transsulfuration portion of the pathway of the metabolism of methionine, which also is associated with methylation reactions and folate metabolism.

Those enzymes that catalyze reactions in the pathway of metabolism of the sulfur containing amino acids involving compounds related to neurotransmitter function are located in different subcellular fractions than the enzymes that make up the remainder of the pathway. Methionine adenosyltransferase and N^5-methyltetrahydrofolic acid-homocysteine methyltransferase were found in soluble fractions. Cystathionine β-synthase, cystathionase, cysteine dioxygenase and cysteine sulfinic acid decarboxylase

were found in particulate fractions. Serine hydroxymethyltransferase, involved in folate metabolism as well as the major site of synthesis of glycine in brain, was found in mitochondrial fractions.

These studies provided an overall view of the localization of the metabolism of methionine rather than an isolated study of a single step. This broad study produced information that may explain how the small amino acid pool necessary for synaptic transmission may be differentiated from the large free amino acid pool that is present in brain.

TO

GLENNDA

MY LOVE

FOR

MEYA AND KEITH

OUR FUTURE

ACKNOWLEDGEMENTS

Dr. Gerald Gaull, Chairman of the Department of Pediatric Research at the Institute for Basic Research in Mental Retardation, has been a friend, advisor and booster without peer. He took me in at the nadir of my educational experience and opened up the world of science for me. I am very grateful for all the opportunities and encouragement he has provided.

Dr. Jack Peter Green, Chairman of the Department of Pharmacology at Mount Sinai, has given me the honor of being associated with his department while allowing me to continue my research at the Institute for Basic Research. Dr. Green's door has always been open, and he has been available to discuss problems and put them in their perspective.

My parents, Dr. and Mrs. Meyer Rassin, have always helped when needed. Their love and support are deeply appreciated.

Working with my colleagues in the Institute for Basic Research has been a gratifying experience. Dr. Victor Whittaker initiated my interest in and provided me with the opportunity to learn the techniques of subcellular fractionation. Dr. Harris Tallan has been a patient encyclopedia of biochemistry and a gentle critic, answering my innumerable questions. I am also in debt to all the other members of the department, past and present who have assisted and encouraged me at one time or another. In particular I would like to thank Cynthis Sargente, Karmela Schneidman, Ram Bhatt and Lucille Donadio for their help.

Learning under the faculty of the Department of Pharmacology has been a pleasure. Their courses were all taught with enthusiasm and pride. Drs. Ed Ronan, Sherwin Wilk and Marian Orlowski have been especially available to me for informal chats about their work and have always been interested in my efforts.

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Four individuals were truly instrumental in getting this thesis to its final form. Senta Frank cheerfully smoothed the bureacratic path through Mount Sinai for myself and many other graduate students. Lena Antonucci, Alexis Marangos and Edna Troiano did the typing, that essential task, with enthusiasm, speed and perfection.

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I. INTRODUCTION

Evidence for the participation of certain amino acids in synaptic function has become convincing, especially in the case of glycine and gamma-aminobutyric acid. Other amino acids which have also been implicated as possible neurotransmitter substances include a number of the sulfur-containing amino acids: cystathionine, cysteine, cysteine sulfinic acid, cysteic acid, hypotaurine and taurine. These amino acids are the substrates and products of one portion of the pathway of metabolism of the sulfur containing amino acids, which also is associated with methylation reactions and folate metabolism. This thesis will show that in rat brain the enzymes that act upon the compounds in that portion of the pathway of sulfur amino acid metabolism related to possible neurotransmitter function are located in different subcellular fractions than the enzymes that make up the remainder of the pathway. This localization may explain how the small amino acid pool necessary for synaptic transmission might be differentiated from the large free amino acid pool that is present in brain.

Amino acids as neurotransmitters

The transmission of nervous impulses across the synaptic cleft at neuronal junctions in the central nervous system is probably accomplished by the release of one of a number of naturally occurring compounds from the presynaptic portion of the synapse, followed by interaction with receptors at the postsynaptic membrane. Compounds may be defined as neurotransmitters if they satisfy the following criteria (Werman, 1966; Dudel, 1968; Phillis, 1970):

1. Presence. The transmitter, its precursor and the enzymes necessary for synthesis should be near the site of action.

2. Collectability. A specific mechanism to release the transmitter at the synapse should be present and it should be possible to collect the transmitter at the synapse after stimulation of the presynaptic neurone.
3. Identity of action. The suspected transmitter must mimic the action of the natural transmitter in its effects on membrane potential, rate of firing, ionic equilibrium potential, and membrane resistance. Interaction with pharmacological agents at the postsynaptic site must be identical for both the natural transmitter and the suspected transmitter.
4. Inactivation. To prevent continuous discharge of the neurone, a means of removal of the transmitter must be available, either by enzymatic breakdown or by rapid reuptake transport mechanisms at the nerve ending.

Possible neurotransmitter compounds may be studied initially by application to the synaptic junction by microiontophoresis (release of a compound from a microelectrode by means of an ionic current). Active neurotransmitter compounds will display properties that may be described as either excitant (depolarization of the synaptic membrane and initiation of nerve impulses) or depressant (hyperpolarization of the existing membrane potential or inhibition of the discharge across the synapse of impulses instigated by a remote stimulus). Compounds that affect electrical activity but are released from resting nerve cells in the course of normal metabolic events, rather than as the end result of nerve stimuli, may be better defined as modulators or regulators of synaptic transmission than as neurotransmitters (Hebb, 1970).

The excitant and depressant actions of some amino acids when iontophoretically applied to central nervous system (CNS) neurones stimulated investigations into their possible function as neurotransmitters (Curtis, Phillis and Watkins, 1959a, 1959b, 1960; Curtis and Watkins 1960; Curtis 1960; Krnjević, 1965). Further evidence for the involvement of certain amino acids in neurotransmission includes their excitant or depressant actions after intraventricular injection into mice (Crawford, 1963), and their release from cerebral cortex after thalamic and brain-stem stimulation in cats (Jasper and Koyama, 1969). Some of the naturally-occurring excitant and depressant amino acids are shown in Table I.

Additional evidence to support a role for amino acids in synaptic function has been sought by attempts to demonstrate storage of amino acids in synaptic vesicles in a manner analogous to storage of acetylcholine (Whittaker, Michaelson and Kirkland, 1964). Acetylcholine also has been demonstrated to have affinity for the synaptic vesicles (Marchbanks, 1968), while dopamine appears to have a much lower affinity for brain particulate fractions (Laverty et al., 1963). Only about 10% of the amino acids found in the synaptosome are localized in synaptic vesicles (Mangan and Whittaker, 1966). These amino acids do not appear to be bound to the synaptic vesicles of the guinea-pig cerebral cortex (Rassin, 1972), although taurine and possibly glutamate may be bound to the synaptic vesicles of the rat brain (DeBelleroche and Bradford, 1973). Synaptic vesicles containing amino acids may be more vulnerable to the osmotic shock used to rupture synaptosomes in these experiments, or some other mechanism may exist for the storage and release of these neurotransmitters.

The electrophysiological properties of subcellular fractions of guinea-pig brain have been studied by direct iontophoretic application and found to be consistent with their content of excitatory and depressant amino acids (Krnjević and Whittaker, 1965). However, the conclusions of

TABLE I

PUTATIVE AMINO ACID NEUROTRANSMITTERS

Putative excitant and depressant amino acid neurotransmitters shown to illustrate their structural relationships (adapted from Curtis and Watkins, 1960; Werman, *et al.*, 1966).

Depressant		Excitant	
Taurine	$SO_3H-CH_2-CH_2-NH_2$	$SO_3H-CH_2-CH(COOH)NH_2$	Cysteic Acid
Hypotaurine	$SO_2H-CH_2-CH_2-NH_2$	$SO_2H-CH_2-CH(COOH)NH_2$	Cysteine Sulfinic Acid
Glycine	$HOOC-CH_2-NH_2$	$HOOC-CH(COOH)NH_2$	Amino Malonic Acid
α-Alanine	$CH_3-CH(COOH)NH_2$		
β-Alanine	$HOOC-CH_2-CH_2-NH_2$	$HOOC-CH_2-CH(COOH)NH_2$	Aspartic Acid
GABA	$HOOC-CH_2-CH_2-CH_2-NH_2$	$HOOC-CH_2-CH_2-CH(COOH)NH_2$	Glutamic Acid
Cystathionine	$NH_2(COOH)CH-CH_2-S-CH_2-CH_2-CH(COOH)NH_2$		

this study must be tempered by the fact that it is not possible to separate excitatory from inhibitory nerve endings (Krnjević and Whittaker, 1965). It has been found that gamma-aminobutyric acid (GABA) is released from a synaptosomal and mitochondrial fraction by osmotic shock (Neal and Iversen, 1969) and that electrical stimulation will cause preferential release of putative transmitter amino acids from synaptosomes, although all amino acids are released to some extent (Bradford, 1970).

The small proportion of amino acids found associated with the synaptic vesicles, whether or not bound, still represents a quantity more than sufficient to fulfill the role of a transmitter compound. It has been calculated that one vesicle from the cerebral cortex of the guinea-pig contains 300 molecules of acetylcholine, which is much less than the number of molecules of glutamate (9300), aspartate (2000), GABA (1900) and glycine (1400) associated with one vesicle (Whittaker, 1968).

The potential importance of amino acids in synaptic transmission can be seen when the quantities of more widely studied neurotransmitters are compared: in the corpus striatum (the richest dopaminergic brain area) only 15% of the nerve terminals contain catecholamines; in the hypothalamus (rich in norepinephrine) only 5% contain catecholamines. There are fewer neurones containing serotonin than norepinephrine, and nerve terminals containing acetylcholine probably represent only about 10% of the total. In contrast 20-40% of nerve terminals in various regions have been found to be associated with GABA (Snyder _et_ _al_., 1973).

The amino acids that have been most closely associated with neurotransmission are glycine and GABA, two putative inhibitory transmitters, and glutamic acid, a putative excitatory transmitter. Glycine (Aprison _et_ _al_., 1968; Werman, 1972) and GABA (Curtis, 1973) both appear to satisfy three of the criteria for neurotransmitters: presence, collectability and identity of action. Glycine has been shown to be specifically blocked by strychnine at inhibitory synapses in the spinal cord and brain-stem (Curtis and Johnston, 1970; Johnson, Roberts and Straughn, 1970), while bicuculline has the same effect upon the inhibitory actions of GABA in the cerebral cortex and other brain areas, but only inhibits spinal motor neurons by its action at central synapses (Curtis, 1973). GABA has been shown not only to block excitatory stimuli but also to cause a hyperpolarization of the cortical neurone similar to that of the natural potential change seen during synaptic inhibition (Krnjević and Schwartz, 1967). The excitatory actions of glutamic acid upon neurones have been demonstrated (Curtis and Watkins, 1960; Zieglgänsberger and Puil, 1973), but it has not been possible to establish this amino acid as an excitatory transmitter in the manner that glycine and GABA have been shown to be inhibitory transmitters. The major difficulties in the study of glutamic acid are its high concentrations and extensive metabolic interrelationships in brain, in particular with the tricarboxylic acid cycle and with aspartic acid (another potential excitatory neurotransmitter) (Johnson, 1972). Also, it has not been possible to find specific pharmacological antagonists for glutamic acid such as strychnine for glycine and bicuculline for GABA (Curtis _et_ _al_., 1972).

Other naturally occurring amino acids that have been demonstrated to have excitatory actions in the CNS include: aspartic acid, cysteic acid and cysteine sulfinic acid (Curtis and Watkins, 1960, 1965). The depressant properties of β-alanine, alanine, taurine (Curtis and Watkins, 1960, 1965) and cystathionine (Werman, Davidoff and Aprison, 1966) make these substances candidates for the role of inhibitory transmitters within the CNS.

There have been two major problems in establishing amino acids as neurotransmitters. One is the necessity for a mechanism of inactivation rapid enough to be consistent with the neurotransmitter function. The second problem is the involvement of the amino acids in general cellular metabolism. Some means must be shown by which the metabolic pool of amino acids is separated from the transmitter pool.

The question of how transmitter amino acids are inactivated may have been answered by the finding that specific high affinity uptake systems exist for GABA, glycine, glutamic acid and aspartate in addition to the low affinity systems that are usually found for amino acids in various CNS preparations. A glutamic acid and aspartic acid high affinity uptake system with structural specificity has been demonstrated in rat brain slices (Balcar and Johnston, 1972a, 1972b). High affinity systems are demonstrable for aspartic acid and glutamic acid in synaptosomes of rat cerebral cortex, and for aspartic acid, glutamic acid and glycine in synaptosomes of rat spinal cord (Logan and Snyder, 1971, 1972). High affinity systems have also been shown for GABA and glutamate in rat brain slices and synaptosomes (Levi and Raiteri, 1973) and for aspartic acid, glutamic acid, glycine and GABA in cat spinal cord (Balcar and Johnston, 1973).

The manner in which the metabolic and transmitter pools of amino acids might be differentiated is less clear. If specific localization of enzymes for the production of transmitters were found in subcellular fractions related to the nerve endings, this might explain how separation of amino acid pools could occur. Glutamate decarboxylase, the enzyme that synthesizes GABA, may be localized in nerve ending particles (Weinstein, Roberts and Kakefuda, 1963; Salganicoff and DeRobertis, 1963; Fonnum, 1965, 1968). Also, aspartate-oxoglutarate transaminase, the glutamic acid synthesizing enzyme, has been found in soluble and mitochondrial forms within synaptosomes (Fonnum, 1965, 1968). The data presented in this thesis support the hypothesis that just such an enzymatic localization exists for portions of the sulfur amino acid metabolic pathway.

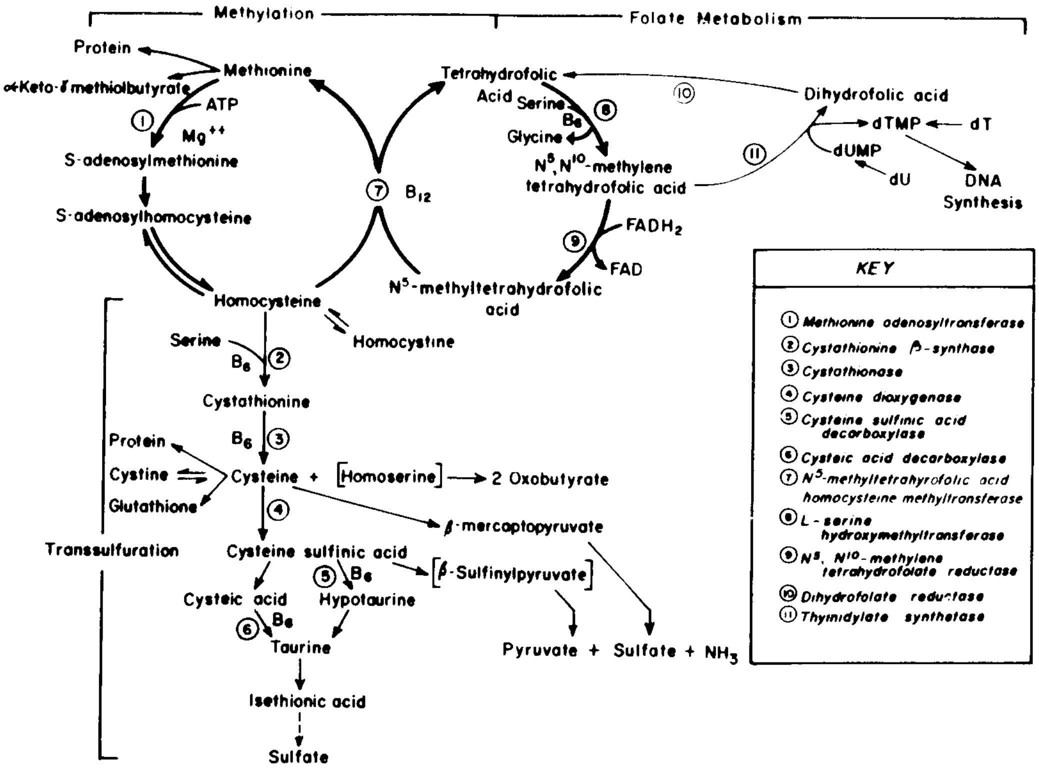
The metabolism of methionine in brain

Methionine may serve as a precursor for protein synthesis or it may enter the methylation-transsulfuration pathway (Figure 1) (reviewed by Meister, 1965; Shapiro and Schlenk, 1965; Jacobsen and Smith, 1968; Blakely, 1969; Gaitonde, 1970). The methylation-transsulfuration reactions involve: 1) a methylation cycle that converts methionine to homocysteine, which is then remethylated to methionine, this cycle producing S-adenosylmethionine (SAM), for use as a methyl group donor; 2) a cycle of reactions involving folate metabolism which is interrelated with the methylation cycle; 3) a series of reactions that may be termed the transsulfuration pathway, which metabolizes homocysteine to other sulfur containing amino acids and sulfate. The methylation and folate cycles may maintain one another by regenerating metabolites for their continued individual function, whereas the transsulfuration pathway draws off methionine for its ultimate breakdown to carbon dioxide and sulfate. Enzymes in each of these three portions of the methionine metabolic pathway have been studied in an effort to determine whether a functional relationship might be correlated with their subcellular localization (the enzymes studied are listed in Table 2).

The first step of the methylation cycle, catalyzed by methionine adenosyltransferase (Reaction I), involves the activation of methionine to SAM by the addition of the adenosine moiety of ATP at the sulfur

FIGURE 1

THE METABOLISM OF METHIONINE IN RAT BRAIN



It should be noted that the interconversion of S-adenosylhomocysteine and homocysteine favors the direction of S-adenosylhomocysteine synthesis. SAM is a cofactor for N^5-methyltetrahydrofolic acid-homocysteine methyltransferase (7).

TABLE 2

ENZYMES AND REACTIONS OF SULFUR AMINO ACID METABOLISM

This table shows the recommended name, the systematic name, the enzyme commission number and the reaction associated with the enzymes upon which this study has been based. The nomenclature reflects the most recent recommendations of the Commission on Biochemical Nomenclature - 1972 (1973). The numbers in parentheses represent the appropriate step in Figure 1.

(1) Methionine adenosyltransferase
ATP:L-methionine S-adenosyltransferase
EC 2.5.1.6
ATP + L-methionine + H_2O = PP_i + P_i + SAM

(2) Cystathionine β-synthase
L-serine hydro-lyase (adding homocysteine)
EC 4.2.1.22
L-serine + L-homocysteine = L-cystathionine + H_2O

(3) Cystathionase (alternate name); Cystathionine γ-lyase
(recommended name)
L-cystathionine cysteine-lyase (deaminating)
EC 4.4.1.1
L-cystathionine + H_2O = L-cysteine + NH_3 + 2-oxobutyrate

(4) Cysteine dioxygenase
L-cysteine: oxygen oxidoreductase
EC 1.13.11.20
L-cysteine + O_2 = L-cysteine sulfinic acid

(5) Cysteine sulfinic acid decarboxylase[1]; Aspartate 4-decarboxylase
(recommended name).
L-Aspartate 4-carboxy-lase
EC 4.1.1.12
L-cysteine sulfinic acid = hypotaurine + CO_2

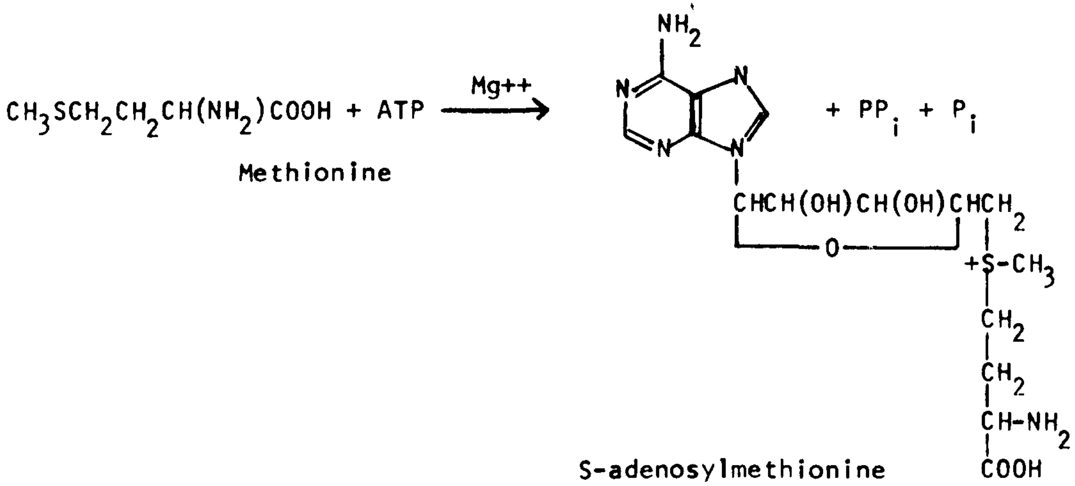
(7) N^5-Methyltetrahydrofolic acid-homocysteine methyltransferase[1];
Tetrahydropteroylglutamate methyltransferase (recommended name)
5-Methyltetrahydropteroyl-L-glutamate:L-homocysteine S-methyltransferase
EC 2.1.1.13
5-methyltetrahydropteroyl-L-glutamate + L-homocysteine = tetrahydropteroylglutamate + L-methionine

(8) Serine hydroxymethyltransferase
5,10-methylenetetrahydrofolate: glycine hydroxymethyltransferase
EC 2.1.2.1
5,10-methylenetetrahydrofolate + glycine = tetrahydrofolate + L-serine

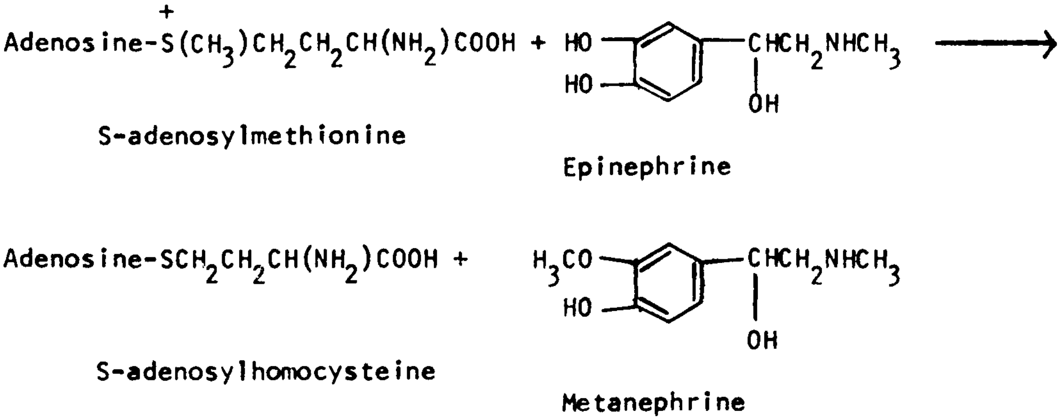
[1] This is not an official or alternative name but indicates more clearly the reaction that is being investigated.

position of methionine. SAM may then be demethylated to S-adenosylhomocysteine by a number of specific enzymes which use SAM as a methyl donor; catechol-O-methyltransferase is one such enzyme found in brain (Reaction II).

(I)



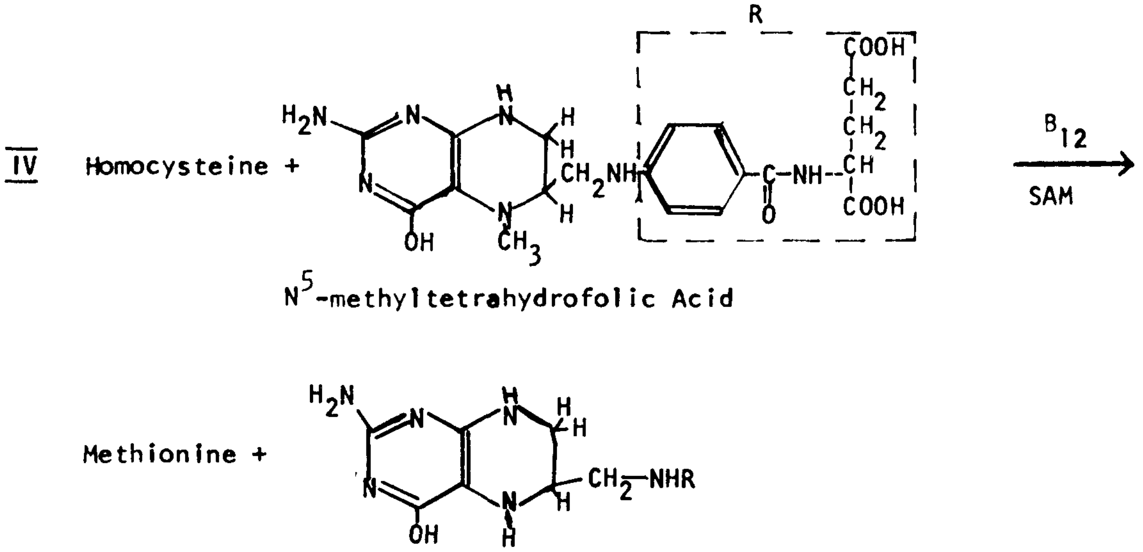
(II)



S-Adenosylhomocysteine is hydrolyzed to homocysteine and adenosine if one product is removed (Reaction III). The equilibrium of this reaction is in the direction of S-adenosylhomocysteine, which may explain why free S-adenosylhomocysteine, and not homocysteine, may be detected in tissue extracts (Gaull and Gaitonde, 1967).

$$\text{(III)}\quad \text{S-adenosylhomocysteine} \rightleftharpoons \text{Adenosine} + \underset{\text{Homocysteine}}{HSCH_2CH_2CH(NH_2)COOH}$$

Homocysteine may then be remethylated to methionine by N^5-methyltetrahydrofolic acid-homocysteine methyltransferase by the transfer of a methyl group from N^5-methyltetrahydrofolic acid in the presence of vitamin B_{12} (Reaction $\overline{\underline{IV}}$).

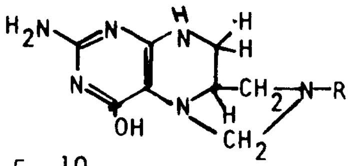


N^5-methyltetrahydrofolic Acid

Tetrahydrofolic Acid

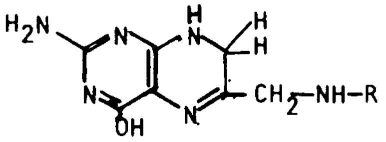
Tetrahydrofolic acid is the precursor for the series of reactions that leads to the regeneration of N^5-methyltetrahydrofolic acid as well as to thymidylate formation for synthesis of DNA. Tetrahydrofolic acid reacts with serine in the presence of pyridoxal phosphate to produce glycine. The β-carbon of serine becomes the methylene group of N^5,N^{10}-methylenetetrahydrofolic acid (catalyzed by the enzyme serine hydroxymethyltransferase)(Reaction $\overline{\underline{V}}$). N^5,N^{10}-Methylenetetrahydrofolic acid may participate in the synthesis of deoxythymidine monophosphate from deoxyuridine monophosphate catalyzed by thymidylate synthetase (Reaction $\overline{\underline{VI}}$). The deoxythymidine monophosphate is a DNA precursor, and the dihydrofolic acid that is produced reenters the folate cycle after reduction to tetrahydrofolic acid in the presence of NADPH by the dihydrofolate reductase (Reaction VII).

V Tetrahydrofolic Acid + $\underset{\text{Serine}}{HOCH_2CH(NH_2)COOH} \overset{B_6}{\rightleftarrows} \underset{\text{Glycine}}{CH_2(NH_2)COOH}$ +



N^5,N^{10}-methylenetetrahydrofolic Acid

VI N^5,N^{10}-methylenetetrahydrofolic Acid + dUMP $\longrightarrow$



\+ dTMP

Dihydrofolic Acid

VII Dihydrofolic Acid $\xrightarrow{NADPH}$ Tetrahydrofolic Acid

VIII N^5,N^{10}-methylenetetrahydrofolic acid $\xrightarrow{FADH_2}$ N^5-methyltetrahydrofolic acid

N^5,N^{10}-methylenetetrahydrofolate reductase catalyzes the reduction of N^5,N^{10}-methylenetetrahydrofolic acid in the presence of $FADH_2$ to regenerate N^5-methyltetrahydrofolic acid which may then donate the methyl group needed for the conversion of homocysteine to methionine once again (Reaction VIII). Thus, the methionine methylation cycle and the folate cycle are interdependent and also have a branch to the pathway for the *de novo* synthesis of DNA. It should be noted here that N^5-methyltetrahydrofolic acid may be a better methyl donor for some reactions than SAM, further emphasizing the importance of this cycle in methylation (Banerjee and Snyder, 1973).

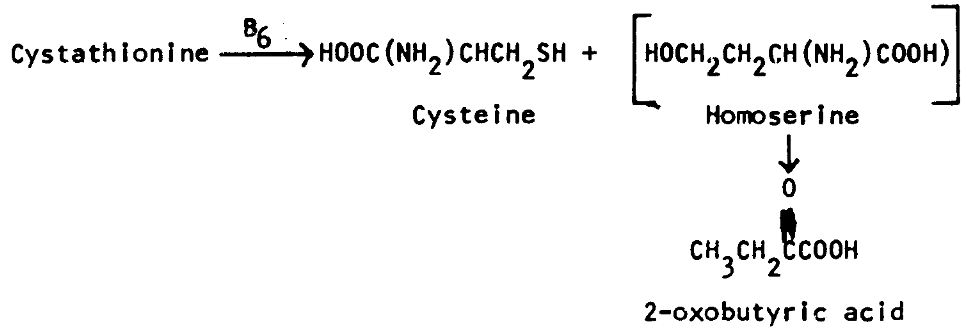
Another branch originating from the methylation cycle at the SAM position is of great importance in brain. S-Adenosylmethionine decarboxylase acts upon SAM in the first of a sequence of reactions that form the polyamines spermidine and spermine. The function of these compounds has not been elucidated, but in liver they appear to be associated with cellular proliferation.

The major route of metabolism away from the methylation-folate cycles for the sulfur moiety of methionine involves the reactions that oxidize homocysteine to sulfate. Homocysteine is first condensed with serine in the presence of pyridoxal phosphate by cystathionine β-synthase to form cystathionine (Reaction IX). This is the second point in the methionine metabolic system in which serine plays a part, the other being with serine hydroxymethyltransferase (Reaction V). Cystathionine is cleaved on the homocysteine moiety side of the sulfur ether to form cysteine (which now contains the carbon skeleton of serine) and 2-oxobutyrate (Reaction X). Homoserine may be a precursor of 2-oxobutyrate but has not been isolated as a direct product of this reaction. Cystathionase is also a pyridoxal phosphate dependent enzyme.

IX Homocysteine + Serine $\xrightarrow{B_6}$ $HOOC(NH_2)CHCH_2CH_2SCH_2CH(NH_2)COOH$

Cystathionine

X



$$\text{Cystathionine} \xrightarrow{B_6} \text{HOOC(NH}_2\text{)CHCH}_2\text{SH} + \left[\text{HOCH}_2\text{CH}_2\text{CH(NH}_2\text{)COOH)}\right] \to \text{CH}_3\text{CH}_2\overset{\text{O}}{\overset{\|}{\text{C}}}\text{COOH}$$

Cysteine; Homoserine; 2-oxobutyric acid

Cysteine may be metabolized in a number of ways, by incorporation into protein, by incorporation into glutathione, by transamination to β-mercaptopyruvic acid, by oxidation to cystine, or by enzymatic oxidation to cysteine sulfinic acid. Cysteine dioxygenase catalyzes the oxidation of cysteine to cysteine sulfinic acid in the presence of NAD$^+$ and Fe^{++} (Reaction XI). The cysteine sulfinic acid is oxidized to cysteic acid or may be decarboxylated to hypotaurine (Reactions XII and XIII) which may then be oxidized to taurine. Taurine may also be formed by the decarboxylation of cysteic acid (Reactions XIV and XV). Cysteine sulfinic decarboxylase and cysteic acid decarboxylase may be the same enzyme. They have been postulated to be the same as glutamic acid decarboxylase (Davison, 1956), although this has been disputed (Jacobsen, Thomas and Smith, 1964). Cysteine sulfinic acid, however, does appear to be a better substrate for the decarboxylase than cysteic acid (Bergeret, Chatagner and Fromageot, 1955).

XI Cysteine $\xrightarrow[\text{Fe}^{++}]{\text{NAD}^+}$ $\text{HO}_2\text{SCH}_2\text{CH(NH}_2\text{)COOH}$

Cysteine Sulfinic Acid

XII Cysteine Sulfinic Acid $\longrightarrow$ $\text{HO}_3\text{SCH}_2\text{CH(NH}_2\text{)COOH}$

Cysteic Acid

XIII Cysteine Sulfinic Aci $\xrightarrow[\text{GSH}]{B_6}$ $\text{HO}_2\text{SCH}_2\text{CH}_2\text{NH}_2$

Hypotaurine

XIV $$\text{Hypotaurine} \longrightarrow \underset{\text{Taurine}}{HO_3SCH_2CH_2NH_2}$$

XV $$\text{Cysteic Acid} \xrightarrow[\text{GSH}]{B_6} \text{Taurine}$$

Cysteine sulfinic acid also may be transaminated to β-sulfinylpyruvic acid which, like the β-mecaptopyruvate formed from cysteine, may be metabolized to pyruvate, ammonia and sulfate. Taurine is further metabolized in heart and brain tissue to isethionic acid (Reaction XVI). Isethionic acid does not appear to be further metabolized and may be an end product. Reports that taurine is metabolized to sulfate may be accounted for by the action of gut bacteria in *in vivo* experiments, for this conversion could be inhibited by prior treatment with antibiotics (Schram and Crokaert, 1957; Boquet and Fromageot, 1965).

XVI $$\text{Taurine} \longrightarrow \underset{\text{Isethionic Acid}}{HOCH_2CH_2SO_3H}$$

Sulfur amino acids and CNS function

The sulfur-containing amino acids have not been established as neurotransmitters in a manner similar to glycine or GABA, but there is considerable evidence for their importance to CNS function and some of them appear to be candidates for neurotransmitter status. Cystathionine, taurine and hypotaurine have been shown to have inhibitory properties (Werman *et al*., 1966, Curtis and Watkins, 1960) and cysteic acid and cysteine sulfinic acid have been shown to have excitatory properties (Curtis and Watkins, 1960).

The initial portion of the methionine metabolic pathway (as shown in Figure 1) is involved in methylation functions. SAM is an important methyl donor for the metabolism of norepinephrine and indoleamines, although recent evidence indicates that N^5-methyltetrahydrofolic acid (MTHF) may be a better methyl donor for serotonin, tyramine (Banerjee and Snyder, 1973) and dopamine (Laduron, 1973). Both SAM and MTHF are involved in the cycle of events that takes methionine to homocysteine and homocysteine back to methionine.

L-Dihydroxyphenylalanine (**L**-DOPA) is methylated to 3-O-methyldihydroxyphenylalanine rapidly after intraperitoneal injection in rats, with an accompanying decrease in brain SAM concentrations (Wurtman *et al.*, 1970). This reduction in rat brain SAM concentration, as well as a reduced concentration of rat blood SAM (Matthysse, Lipinski and Shih, 1971), has been reported to be accompanied by increased (Liu, Ombani and van Woert, 1972), unchanged (Ordonez and Wurtman, 1973) or decreased (Cotzias *et al.*, 1971) rat brain methionine concentrations after a single intraperitoneal injection of **L**-DOPA. Repeated intraperitoneal administration of **L**-DOPA did cause the methionine concentration in brain to decrease to 69% of control values (Ordonez and Wurtman, 1973). Single intraperitoneal injections of **L**-DOPA caused significant decreases in brain methionine concentrations in folic acid deficient rats, implying that maintenance of methionine brain concentrations is dependent upon remethylation of homocysteine by the folate cycle (Ordonez and Wurtman, 1974). Intraperitoneal injections of **L**-DOPA given every 24 hours for 7 days to rats have been reported to result in increased concentrations of cystathionine in brain and kidney (Brown and DeFoor, 1974).

L-DOPA also inhibits the uptake of methionine by synaptosomal particles in vitro (Baldessarini and Karobath, 1972). In the converse situation of high methionine concentrations, dopamine and its metabolites might be affected since methylation reactions may be increased. Worsened behavior has been observed in schizophrenic patients given methionine or cysteine loads with a monoamine oxidase inhibitor (Spaide, Davis and Himwich, 1971). Cysteine loading together with administration of tranylcypromine caused worsened behavior accompanied by excretion of the psychotomimetic methylated tryptamines (N-dimethyltryptamine, 5-hydroxy-N-dimethyltryptamine and 5-methoxy-N-dimethyltryptamine) (Narasimhachari et al., 1970). The increased excretion of these methylated derivatives may be a reflection of the methionine-sparing effects of cysteine, a favoring of the use of methionine for methylation and protein synthesis as opposed to its metabolism by the transsulfuration pathway to sulfate (Finkelstein and Mudd, 1967).

The first metabolite formed in the transsulfuration route away from the methylation cycle is cystathionine, a compound found in relatively high concentrations in human brain (Tallan, Moore and Stein, 1958). Cystathionine has also been found in spinal cord, where it has been implicated as a neurotransmitter because of its depressant actions on neurons after application by microiontophoresis (Werman et al., 1966). The importance of cystathionine as a putative transmitter has been questioned because of the nature of its distribution in spinal cord; the spinal gray matter does not appear to have significantly higher concentrations than the spinal white matter (Johnston, 1968), as has been found for glycine (Aprison et al., 1968).

Cat electrocorticogram patterns may be affected by cystathionine and by cysteine, one of the cleavage products of cystathionine.

Cystathionine caused increased synchrony and reduced blood pressure in *encéphale isolé* cats with ventricular cannulae. Cysteine caused desynchrony of the electrocorticogram and raised blood pressure in this type of preparation. Cystathionine (2 mg intraventricularly) given two minutes before cysteine (1 mg intraventricularly) abolished the usual desynchronous effect of the latter compound, although if cysteine were given eleven minutes after the cystathionine the characteristic desynchronous response was observed (Key and White, 1970). Cats with chronically implanted ventricular cannulae but no spinal transection in whom cysteine was injected showed hyperactivity, increased onset time before sleep and desynchrony of the electrocorticogram. The only effect of injected cystathionine in this group of animals was to shorten the time until the onset of sleep (Key and White, 1970). These effects are compatible with the suggested inhibitory transmitter properties of cystathionine (Werman *et al.*, 1966) and the possible excitatory properties of cysteine or its metabolites (Olney, Ho and Rhee, 1971).

The interactions of cystathionine and cysteine may be important during the development of the human fetus. The cystathionine concentration in human fetal brain is lower than that of the mature human brain; further cystathionase, the enzyme that cleaves cystathionine to cysteine, is virtually absent in fetal human brain (Sturman, Gaull and Raiha, 1970). Cystine may be an essential amino acid for the immature human (Sturman, Gaull and Raiha, 1970), but cystine is the only plasma free amino acid found in concentrations equal to or lower than that of the maternal plasma (Gaull *et al.*, 1973a). L-Methionine, L-leucine and L-ornithine are rapidly transferred to the fetus by the placenta against concentration gradients, but cystine appears slowly in the fetus after L-cysteine or L-cystine loads, suggesting that the fetus may be protected from excessive quantities of this metabolite (Gaull *et al.*, 1973a). Cystine concentrations may be regulated in this unique way by the placenta to ensure the correct balance between cystine and cystathionine, for cysteine (or its metabolites) may be neurotoxic (Olney, Ho and Rhee, 1971), especially in the presence of low concentrations of cystathionine. The reduced concentrations of cystathionine in the absence of its cleavage enzyme, cystathionase, may be explained by the high specific activities of N^5-methyltetrahydrofolic acid-homocysteine methyltransferase and serine hydroxymethyltransferase found in human fetal brain during the period of neuroblast proliferation (Gaull *et al.*, 1973b). The N^5-methyltetrahydrofolic acid-homocysteine methyltransferase also, has a higher affinity for homocysteine (Km = 10^{-5}M) than does cystathionine β-synthase (Km = 10^{-3}M) (Finkelstein, 1971) or S-adenosylhomocysteine hydrolase (Km = 10^{-3}) (J. Duerre, personal communication), two other enzymes for which homocysteine is a substrate.

Cysteine sulfinic acid and cysteic acid are two oxidation products of cysteine to which excitatory activity has been attributed (Curtis and Watkins, 1960). These compounds may also be responsible for the apparently excitatory neurotoxic effects observed after subcutaneous injection of L-cysteine because of *in vivo* conversion of its sulfhydryl group to the sulfinic or sulfonic groups (Olney, Ho and Rhee, 1971). Cysteic acid and cysteine sulfinic acid are the most potent competitive inhibitors of the high affinity transport system that has been suggested as the mechanism by which glutamic acid may be removed from receptor sites at the synapse (Balcar and Johnston, 1972a, 1972b). This inhibition may reflect a mechanism by which cysteic acid and cysteine sulfinic acid modulate the excitatory effects of glutamic acid. Alternatively this high affinity transport system may be a general reuptake mechanism for amino acids that function as excitatory neurotransmitters. Rat brain is the only tissue in which cysteine sulfinic acid has been detected without artificially raising concentrations of this metabolite by preloading (Bergeret and Chatagner, 1954). Rat brain has the capacity to synthesize cysteine sulfinic acid from cysteine (Yamaguchi et al., 1973).

Taurine, the decarboxylation product of cysteine sulfinic acid via hypotaurine or by direct decarboxylation of cysteic acid, is involved in a variety of physiological functions (Table 3) (Jacobsen and Smith, 1968). Taurine has recently been suggested as a transmitter as it satisfies the requirements of presence, both the compound and its synthesizing enzyme having been found in nerve ending particles (Agrawal, Davison and Kaczmarek, 1971). Uptake mechanisms capable of inactivating taurine are necessary because of the slow catabolism of taurine to isethionic acid (Peck and Awapara, 1967) and have been described (Davison and Kaczmarek, 1971; Kaczmarek and Davison, 1972). Conflicting results from experiments involving the transport of taurine in brain slices fail to confirm this transmitter role for taurine. Release of taurine after electrical stimulation in a manner analogous to presynaptic excitation has been observed (Hammerstad, Murray and Cutler, 1971; Davison and Kaczmarek, 1971; Kaczmarek and Davison, 1972). However, other experiments have failed to show the presence of either the high affinity uptake mechanism (Oja, 1971; Honegger et al., 1973) or the release of taurine by electrical stimulation (Lähdesmaki and Oja, 1972). An unexplained, electrically-stimulated influx of taurine in brain slices has been

TABLE 3

PHYSIOLOGICAL PROPERTIES OF TAURINE

Some of the physiological functions and phenomena with which taurine has been associated (adapted from Jacobsen and Smith, 1968) and others as cited in the text).

Possible physiological functions of taurine:

1. The inhibition of nerve impulses in invertebrate and vertebrate nerve tissue, neurotransmitter or modulator.
2. The regulation of excitability in cardiac and skeletal muscle.
3. The synthesis of bile salts.
4. Osmoregulation in marine invertebrates.
5. Energy storage in worms.

Unexplained phenomena involving taurine:

1. The high concentration of taurine in:
    - a) Brain
    - b) Cardiac Muscle
    - c) Skeletal Muscle
    - d) Platelets
    - e) Mast Cells
    - f) Pineal Body
    - g) Retina
2. The high concentration of taurine in fetal brain and liver and the rapid postnatal decrease in concentration.
3. The decreased excretion of taurine in mongoloids.
4. The increased excretion of taurine in X-irradiated humans.
5. The relationship of taurine to epilepsy.
6. The relationship of taurine to muscular dystrophy.
7. The relationship of taurine to retinal function.

observed (Lähdesmaki and Oja, 1972). Also, electrical stimulation apparently increases the rate at which taurine is synthesized by the decarboxylation of cysteine sulfinic acid (Oja, Karvonen and Lähdesmaki, 1973).

The depressant actions of taurine on spinal neurones, brain stem neurones, and cortical neurones have been observed, and pharmacological interactions with strychnine and bicuculline have been examined in order to find a specific antagonist. In brain stem neurones, strychnine blocks the depressant effect of taurine and glycine but not GABA (Haas and Hösli, 1973; Hösli, Haas and Hösli, 1973). The antagonism of glycine by strychnine in spinal neurones while the action of GABA continues unaffected has led to the classification of these inhibitory neurotransmitters as "glycine-like" or "GABA-like" (Curtis, Hösli and Johnston, 1967). The failure of bicuculline to antagonize taurine while it antagonizes GABA and the antagonistic effect of strychnine on glycine and taurine but not GABA in the brain stem neurones have led to classification of taurine with the "glycine-like" amino acids (Haas and Hösli, 1973). These pharmacological interactions are positive evidence for specific taurine post-synaptic receptor sites and favor a role as a possible neurotransmitter.

Taurine has been implicated in retinal function because of its depressant activity there (Pasantes-Morales _et_ _al_., 1972). Also, light-stimulation was found to cause release of taurine from the retina of dark-adapted chickens (Pasantes-Morales _et_ _al_., 1973). Studies of taurine transport and metabolism in rat retina failed to reveal a mechanism which was sufficiently finely-tuned to explain how taurine could be inactivated after release (Starr and Voaden, 1972), although a specific transport system appears to exist for taurine and another for GABA (Starr, 1973). It has been suggested that although taurine may not be a transmitter in the retina it is quite possible that taurine may be a modulator of synaptic function in this neural tissue (Starr and Voaden, 1972).

Study of the amino acid content in brain tissue from patients undergoing neurosurgery for the treatment of focal epilepsy has revealed that the focal region has low concentrations of taurine and glutamic acid and a high glycine concentration, accompanied by a decrease in concentration of GABA and aspartic acid in the cortex (Van Gelder, Sherwin and Rasmussen, 1972). These changes in amino acid concentration also have been found in the focal region of cats made epileptic by application of cobalt powder to the cortex (Van Gelder and Courtois, 1972). Taurine treatment of cats and mice made epileptic by topical application of cobalt reduced the seizure activity and, also restored the concentrations of the affected amino acids to normal (Van Gelder, 1972). Taurine has been reported to have an anticonvulsant effect in humans (Barbeau and Donaldson, 1973, 1974). Intraventricular oubain injections have been used as a model of epilepsy, and the resultant seizures may be decreased far more efficiently by taurine than by GABA (Izumi et al., 1973). It has been suggested that taurine may function to stabilize excitable membranes in the CNS, and it is this action that gives it anticonvulsant properties rather than an action as a neurotransmitter (Barbeau and Donaldson, 1974).

Intracisternal injections of taurine in the rat have been found to cause slow recovery of the righting reflect, a drop and slow recovery of colonic temperature, and a fall in arterial blood pressure. These effects were similar to but more dramatic than those of the inhibitory transmitters GABA and glycine when they were administered in the same way (Sgaragli and Pavan, 1972). Taurine and its metabolite isethionic acid have been shown to influence the excitable tissue of the heart, through direct and indirect effects upon calcium and potassium flux during irregularities associated with cardiac glycosides (Read and Welty, 1963, 1965; Welty and Read, 1964). Taurine has been shown to potentiate the positive inotropic effect of strophanthin-K on guinea-pig auricles (Guidotti, Badiani and Giotti, 1971) and to exert negative inotropic effects in rat heart and positive inotropic effects in guinea-pig heart similar to those of ouabain (Dietrich and Diacono, 1971). The function of taurine in muscle may be as a membrane stabilizer rather than a transmitter (Huxtable and Bressler, 1973) a mechanism that has also been suggested for the brain (Barbeau and Donaldson, 1974). Failure of this mechanism in muscle may be a part of the biochemical basis of muscular dystrophy (Banks, Rowland and Ipsen, 1971; Baskin and Dagirmanjian, 1973).

Homocysteine and folic acid also have been found to have some CNS effects. Homocysteine given in large concentrations to rats by intraperitoneal injection has been shown to have a convulsant action. These concentrations are admittedly excessive from either a physiological or pharmacological viewpoint but other metabolites of the pathway (methionine, cystine, serine, homoserine, cysteine) did not have such an effect in similar doses. The convulsant effect could be eliminated by prior administration of homoserine, serine, betaine, glycine or glucose (Sprince _et_ _al_., 1969; Sprince, Parker and Josephs, 1969). Folate and folinate have been shown to facilitate the excitatory effects of glutamate on single neurones of cat cerebral cortex and have weak excitatory effects of their own when applied to quiescent neurones (Davies and Watkins, 1973).

The evidence cited neither establishes these sulfur containing compounds as neurotransmitters nor does it establish any other specific CNS function. However, these metabolites do seem to be important to the CNS, and eventually they may be defined as transmitters or modulators of synaptic function when enough evidence has been accumulated.

It is also germane that inborn errors of metabolism involving the pathway of metabolism of sulfur amino acids have been reported. These diseases are sometimes associated with mental retardation, implying that normal CNS function is dependent upon normal sulfur amino acid metabolism. Some of the findings related to these disorders are relevant to this discussion of sulfur amino acids and CNS function. Homocystinuria associated with deficient activity of cystathionine β-synthase is the most common of these disorders (Mudd et al., 1964, 1965). This defect prevents the metabolism of homocysteine to cystathionine, causing increased plasma concentrations of methionine and homocystine and decreased concentrations or absence of plasma cystine. Post-mortem studies of the brains of affected individuals have demonstrated an absence of cystathionine (Brenton, Cusworth and Gaull, 1964, 1965; Gerritsen and Waisman, 1964) in contrast to the relatively high concentrations reported in normal brain (Tallan, Moore and Stein, 1958). A tentative conclusion is that an inability to synthesize cystathionine in brain may be associated with CNS dysfunction.

Homocystinuria may also be associated with enzymatic defects of N^5-methyltetrahydrofolic acid-homocysteine methyltransferase and $N^{5,10}$-methylenetetrahydrofolate reductase. Since remethylation is impaired these defects are accompanied by high plasma homocystine concentrations but low methionine concentrations. The methyltransferase deficiency was also accompanied by a cystathioninemia (Mudd, Levy and Morrow, 1970; Mudd et al., 1972). The clinical picture in the last two types of homocystinuria is not clear, because so few cases have been identified. However, it is likely that some have been associated with brain dysfunction (Mudd et al., 1972).

A defect in the conversion of cystathionine to cysteine associated with cystathioninuria (Harris, Penrose and Thomas, 1959) has been found to be due to a deficiency of the enzyme cystathionase (Frimpter, 1965). The associatbn of this disease with mental retardation has been questioned (Gaull, 1972), and its relevance to CNS function remains unclear.

Subcellular fractionation

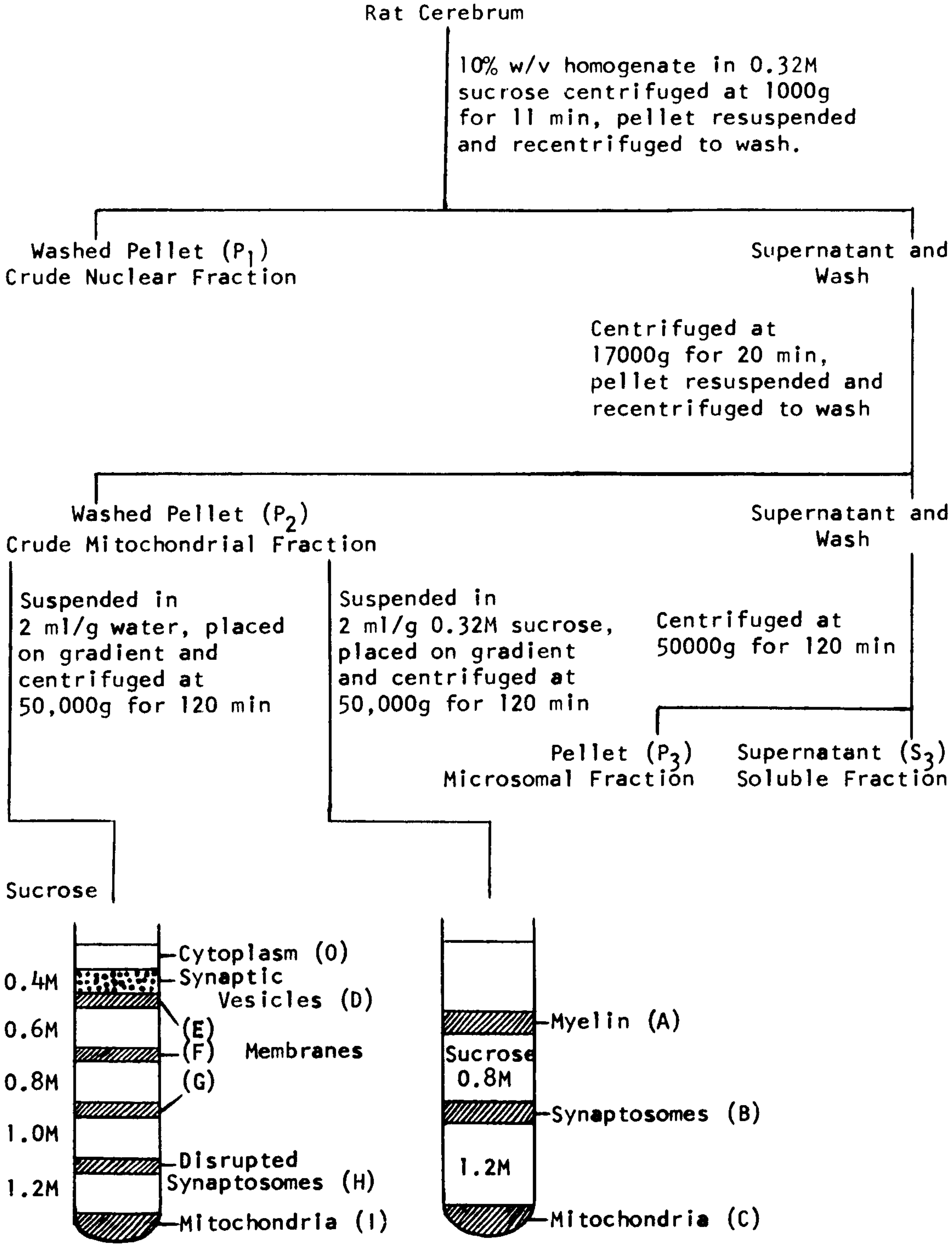
The application of subcellular fractionation techniques to brain tissue and the discovery that isolated nerve ending particles could be isolated by these methods has laid the foundation for many recent neurochemical and neuropharmacological investigations (Gray and Whittaker, 1960, 1962; De Robertis et al., 1961, 1962). The uses to which these isolated populations of brain subcellular organelles and structures may be put have been extensively reviewed by the two major investigators in this area (Whittaker, 1965, 1969; De Robertis, 1967, 1969).

The use of differential centrifugation and discontinuous sucrose density gradients has made it possible to isolate the major subcellular organelles of mammalian brain in fairly homogeneous populations (Figure 2). Slow-speed differential centrifugation yields crude nuclear and crude mitochondrial fractions and a supernatant solution. High-speed centrifugation of the supernatant solution yields a microsomal pellet and a final supernatant that contains ribosomes. Examination of the crude mitochondrial fraction revealed that this material contained particles that resembled sheared off nerve endings. These nerve ending particles or synaptosomes (Whittaker, Michaelson and Kirkland, 1964) contain those organelles usually found at the nerve ending, _i.e._ mitochondria and synaptic vesicles. If the crude mitochondrial fraction is layered on a discontinuous density gradient of 0.8 M and 1.2 M sucrose, the equilibrium density differences of myelin, synaptosomes and mitochondria allow populations of these structures to be isolated at the top of the 0.8 M layer, the top of the 1.2 M layer and in the pellet respectively.

The isolated synaptosome has been described as a miniature unnucleated cell (Whittaker, 1969), containing cell cytoplasm and small mitochondria. Synaptosomes have been shown to respire (Bradford, 1969) and to release amino acids upon electrical stimulation (Bradford, 1970). They contain transmitter substances such as acetylcholine, norepinephrine, dopamine, 5-hydroxytryptamine and the amino acids. Structural proteins and lipids, enzymes of the intraterminal mitochondria, enzymes of the soluble cytoplasm and enzymes associated with the external synaptosomal membrane have been associated with the isolated nerve ending particles. The isolated

FIGURE 2

SUBCELLULAR FRACTIONATION SCHEME FOR BRAIN



synaptosome has also been used to study the transport of putative neurotransmitters (Logan and Snyder, 1972; Levi and Raiteri, 1973). Synaptosomal preparations have provided a means of studying the events that take place at the synapse in a preparation of relatively homogeneous morphological nature. The limitation of this preparation is of course that more than one type of transmitter-containing synaptosomes is probably present, *i.e.* the population of these nerve endings is probably chemically heterogeneous. Attempts to isolate different transmitter-containing synaptosomes have been made, and it has been claimed that synaptosomes containing different amino acids may be separated from one another (Snyder *et al.*, 1973).

The contents of the synaptosome may be more closely studied by rupture of the external membrane using hypo osmotic shock (resuspension of the crude mitochondrial fraction in water) and layering this preparation on a discontinuous sucrose density gradient. This preparation separates the synaptosomal soluble components, the synaptic vesicles, the membrane fragments and the intraterminal mitochondria. A compound or enzyme under investigation that is found in the crude mitochondrial fraction may then be specifically located within the synaptosome as either bound to an organelle or merely occluded within the synaptosome. Synaptic vesicles may be isolated and their contents examined for various transmitter candidates, although these also represent a heterogeneous population probably containing various different transmitters.

These subcellular fractions of brain tissue have been invaluable in providing much of the evidence necessary to demonstrate that some compounds are neurotransmitters. The criteria that a neurotransmitter must satisfy may be studied in these preparations: The presence of the suspected transmitter and its synthesizing enzymes may be determined. The inactivation of the transmitter may be studied by examining the catabolic enzymes and reuptake transport mechanisms of the synaptosome. The collectability of transmitters after stimulation of synaptosomal particles has been observed. The identity of action of the suspected transmitter with the natural transmitter may be studied by examining effects on ionic fluxes in these preparations.

Some components of the pathway of methionine metabolism have been studied by these techniques: Cystathionine β-synthase has been associated with a mitochondrial fraction (Kashiwamata 1971a, 1971b), although it has also been stated that this enzyme is found entirely in the soluble fraction after brain homogenization and centrifugation (Volpe and Laster, 1973). Cysteine sulfinic acid decarboxylase has been found to be occluded within synaptosomal particles, but not in association with the intraterminal mitochondria (Agrawal, Davison and Kaczmarek, 1971). Serine hydroxymethyltransferase has been associated with mitochondrial and cytoplasmic fractions of the central nervous system (Davies and Johnston, 1973). Lastly, there is some evidence that taurine may be associated with synaptic vesicles of the rat brain (DeBelleroche and Bradford, 1973).

In the present work, these methods of subcellular fractionation have been used to study the location of the enzymes of the sulfur amino acid metabolic pathway in subcellular fractions of rat brain. In addition, crude separations of particulate and soluble material have been made to examine in a preliminary manner the association of these enzymes with particles. Such a simple separation procedure initiated the studies that led to the mitochondrial localization of cystathionine β-synthase (Kashiwamata, 1971a); however similar preparations have been used and it was claimed that all the cystathionine β-synthase was recoverable in the soluble fraction (Volpe and Laster, 1973). The present experiments will clarify these discrepant findings.

This study will allow some generalization about the subcellular localization of pathways of the metabolism of methionine, rather than just individual reactions. These relationships may help clarify the physiological roles of some of the intermediates of methionine metabolism.

2. MATERIALS AND METHODS

Materials

Amino acids were obtained as L-isomers, unless otherwise noted, from Calbiochem (Los Angeles, Calif.) and Sigma Chemical Co. (St. Louis, Mo.). L-Cystathionine-^{35}S, N^5-methyltetrahydrofolate-$^{14}CH_3$ and L-serine-3-^{14}C were obtained from Amersham/Searle Co. (Arlington Heights, Ill.). DL-Cystine-1-^{14}C was obtained from New England Nuclear (Boston, Mass.). Ion exchange resins were obtained from Bio-Rad (Richmond, Calif.). Adult male Nelson-Wistar rats weighing 100-250g were supplied by Carworth (New City, N.Y.). Analytical grade reagents and glass-distilled water were used throughout. All experiments were carried out in a 4° cold room or with all materials kept on ice. All fractions were stored at -70°.

Tissue Preparation

Preliminary fractions of rat tissue were prepared by decapitating the animal and excising the brain and liver. Both tissues were homogenized (10% w/v) in 0.03 M potassium phosphate buffer (pH 6.9) using a teflon-pestled glass homogenizer. The crude homogenate was centrifuged at 12,000 x g for 30 min and the supernatant solution and pellet separated. The pellet was resuspended in the homogenizing buffer and aliquots of the crude homogenate, supernatant solution and pellet suspension were stored for enzymatic analysis. The release of enzymes from particulate material in these fractions was studied by adding Triton X-100 to a final concentration of 1% to the buffer used for homogenization. Rat brain was also homogenized in 1.15% KCl, 2 volumes, for the cystathionine β-synthase assay in order to reproduce the initial conditions of Kashiwamata (1971a).

Subcellular fractions of rat brain were prepared using the methodology developed in the laboratory of Whittaker (Gray and Whittaker, 1962; Whittaker, Michaelson and Kirkland, 1964; Mangan and Whittaker, 1966) (see Figure 2). Three rats were killed by decapitation, their brains dissected out and pooled for each fractionation. The pooled brains were homogenized (fraction Hom) in 0.32 M sucrose, 10 ml per gram of tissue, in a teflon-pestled glass homogenizer. The crude nuclear fraction (P_1) was sedimented by centrifugation at 1000 x g for 11 min and washed once. The combined supernatant solutions (S_1) were centrifuged at 17,000 x g for 20 min and the pellet was washed once to obtain the crude mitochondrial fraction (P_2). The combined supernatant solutions (S_2) were centrifuged at 50,000 x g for 120 min to separate the soluble supernatant (S_3), containing cytoplasm, from the pellet (P_3) containing microsomes.

The crude mitochondrial fraction (P_2) was resuspended in water (P_2W), 2 ml/g of tissue, to disrupt the synaptosomal particles and was layered over a discontinuous density gradient consisting of 6 ml each of 0.4, 0.6, 0.8, 1.0 and 1.2 M sucrose. The gradient was centrifuged at 50,000 x g for 120 min. The following subcellular fractions were obtained: soluble cytoplasmic constituents (O), synaptic vesicles (D), membrane fragments and microsomes (E, F and G), incompletely disrupted synaptosomes (H) and mitochondria (I).

In other experiments the crude mitochondrial fraction (P_2) was resuspended in 0.32 M sucrose, 2 ml/g of tissue and layered over a discontinuous density gradient consisting of 12 ml each of 0.8 and 1.2 M sucrose. The gradient was centrifuged at 50,000 x g for 120 min and the following subcellular fractions isolated: myelin (A), synaptosomes (B) and mitochondria (C).

Fractions to be used for enzymatic analysis were sampled during the isolation procedure. Pellets were resuspended in 0.32 M sucrose and frozen; soluble fractions also were frozen. Assays for each particular enzyme were done at the same time on all the fractions of any one preparation to ensure the same relative activities between fractions in case of storage effects.

Analytical Procedures

Methionine adenosyltransferase was assayed by the method of Gaull, Rassin and Sturman (1969). The reaction mixture contained (in μmoles): Tris-HCl buffer (pH 7.2), 60; KCl, 80; $MgCl_2$, 120; ATP, 12; L-methionine, 10. Samples from the various tissue fractions were added and the total reaction mixture of 0.5 ml was incubated for 120 min at 37°. The reaction was stopped by the addition of an equal volume of 6% (w/v) perchloric acid and the precipitated protein removed by centrifugation at 10,000 x g for 10 min at 4°. The SAM produced was measured directly in the supernatant solution by ion-exchange chromatography on a specially adapted column of an amino acid analyzer as described previously (Gaull, Rassin, and Sturman, 1969).

N^5-Methyltetrahydrofolic acid-homocysteine methyltransferase was assayed by the method of Mudd, Levy and Morrow (1970). The reaction mixture contained (in nmoles): Potassium phosphate buffer (pH 7.4), 20,000; SAM 10; Cyano-B_{12}, 10; L-methionine, 100; N^5-methyltetrahydrofolate-$^{14}CH_3$ (0.05μCi), 31; 2-mercaptoethanol, 45,000; L-homocysteine, 50. Samples from the various tissue fractions were added and the total reaction mixture of 0.2 ml was incubated for 60 min under nitrogen at 37°. The reaction was stopped by the addition of 0.4 ml ice-cold absolute ethanol containing 1.3 mM L-methionine and the precipitated protein was removed by centrifugation as described above. A portion of the supernatant solution was applied to a strip of Whatman No. 1 chromatography paper and developed for 24 hours in n-butanol: concentrated ammonia: water (75:5:20 by volume). The methionine was located by spraying the paper with ninhydrin and the appropriate area was cut out and counted in a triton-toluene scintillation cocktail (5.5 g 2,5-diphenyloxazole (PPO), 0.1 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 333 ml Triton X-100 and toluene added to a total volume of 1 liter).

Cystathionine β-synthase was assayed by the method of Gaull, Rassin, and Sturman (1969). The reaction mixture contained (in μmoles): Tris-HCl buffer (pH 8.3), 60; pyridoxal-5'-monophosphate, 0.015; ethylenediaminetetraacetate, 1; L-serine, 8; DL-homocysteine, 10. Samples from the various tissue fractions were added and the total reaction mixture of 0.4 ml was incubated for 135 min at 37°. The reaction was stopped by the addition of 0.4 ml 10% trichloroacetic acid and the precipitated protein removed by centrifugation as described above. An aliquot of the supernatant solution was applied to a column of Dowex 50-X4(H^+) resin to separate the product, cystathionine, from the substrate, serine. After acid and water washes to remove serine, the cystathionine was collected in an ammonia fraction, the ammonia was removed by evaporation and the sample redissolved in 0.1 N HCl. Appropriate amounts of the final acid solution were chromatographed on a 6-cm column of Beckman UR-30 cation exchange resin on an amino acid analyzer to measure directly the cystathionine formed, as previously described (Gaull, Rassin and Sturman, 1969).

Cystathionase was assayed using the incubation conditions of Gaull, Rassin and Sturman (1969), but the product, cysteine, was measured by the formation of ^{35}S-labeled substance, rather than by the ninhydrin method (Gaitonde, 1967). L-Cystathionine-^{35}S was prepared by chromatography of the commercial preparation on a 58 X 0.9 cm cation exchange column of an amino acid analyzer. A Packard Tri-Carb Flow Analyzer was used as a detector. As the cystathionine peak appeared on the chart of the flow analyzer collection was begun directly distal to the detection flow cell. Collection ceased after the chart pen returned to baseline and an aliquot of the collected sample was rechromatographed through both the flow cell and the amino acid analyzer to determine the specific activity and ensure that only one peak was present. Non-radioactive cystathionine was added to this preparation to attain the required concentration for the enzyme assay. The reaction mixture contained (in μmoles): Tris-HCl buffer (pH 8.4), 50; pyridoxal 5'-monophosphate, 0.125; L-cystathionine-^{35}S (0.21μCi), 2. Samples from the various tissue fractions were added and the total reaction mixture of 0.5 ml was incubated for 120 min (liver fractions were incubated for 30 min) at 37°. The reaction was stopped by placing the tubes in ice, 0.25 ml of 0.1 M dithiothreitol was added to maintain all the cysteine produced in the sulfhydryl form and after 30 min 0.5 ml of 10% TCA was added, and the tubes were centrifuged as above to remove the precipitated protein. Aliquots of the supernatant solution were chromatographed in the same system as described for the isolation of L-cystathionine-^{35}S; the amount of cysteine produced was calculated from the area of the peak by triangulation.

Cysteine dioxygenase was assayed by the method of Yamaguchi et al. (1971). The reaction mixture contained (in μmoles): Potassium phosphate buffer (pH 6.8), 100; NAD$^+$, 4; ferrous ammonium sulfate ($Fe_2(NH_4)_2(SO_4)_3$), 0.5; hydroxylamine ·HCl, 10; DL-cysteine-1-^{14}C (0.14μCi), 20. Samples from the various tissue fractions were added, and the total reaction mixture of 2 ml was incubated for 60 min at 37°. The reaction was stopped by the addition of 1 ml of 8% trichloroacetic acid and the precipitated protein removed by centrifugation as above. A 2-ml aliquot of the supernatant was applied to a 1 x 5 cm column of Dowex 50 X-8 (H^+) (200-400 mesh) resin and the reaction product eluted with 10 ml of water. Cysteine sulfinic acid was determined in samples of the column eluate either by the reaction with ninhydrin (Rosen, 1957) or by counting in the triton-toluene scintillation cocktail described above.

Cysteine sulfinic acid decarboxylase was assayed using the conditions of Jacobsen, Thomas and Smith (1964). However, a different method for the synthesis of cysteine sulfinic acid-1-^{14}C was utilized. DL-Cystine-1-^{14}C was incubated in 3 ml of 0.033 N NaOH containing 1 μmole of $CuCl_2$ for 120 min at 38° (DeMarco, Coletta and Cavallini, 1963). After the addition of 1 ml of 2 N HCl, cysteine sulfinic acid-1-^{14}C was separated from the substrate and other products, in particular cysteic acid, by chromatography on a 0.9 x 10 cm column of Dowex 50 X-8 (H^+) (200-400 mesh) resin, using water as an eluant. The first 20 ml were collected in 1-ml fractions and 10-μl aliquots were applied to Whatman 3 MM paper for high voltage electrophoresis. Non-radioactive cysteine sulfinic acid and cysteic acid were applied to each spot and the paper was electrophoresed at 1400 volts for 90 min at 5° in 6.8% formic acid (Schneider, Bradley and Seegmiller, 1968). The paper was then sprayed with ninhydrin, and the cysteic acid and cysteine sulfinic spots were cut out and counted in the aforementioned triton-toluene scintillation cocktail. Those fractions containing cysteine sulfinic acid, usually the 11-13 ml fractions, were combined and non-radioactive cysteine sulfinic acid added to the correct substrate concentration.

The purity of the isolated cysteine sulfinic acid was checked by electrophoresis as above, 0.5- and 1-cm sections of the entire strip being counted, and also by chromatography on Whatman No. 1 paper, using aqueous phenol (72% w/v) containing 0.3% ethylenediaminetetraacetate and butanol: propionic acid: water (3:2:2) as solvents. Both chromatographic systems separate cysteine sulfinic acid, cysteic acid and cysteine. The cysteine sulfinic acid was kept frozen at -70°. Electrophoresis was performed on stored samples to check decomposition and usually less than 5% cysteic acid was found in these preparations.

The reaction mixture contained (in μmoles): Potassium phosphate buffer (pH 7.35), 250; pyridoxal 5'-phosphate, 0.2; glutathione (reduced), 10; DL-cysteine sulfinic acid-1-^{14}C (0.002μCi), 20; Triton X-100, 100 μl of a 10% solution (v/v). Samples from the various tissue fractions were added and the total reaction mixture of 1 ml was incubated for 60 min at 37° in a 15-ml test tube with a Kontes stopper and disposable centerwell containing 0.25 ml of 20% KOH (w/v) to trap the CO_2 produced by the reaction. In some experiments the reaction mixture was incubated in the flasks of a Warburg apparatus and the evolution of CO_2 measured by the change in manometric pressure. The reaction was stopped by injecting 1 ml of 2 N H_2SO_4 through the stopper and the tubes were allowed to stand at room temperature for 60 min. The center wells containing $^{14}CO_2$ were then dropped into counting vials with 10 ml of the previously described triton-toluene scintillation cocktail; 1 ml of H_2O was added to achieve the correct aqueous volume to ensure a clear counting solution in the presence of the alkali (Murray, 1971).

Serine hydroxymethyltransferase was assayed by the method of Taylor and Weissbach (1965) as modified by Gaull *et al*. (1973b). The reaction mixture contained (in µmoles): potassium phosphate buffer (pH 7.4), 36; pyridoxal 5'-phosphate, 0.1; *dl*-*L*-tetrahydrofolate, 0.8; 2-mercaptoethanol, 4; L-serine-3^{14}C, (0.04µCi), 0.333 (the original method used 0.1 µmole of *L*-serine). Samples from the various tissue fractions were added and after a 5 min preincubation at 37°, the reaction was started by addition of the substrate, serine. The 0.4 ml reaction mixture was incubated for 15 min at 37° and the reaction stopped by addition of 0.3 ml 1.0 M sodium acetate, pH 4.5. After the reaction was terminated, 0.2 ml of 0.1 M formaldehyde and 0.3 ml of 0.4 M dimedon were added in succession. The tubes were heated in a boiling water bath for 5 min to accelerate formation of the dimedon-formaldehyde derivative, then cooled for 5 min in an ice bath. The dimedon derivative was then extracted into toluene by vigorous shaking at room temperature. The two phases were separated by centrifugation, 2 min at 1800 x g, and 3.0 ml of the upper phase was counted in the usual scintillation fluid.

Lactate dehydrogenase and *fumarase* were used for cytoplasmic and mitochondrial markers, respectively, as suggested by Whittaker and Barker (1972). Fumarase was determined by the change in absorbance measured at 250 nm caused by the formation of the double bond in fumarate (Racker, 1950). Lactate dehydrogenase was determined by the change in absorbance at 340 nm due to the transfer of hydrogen from NADH$_2$ to pyruvate (Johnson, 1960).

Protein was determined by the method of Lowry *et al*. (1951).

Electron Microscopy

The morphology of the following fractions was checked by electron microscopy: crude nuclear (P_1), crude mitochondrial (P_2), microsomal (P_3), supernatant (S_3), mitochondrial (C), synaptic vesicle (D), mitochondrial (I), synaptosomal (B) and myelin (A). Pellets were resuspended in the sucrose solution from which they had been derived and an equal volume of fixative added to give a final concentration of 2% glutaraldehyde and 2% paraldehyde. This solution was shaken at room temperature for 10 minutes and allowed to stand overnight (except for the synaptic vesicles, which were examined by negative staining), then centrifuged at 100,000 x g for 30 minutes. The supernatant solution was discarded and the pellets were dehydrated in ethanol and cut into oriented pieces to allow differentiation of the top and bottom of the pellet. These pieces were flat-embedded in araldite and thin sections were cut on a diamond knife in such a way that a complete cross-section of each pellet was sampled in each section. The sections were mounted on parallel bar grids to facilitate examination of the entire cross-section of the pellet and studied with a Phillips 300 electron microscope operating at 80 kV fitted with a 25μm objective aperture (after Cotman, 1972).

Calculations

The enzymatic activities are expressed as μmole or nmole/fraction/hour or min. Recoveries are presented as percentages of the amount of the fraction of origin (P_1+P_2+P_3+S_3 as a percent of Hom, A+B+C as a % of P_2, and O+D+E+F+G+H+I as a % of P_2W) and distributions as percentages of the amount of material recovered.

As an indication of enzyme enrichment relative specific activity was calculated by dividing the percent of recovered activity by the percent of recovered protein.

$$\text{Relative specific activity} = \frac{\text{\% of total Recovered Activity found in fraction}}{\text{\% of total Recovered Protein found in fraction}}$$

3. RESULTS

Development of analytical procedures

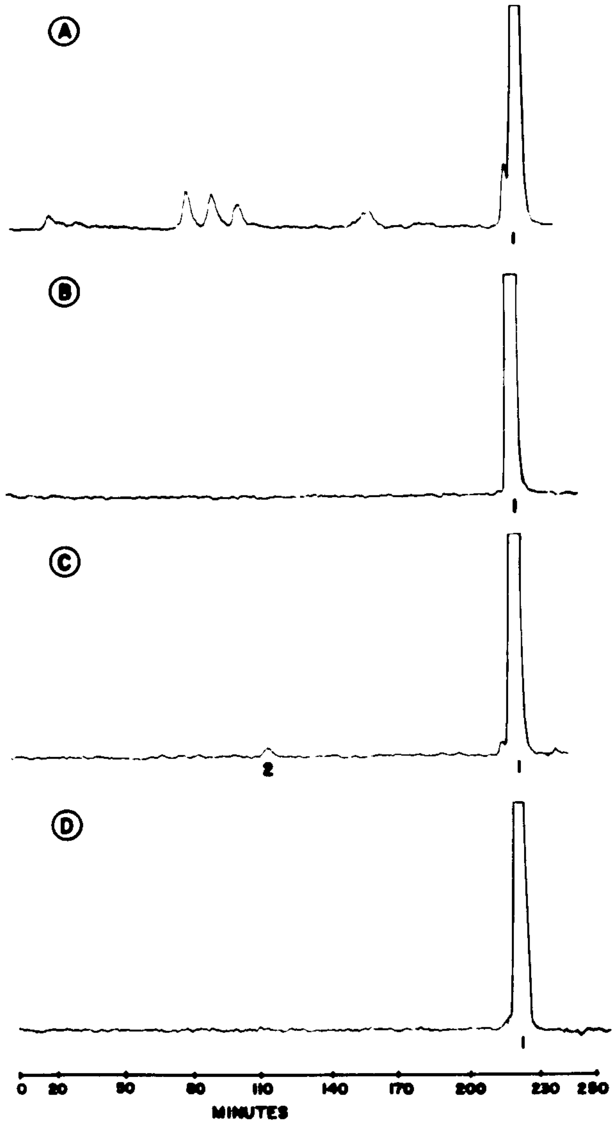
The various techniques used in this investigation generally were not different from the methods described in the literature, as presented in the Methods section, with the exception of the assays for cystathionase and cysteine sulfinic acid decarboxylase.

The low activity of cystathionase in rat brain — 9 nmoles of cysteine formed/mg protein/hour (Sturman, Rassin and Gaull, 1970) — represents only a small difference between the absorbance values obtained from unincubated enzyme blanks versus those of complete reaction mixtures incubated with brain tissue extracts (usually a difference in absorbance of approximately 0.010 to 0.020), employing the specific colorimetric reaction of cysteine with ninhydrin at acid pH (Gaull, Rassin and Sturman, 1969). In order to increase the sensitivity of the assay, cystathionine-^{35}S was used as the substrate. Chromatography on the acidic and neutral system of an amino acid analyzer using a radioactivity flow-cell analyzer as a detector showed that a number of impurities were present in the commercial cystathionine-^{35}S preparation (Figure 3A). The cystathionine-^{35}S was separated from these contaminants by chromatography as described and collection of the eluant just distal to the flow-cell; a sample of the collected eluant was rechromatographed and found to be free of contaminants (Figure 3B).

The cysteine-^{35}S produced by rat brain cystathionase from cystathionine-^{35}S was separated by chromatography and detected by

FIGURE 3

EXPERIMENTS USING CYSTATHIONINE-^{35}S



1. Cystathionine, 2. Cysteine

A. Chromatogram of a commercial preparation of cystathionine-^{35}S.
B. Rechromatographed isolated cystathionine-^{35}S.
C. Activity of cystathionase in a rat brain preparation.
D. Chromatogram of the unincubated enzyme blank from the cystathionase assay.

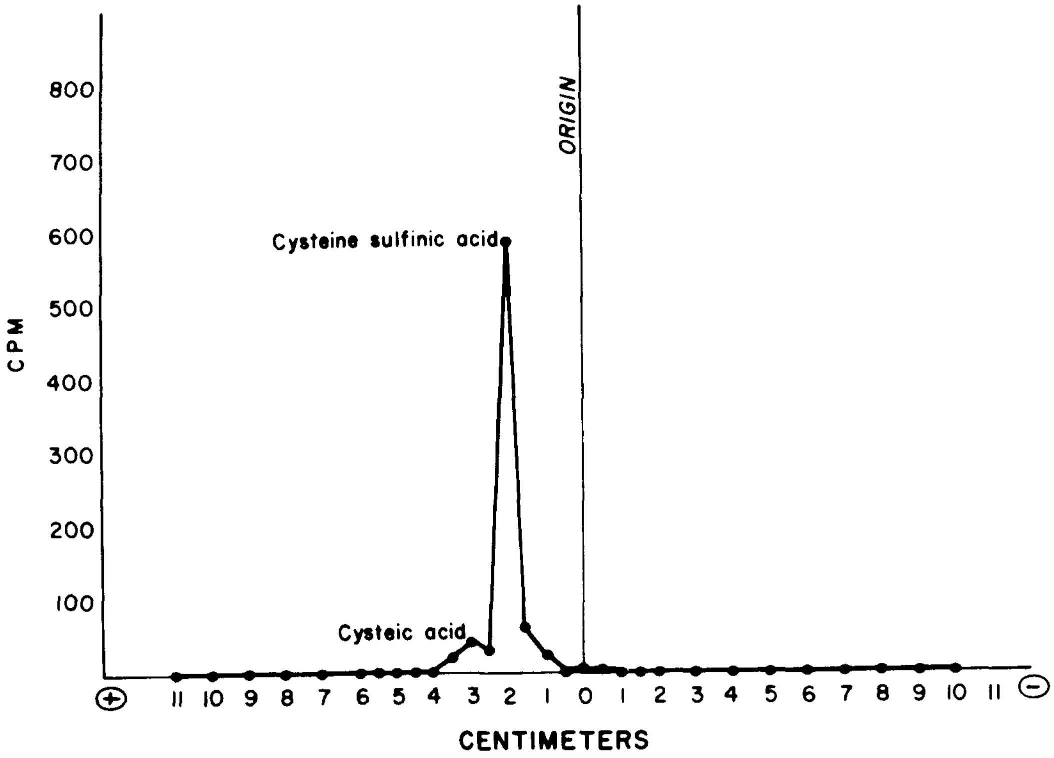
the flow-cell analyzer in the same manner (Figure 3C). In no instance was any cysteine-^{35}S detected in the unincubated enzyme blanks (Figure 3D). Even with this sensitive method only very small peaks representing cysteine-^{35}S produced enzymatically from cystathionine-^{35}S were found.

The method of Jacobsen, Thomas and Smith (1964), based on the evolution of $^{14}CO_2$, was used to measure cysteine sulfinic acid decarboxylase activity, but a different technique for the preparation of cysteine sulfinic acid-1-^{14}C was devised. Previously the hazardous procedure of oxidation of cystine-1-^{14}C to cystine disulfoxide-1-^{14}C with perbenzoic acid and dismutation to cysteine sulfinic acid-1-^{14}C in ammonia had been employed (Jacobsen, Thomas and Smith, 1964). Oxidation of cystine to a number of acidic products in the presence of cupric chloride in an alkaline medium has been used to study various possible reaction routes from cystine (DeMarco, Coletta and Cavallini, 1963). Thus, cystine-1-^{14}C was incubated in alkali with cupric chloride and cysteine sulfinic acid-1-^{14}C was isolated from the other products on an ion exchange column. Only trace amounts of cysteic acid could be detected when the product was examined using high voltage electrophoresis (Figure 4).

The assay for cysteine sulfinic acid decarboxylase was checked by comparing the change in pressure due to CO_2 evolved, as measured in a Warburg apparatus, with the radioactivity absorbed in the alkali of the center well when labelled substrate was utilized. The manometric measurement of the decarboxylase gave an activity of 467 ± 70 nmoles/mg protein/hour (±SEM, n=6), whereas the radioactive method gave an activity of 587 ± 107 nmoles/mg protein/hour (±SEM, n=6). The difference between the two methods of assay is not significant ($0.25 > p > 0.20$ using Student's t-test).

FIGURE 4

ELECTROPHORESIS OF SYNTHESIZED CYSTEINE SULFINIC ACID-1-^{14}C



The pattern of radioactivity from synthesized and isolated cysteine sulfinic acid-1-^{14}C after high voltage electrophoresis, 1400 volts for 90 min at 5° in 6.8% formic acid.

Characterization of the subcellular fractions

Protein content, fumarase activity and lactate dehydrogenase activity were measured to determine the biochemical character of the fractions and the electron microscope was used to study the morphology of the fractions. The distribution of protein in the preliminary fractions is shown in Table 4. There is more protein in the particulate matter (pellet) of the brain (63.9%) than in the pellet from the liver (48.2%). Triton X-100 added to the homogenizing buffer solubilizes some of the particulate protein; there is approximately the same percent change in the brain (the pellet is reduced by 27.4%) as in the liver (the pellet is reduced by 29.5%). The protein distribution in the subcellular fractions (Table 5) is similar to the total nitrogen distribution found by Mangan and Whittaker (1966). Most of the protein is found in the crude mitochondrial fraction (47.2%) of the primary fractions. In the secondary fractions the protein appears to be concentrated in the synaptosomes of the A to C gradient (47.9%) and about equally in the cytoplasm and mitochondria of the 0 to I gradient (20.1 and 20.7% respectively).

The distribution of lactate dehydrogenase, a cytoplasmic marker, is shown in Tables 6 and 7. In the preliminary fractionation, a significant proportion of the enzyme is found in the brain particulate matter, from which it is released by Triton X-100. There is also a significant amount of lactate dehydrogenase in the crude mitochondrial fraction of the subcellular fractions. The presence of lactate dehydrogenase in particulate matter of brain is probably because of its occlusion within synaptosomes. In the A to C gradient most of the lactate dehydrogenase activity is located in the synaptosomal fraction (64.9%). When the

TABLE 4

PROTEIN CONTENT OF THE PRELIMINARY FRACTIONS

		-Triton X-100			+Triton X-100		
Tissue	Fraction	Protein (mg/fraction)	Recovery (%)	Distribution[2] (%)	Protein (mg/fraction)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	133.6			217.4		
	Pellet	88.0		63.9	85.7		36.5
	Supernatant	49.6		36.1	148.9		63.5
	Total	137.6	103.0		234.6	107.9	
Liver	Homogenate	300.1			355.7		
	Pellet	145.2		48.2	81.7		18.7
	Supernatant	155.7		51.7	355.9		81.3
	Total	300.9	100.3		437.6	123.0	

[1] The homogenates were prepared from 2.7 g (-Triton X-100) and 2.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of the total recovered protein.

TABLE 5

PROTEIN CONTENT OF THE SUBCELLULAR FRACTIONS

Subcellular Fractions[1]:		Protein (mg/Fraction ± SEM)	Distribution[3] (%)
Primary Fractions			
(HOM)	Homogenate[2]	472.8 ± 24.2	
(P_1)	Crude Nuclear	123.6 ± 7.3	20.2
(P_2)	Crude Mitochondrial	288.8 ± 19.2	47.2
(P_3)	Microsomal	23.1 ± 1.9	5.4
(S_3)	Supernatant	166.4 ± 4.8	27.2
	(% Recovered (±SEM) from Hom = 130.8 ± 7.0)		
Secondary Fractions			
(P_2)	Crude Mitochondrial Layered	288.8 ± 7.3	
(A)	Myelin	66.6 ± 4.3	30.7
(B)	Synaptosomes	103.9 ± 3.6	47.9
(C)	Mitochondria	46.2 ± 1.4	21.4
	(% Recovered (±SEM) from P_2 = 76.0 ± 3.4)		
(P_2W)	Crude Mitochondrial Layered	31.5 ± 1.2	
(O)	Cytoplasm	6.3 ± 1.1	20.1
(D)	Synaptic Vesicles	2.4 ± 0.2	7.5
(E)	Membranes	2.8 ± 1.0	9.0
(F)		4.4 ± 0.7	14.0
(G)		3.7 ± 0.5	11.7
(H)	Partially Disrupted Synaptosomes	5.4 ± 0.9	17.0
(I)	Mitochondria	6.5 ± 0.4	20.7
	(% Recovered (±SEM) from P_2W = 95.0 ± 5.5)		

[1] The data represent 6 fractionations.

[2] The homogenate was prepared from an average of 3.8 g of rat brain.

[3] The distribution is calculated as a percent of the total recovered protein.

TABLE 6

PRELIMINARY FRACTIONATION OF LACTATE DEHYDROGENASE

		-Triton X-100			+Triton X-100		
Tissue	Fraction	Activity (µmole/fraction/min)	Recovery (%)	Distribution[2] (%)	Activity (µmole/fraction/min)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	153.7			162.4		
	Pellet	34.6		22.9	10.1		5.3
	Supernatant	116.5		77.1	181.5		94.7
	Total	151.1	98.3		191.6	118.0	
Liver	Homogenate	623.9			604.8		
	Pellet	26.0		3.8	11.6		1.5
	Supernatant	660.1		96.2	773.4		98.5
	Total	686.1	110.0		785.0	129.8	

[1] The homogenates were prepared from 2.7 g (-Triton X-100) and 2.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of the total recovered activity.

TABLE 7

SUBCELLULAR LOCALIZATION OF LACTATE DEHYDROGENASE

Subcellular Fractions[1]:	Activity (µmole/fraction/min ± SEM)	Distribution[3] (%)	Relative Specific Activity
Primary Fractions			
(HOM) Homogenate[2]	297.1 ± 21.1		
(P_1) Crude Nuclear	32.6 ± 7.2	14.4	0.42
(P_2) Crude Mitochondrial	76.1 ± 10.7	33.5	0.42
(P_3) Microsomal	7.3 ± 1.2	3.2	0.35
(S_3) Supernatant	111.0 ± 16.2	48.9	1.05
(% Recovered (±SEM) from Hom = 76.9 ± 9.5)			
Secondary Fractions			
(P_2) Crude Mitochondrial Layered	76.1 ± 10.7		
(A) Myelin	7.1 ± 1.5	16.0	0.39
(B) Synaptosomes	29.0 ± 2.5	64.9	1.10
(C) Mitochondria	8.5 ± 1.0	19.1	0.72
(% Recovered (±SEM) from P_2 = 61.2 ± 4.4)			
(P_2W) Crude Mitochondrial Layered	20.2 ± 2.3		
(O) Cytoplasm	6.8 ± 2.3	26.8	1.85
(D) Synaptic Vesicles	3.9 ± 0.4	15.3	2.71
(E)	2.9 ± 0.7	11.3	1.66
(F) Membranes	3.1 ± 0.6	12.2	1.28
(G)	3.6 ± 0.5	14.1	1.57
(H) Partially Disrupted Synaptosomes	3.2 ± 0.4	12.8	1.10
(I) Mitochondria	1.9 ± 0.2	7.4	0.05
(% Recovered (±SEM) from P_2W = 128.1 ± 20.3)			

[1] The data represent 6 fractionations.

[2] The homogenate was prepared from an average of 3.8 g of rat brain.

[3] The distribution is calculated as a percent of the total recovered activity.

crude mitochondrial fraction is disrupted and layered on the 0 to I gradient the single fraction with the most lactate dehydrogenase activity is the cytoplasmic fraction (26.8%), approximately twice the percentage of any other fraction.

Fumarase activity has been studied as an indication of the presence of mitochondria (Tables 8 and 9). In the preliminary fractionation a considerable proportion of the fumarase was associated with the pellets from both brain and liver and could be solubilized with Triton X-100. The distribution of fumarase in the subcellular fractions is consistent with its association with mitochondria. In the preliminary fractions 76.2% of the activity was found in the crude mitochondrial fraction. When the crude mitochondrial fraction was disrupted and layered on the 0-to-1 gradient, 66.2% of the activity was found in the mitochondrial pellet. The A to C gradient of the undisrupted crude mitochondrial fraction yielded most of the activity (53.5%) in the synaptosomal fraction. This is consistent with the presence of mitochondria within the synaptosomes; a similar distribution of fumarase in these fractions has been reported elsewhere -- A (myelin):0.2%; B (synaptosomes):58% and C (mitochondria):42% (Broch and Fonnum, 1972).

Examination of the fractions by electron microscopy confirmed the biochemical evidence concerning the distribution of the mitochondria and also provided information about the character of some of the other fractions studied. This study of the morphology illustrated that the brain subcellular fractions are enriched with certain organelles but do not represent completely homogeneous preparations. All the fractions studied were enriched with the particular organelle with which each is identified.

TABLE 8

PRELIMINARY FRACTIONATION OF FUMARASE

		-Triton X-100			+Triton X-100		
Tissue	Fraction	Activity (μmole/fraction/min)	Recovery (%)	Distribution[2] (%)	Activity (μmole/fraction/min)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	276.0			413.1		
	Pellet	219.8		67.5	97.9		19.7
	Supernatant	106.1		32.5	397.5		80.3
	Total	325.9	118.0		495.4	119.9	
Liver	Homogenate	286.4			178.1		
	Pellet	90.7		46.5	10.5		5.9
	Supernatant	104.5		53.5	167.9		94.1
	Total	195.2	68.2		178.4	100.2	

[1] The homogenates were prepared from 2.7 g (-Triton X-100) and 2.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of the total recovered activity.

TABLE 9

SUBCELLULAR LOCALIZATION OF FUMARASE

Subcellular Fractions[1]:	Activity (μmole/fraction/min ± SEM)	Distribution[3] (%)	Relative Specific Activity
Primary Fractions			
(HOM) Homogenate[2]	407.0 ± 54.1		
(P_1) Crude Nuclear	38.3 ± 5.4	12.5	0.42
(P_2) Crude Mitochondrial	232.9 ± 37.9	76.2	1.08
(P_3) Microsomal	5.7 ± 1.1	1.9	0.21
(S_3) Supernatant	28.6 ± 9.7	9.4	0.24
(% Recovered (±SEM) from Hom = 87.7 ± 21.3)			
Secondary Fractions			
(P_2) Crude Mitochondrial Layered	232.9 ± 37.9		
(A) Myelin	23.8 ± 6.1	19.8	0.57
(B) Synaptosomes	64.1 ± 10.9	53.5	0.92
(C) Mitochondria	32.0 ± 10.2	26.7	1.15
(% Recovered (±SEM) from P_2 = 65.5 ± 21.0)			
(P_2W) Crude Mitochondrial Layered	74.8 ± 10.5		
(O) Cytoplasm	1.5 ± 1.1	3.0	0.11
(D) Synaptic Vesicles	1.3 ± 0.9	2.5	0.28
(E) Membranes	3.4 ± 2.1	6.7	0.49
(F)	4.1 ± 3.3	8.1	0.37
(G)	3.4 ± 2.6	6.8	0.33
(H) Partially Disrupted Synaptosomes	3.3 ± 1.3	6.6	0.37
(I) Mitochondria	33.4 ± 3.6	66.2	2.47
(% Recovered (±SEM) from P_2W = 72.8 ± 17.3)			

[1] The data represent 6 fractionations.
[2] The homogenate was prepared from an average of 3.8 g of rat brain.
[3] The distribution is calculated as a percent of the total recovered activity.

The crude nuclear fraction (P_1) contained nuclei, some broken cells, a few synaptosomes, a few mitochondria and some vesicles of unknown origin. The microsomal fraction (P_3) contained smooth membranes, a few rough membranes, some particulate and vesicular material and some broken nuclear material. The supernatant fraction (S_3) had some nuclear material, a few synaptosomes at the bottom of the pellet and some mitochondria. The synaptic vesicle fraction (D) was enriched with vesicles but also had some unidentified filament-like material present.

The crude mitochondrial fraction (P_2) was enriched with mitochondria and synaptosomes but also contained some myelin and torn off nuclear material (Figure 5). Some free mitochondria and uncharacterized membranes were found in the synaptosomal fraction (B), which was enriched with synaptosomes that contained intraterminal mitochondria and synaptic vesicles (Figure 6). The two mitochondrial fractions (C and I) were enriched with the expected mitochondria but also contained some nuclear material (Figures 7 and 8). Some synaptosomes and both swollen and compact mitochondria were also observed in fraction C. The synaptosomal and mitochondrial fractions were typical of this type of preparation, being enriched in the expected component but not homogeneous.

The enzymes of methylation

The preliminary fractionation of methionine adenosyltransferase (Table 10) showed little activity associated with particulate material in either the brain or the liver. Subcellular fractionation of the brain tissue confirmed the preliminary observation, 77.2% of the

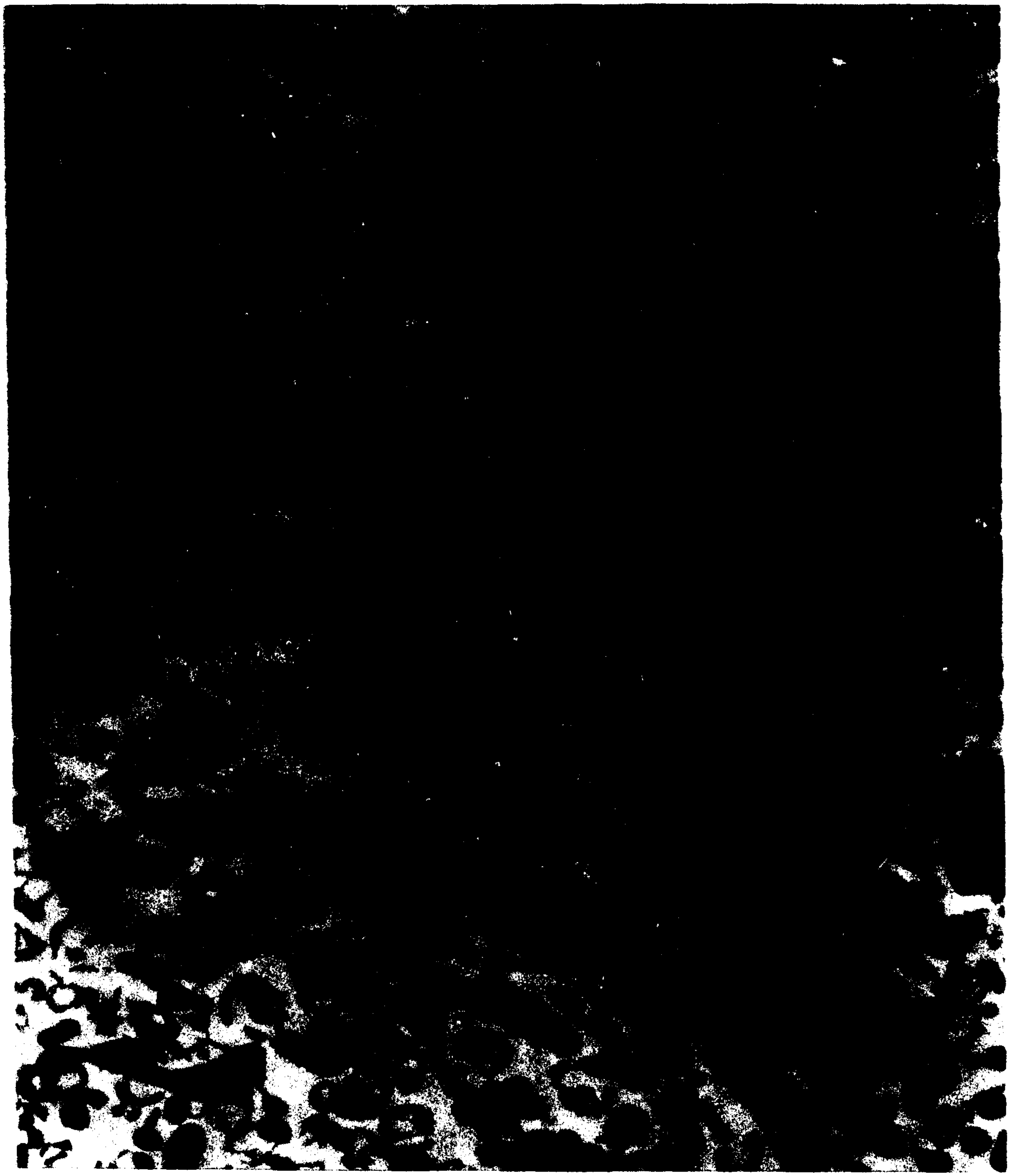
FIGURE 5

AN ELECTRON MICROGRAPH OF THE CRUDE MITOCHONDRIAL FRACTION (P_2)

A representative picture of the crude mitochondrial fraction showing synaptosomes (S), mitochondria (M) and myelin (MY). Magnification x 21000.

FIGURE 6A

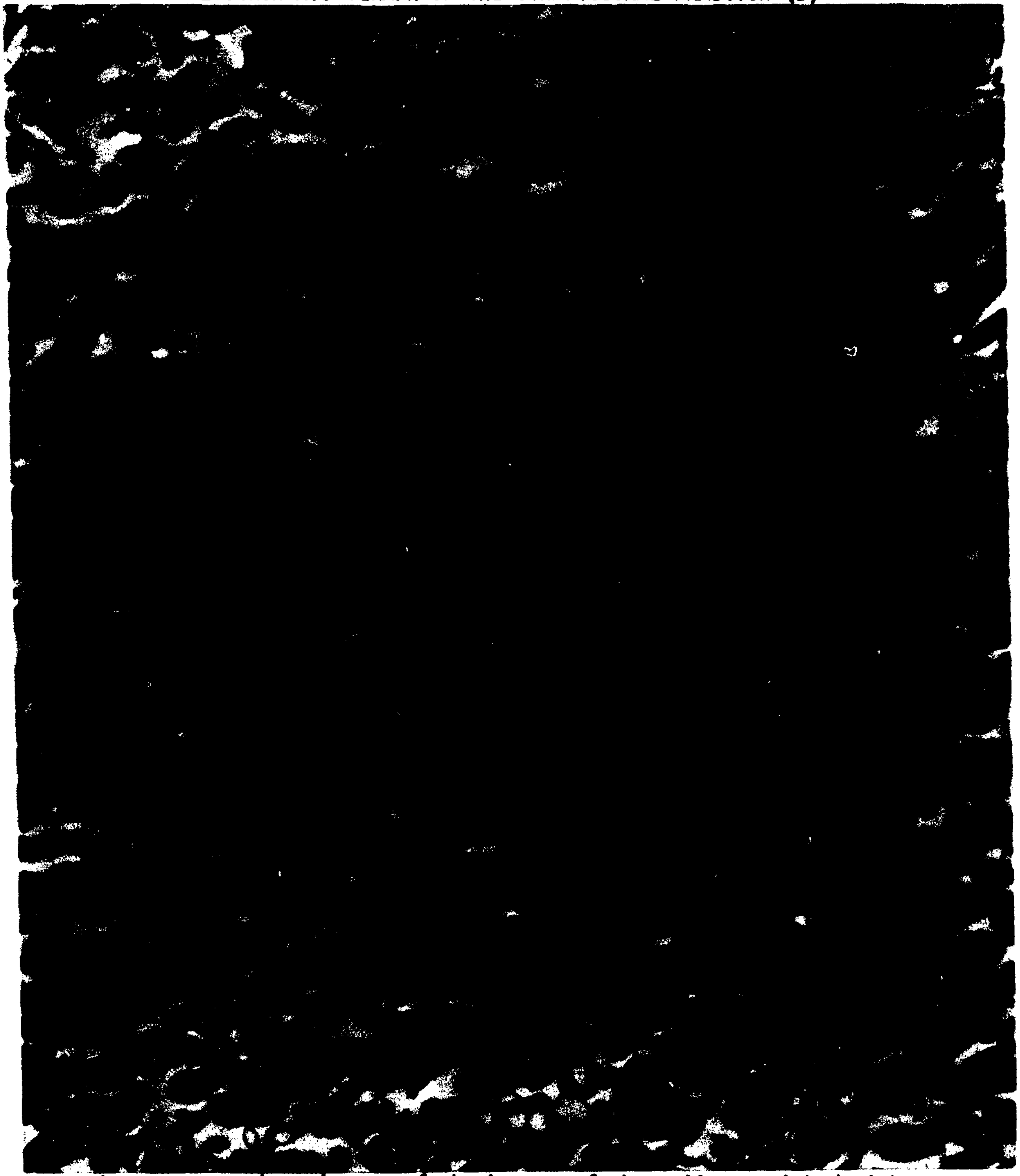
AN ELECTRON MICROGRAPH OF THE SYNAPTOSOMAL FRACTION (B)



A representative picture of the top of the pellet enriched with synaptosomes, some contain mitochondria (SM). Magnification x 21000.

FIGURE 6B

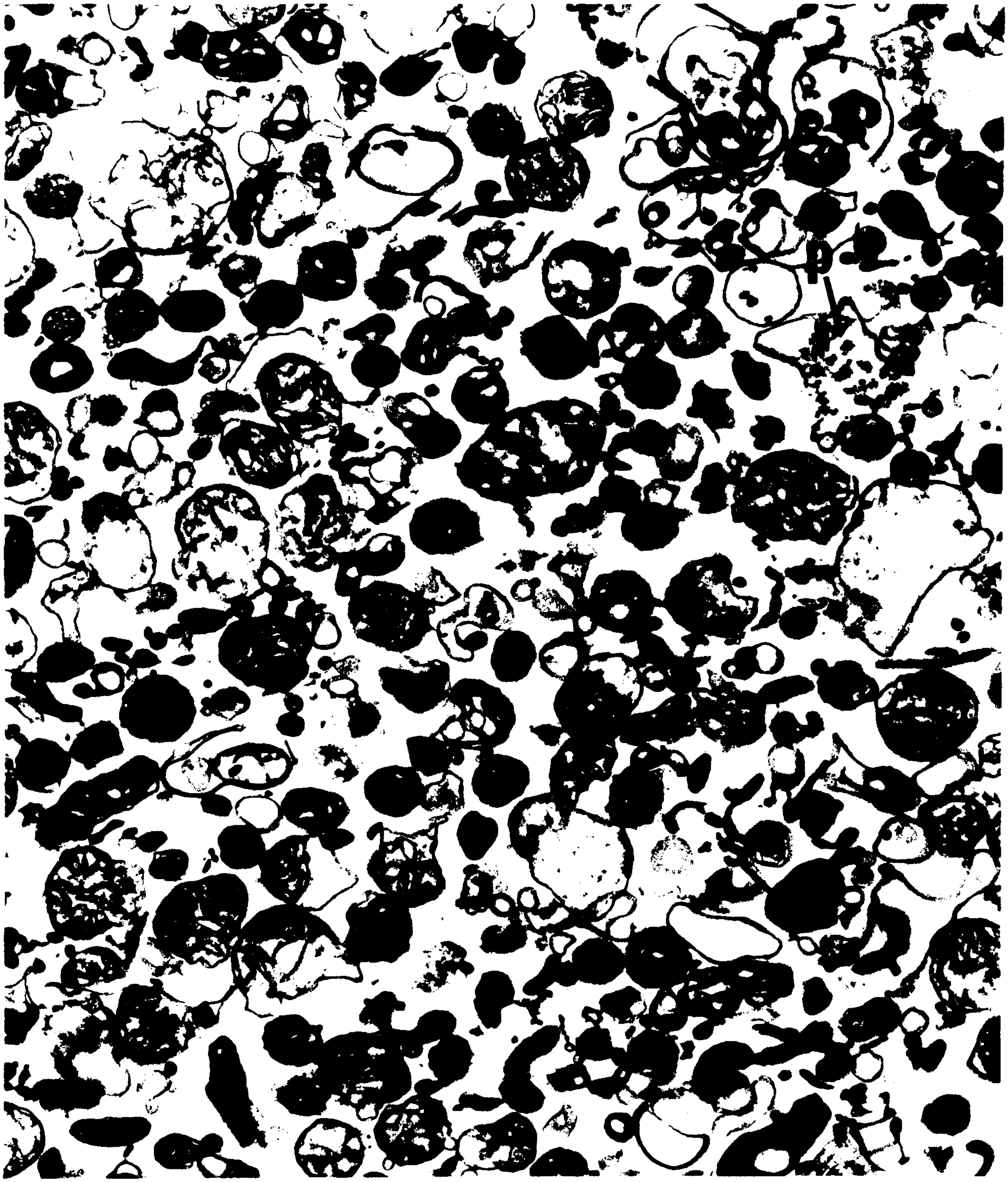
AN ELECTRON MICROGRAPH OF THE SYNAPTOSOMAL FRACTION (B)



A representative picture of the bottom of the pellet enriched with synaptosomes, an occasional mitochondrion (M) is present. Magnification x 21000.

FIGURE 7A

AN ELECTRON MICROGRAPH OF THE MITOCHONDRIAL FRACTION (C)



A representative picture of the top of the pellet enriched with mitochondria, polysomes (P) and some synaptosomes (S) are present. Magnification x 21000.

FIGURE 7B

AN ELECTRON MICROGRAPH OF THE MITOCHONDRIAL FRACTION (C)

A representative picture of the bottom of the pellet enriched with mitochondria. There is some nuclear material (N), an occasional synaptosome (S) and a centriole (C) present. Magnification x 21000.

FIGURE 8B

AN ELECTRON MICROGRAPH OF THE MITOCHONDRIAL FRACTION (I)

A representative picture of the bottom of the pellet enriched with mitochondria. There is some rough endoplasmic reticulum (ER) and an occasional synaptosome (S) present. Magnification x 21000.

TABLE 10

PRELIMINARY FRACTIONATION OF METHIONINE ADENOSYLTRANSFERASE

		-Triton X-100			+Triton X-100		
Tissue	Fraction	Activity (nmole/fraction/hour)	Recovery (%)	Distribution[2] (%)	Activity (nmole/fraction/hour)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	434.5			284.4		
	Pellet	26.8		6.7	0		0
	Supernatant	374.6		93.3	282.4		100
	Total	401.4	92.4		282.4	99.3	
Liver	Homogenate	17655			14083		
	Pellet	54		0.4	95		0.7
	Supernatant	13794		99.6	13138		99.3
	Total	13848	78.4		13233	94.0	

[1] The homogenates were prepared from 2.7 g (-Triton X-100) and 2.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of the total recovered activity.

recovered material being localized in the supernatant fraction (Table 11). Further fractionation of the crude mitochondrial fraction was not possible because of the small amount of activity present in this fraction. It is clear that a significant amount of methionine adenosyltransferase activity is not associated with the particulate fractions.

N^5-Methyltetrahydrofolic acid-homocysteine methyltransferase has a distribution similar to that of methionine adenosyltransferase. In the preliminary fractions the major proportion of the activity is present in the supernatant fractions (Table 12). The subcellular fractionation of N^5-methyltetrahydrofolic acid-homocysteine methyltransferase localizes 76.1% of the recovered activity in the supernatant fraction (Table 13), similar to the 77.2% of methionine adenosyltransferase found in this fraction. The secondary fractions derived from the crude mitochondrial fraction confirm the soluble nature of N^5-methyltetrahydrofolic acid-homocysteine methyltransferase. Poor recoveries because of the low amount of activity layered on the A to C gradient (14.2% of the total recovered activity), make these particular data questionable (32.4% recovery) but 35.8% of the recovered material apparently is in the synaptosomal fraction. When the disrupted crude mitochondrial fraction was layered on the 0 to I gradient, the bulk of the activity was recovered in the cytoplasmic fraction.

The enzymes of transsulfuration

The preliminary fractionation of cystathionine β-synthase showed that 43.7% of the recovered activity was associated with the brain pellet, in contrast to only 5.1% in the liver pellet (Table 14). These results are similar to those of Kashiwamata (1971a), who, after homogenizing in

TABLE 11

SUBCELLULAR LOCALIZATION OF METHIONINE ADENOSYLTRANSFERASE

Subcellular Fractions[1]:		Activity (nmole/fraction/hour ±SEM)	Distribution[3] (%)	Relative Specific Activity
Primary Fractions				
(HOM)	Homogenate[2]	218.9 ± 32.6		
(P_1)	Crude Nuclear	25.0 ± 7.9	16.9	0.19
(P_2)	Crude Mitochondrial	5.9 ± 4.4	4.0	0.13
(P_3)	Microsomal	2.9 ± 1.4	1.9	0.33
(S_3)	Supernatant	114.2 ± 26.3	77.2	1.37
	(% Recovered (±SEM) from Hom = 65.3 ± 8.4)			

[1] The data represent 13 fractionations.

[2] The homogenate was prepared from an average of 3.5 g of rat brain.

[3] The distribution is calculated as a percent of the total recovered activity.

TABLE 12

PRELIMINARY FRACTIONATION OF N^5-METHYLTETRAHYDROFOLATE–HOMOCYSTEINE METHYLTRANSFERASE

		-Triton X-100			+Triton X-100		
Tissue	Fraction	Activity (nmole/fraction/hour)	Recovery (%)	Distribution[2] (%)	Activity (nmole/fraction/hour)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	489.5			126.9		
	Pellet	80.3		19.4	16.4		12.5
	Supernatant	333.0		80.6	115.2		87.5
	Total	413.3	84.4		131.6	103.7	
Liver	Homogenate	137.6			134.4		
	Pellet	23.5		11.5	1.3		0.8
	Supernatant	181.3		88.5	165.6		99.2
	Total	204.8	148.9		166.9	124.2	

[1] The homogenates were prepared from 2.7 g (-Triton X-100) and 2.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of the total recovered activity.

TABLE 13

SUBCELLULAR LOCALIZATION OF N^5-METHYLTETRAHYDROFOLIC ACID–HOMOCYSTEINE METHYLTRANSFERASE

Subcellular Fractions[1]:		Activity (nmole/fraction/hour ± SEM)	Distribution[3] (%)	Relative Specific Activity
Primary Fractions				
(HOM)	Homogenate[2]	484.2 ± 78.9		
(P_1)	Crude Nuclear	33.0 ± 8.1	8.1	.24
(P_2)	Crude Mitochondrial	57.8 ± 15.6	14.2	.29
(P_3)	Microsomal	6.6 ± 2.0	1.6	.15
(S_3)	Supernatant	309.6 ± 53.5	76.1	1.77
	(% Recovered (±SEM) from Hom = 88.2 ± 5.6)			
Secondary Fractions				
(P_2)	Crude Mitochondrial Layered	57.8 ± 15.6		
(A)	Myelin	3.4 ± 1.3	18.2	.78
(B)	Synaptosomes	6.7 ± 2.6	35.8	1.00
(C)	Mitochondria	8.6 ± 2.4	46.0	2.38
	(% Recovered (±SEM) from P_2 = 32.4 ± 10.9)			
(P_2W)	Crude Mitochondrial Layered	22.7 ± 2.4		
(O)	Cytoplasm	16.0 ± 2.3	69.0	3.61
(D)	Synaptic Vesicles	2.1 ± 0.9	9.1	1.29
(E)	Membranes	0.6 ± 0.2	2.6	0.28
(F)		1.2 ± 0.5	5.2	0.40
(G)		0.8 ± 0.6	3.4	0.28
(H)	Partially Disrupted Synaptosomes	1.3 ± 1.0	5.6	0.38
(I)	Mitochondria	1.2 ± 0.6	5.2	0.29
	(% Recovered (±SEM) from P_2W = 104.0 ± 25.0)			

[1] The data represent 6 fractionations.
[2] The homogenate was prepared from an average of 3.8 g of rat brain.
[3] The distribution is calculated as a percent of the total recovered activity.

TABLE 14

PRELIMINARY FRACTIONATION OF CYSTATHIONINE β-SYNTHASE

		-Triton X-100			+Triton X-100		
Tissue	Fraction	Activity (nmole/fraction/hour)	Recovery (%)	Distribution[2] (%)	Activity (nmole/fraction/hour)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	4405			6443		
	Pellet	1362		43.7	282		4.6
	Supernatant	1751		56.3	5885		95.4
	Total	3113	70.7		6167	95.7	
Liver	Homogenate	68293			98616		
	Pellet	2615		5.1	841		1.0
	Supernatant	48798		94.9	86525		99.0
	Total	51413	75.3		87366	88.6	

[1] The homogenates were prepared from 1.9 g (-Triton X-100) and 1.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of the total recovered activity.

KCl solution and centrifuging at 12,000 x g, found 57.0% of the enzyme activity of brain in the pellet, compared to 7.0% of the liver activity in the pellet. Preliminary fractionation of the brain after homogenization in KCl solution was also carried out in the present work, with results comparable to those obtained after homogenization in potassium phosphate buffer; 46.5% of the recovered activity was found in the brain pellet and 53.5% in the brain supernatant (with a recovery of 78.4%). The material associated with the brain pellet could be released into the supernatant by treatment with Triton X-100.

Analysis of the subcellular fractions showed the major portion of the enzyme to be in the crude mitochondrial fraction (P_2) (Table 15). Further fractionation of the crude mitochondrial fraction on the A to C gradient showed that most of the activity was associated with the synaptosomal fraction (68.2%) and the mitochondrial fraction (23.3%). Disruption of the crude mitochondrial fraction and separation of the components on the 0 to I gradient showed the major part of the activity to be in the cytoplasm (26.3%), undisrupted synaptosomes (23.4%) and mitochondria (24.2%). The percent of the activity found in the mitochondria in this latter secondary fractionation (24.2%) was similar to that found in the A to C gradient mitochondrial fraction (23.3%). There may be both mitochondrial and cytoplasmic components of cystathionine β-synthase occluded within the synaptosomes.

Kashiwamata (1971b) found a distribution of activity in the primary subcellular fractions of 37.0% in P_2 and 43.0% in S_3, similar to the 46.4% in P_2 and 34.4% in S_3 found in this work. However, the results obtained with the secondary fractions in the present work do not agree with the results of Kashiwamata (1971b), who found most of the activity

TABLE 15

SUBCELLULAR LOCALIZATION OF CYSTATHIONINE β-SYNTHASE

Subcellular Fractions[1]:	Activity (nmole/fraction/hour ± SEM)	Distribution[3] (%)	Relative Specific Activity
Primary Fractions			
(HOM) Homogenate[2]	11115 ± 767		
(P_1) Crude Nuclear	899 ± 251	17.4	0.31
(P_2) Crude Mitochondrial	2405 ± 203	46.4	0.36
(P_3) Microsomal	94 ± 15	1.8	0.12
(S_3) Supernatant	1781 ± 346	34.4	0.47
(% Recovered (±SEM) from Hom = 47.9 ± 6.9)			
Secondary Fractions			
(P_2) Crude Mitochondrial Layered	2405 ± 203		
(A) Myelin	125 ± 28	8.5	0.22
(B) Synaptosomes	999 ± 91	68.2	1.14
(C) Mitochondria	341 ± 25	23.3	0.90
(% Recovered (±SEM) from P_2 = 61.4 ± 4.6)			
(P_2W) Crude Mitochondrial Layered	319.2 ± 14.2		
(O) Cytoplasm	59.5 ± 19.0	26.3	0.98
(D) Synaptic Vesicles	12.0 ± 5.5	5.3	0.51
(E) Membranes	10.2 ± 7.5	4.5	0.36
(F) Membranes	18.7 ± 8.7	8.3	0.43
(G) Membranes	18.2 ± 10.3	8.0	0.49
(H) Partially Disrupted Synaptosomes	52.9 ± 9.5	23.4	0.99
(I) Mitochondria	54.7 ± 10.9	24.2	0.82
(% Recovered (±SEM) from P_2W = 70.9 ± 12.6)			

[1] The data represent 6 fractionations.

[2] The homogenate was prepared from an average of 3.8 g of rat brain.

[3] The distribution is calculated as a percent of the total recovered activity.

(83.0%) in the C or mitochondrial fraction while only 23.3% was found in this work. The cause of this difference is not clear. The fractionation techniques used by Kashiwamata (1971b) and in this work were the same, and both studies were monitored by use of enzyme markers and electron microscopy. The studies differ in the conditions used in the enzymatic assays and in the methods for measuring cystathionine (a colorimetric method versus the amino acid analyzer used here), which would not seem to offer an explanation for the varying results obtained. The two studies agree on the particulate nature of cystathionine β-synthase, but differ in the nature of the organelle involved. Kashiwamata (1971b) did not do fractionations of ruptured synaptosomes (the 0 to I gradient) as was done in the present work. These fractionations show a release of cystathionine β-synthase which shows that this enzyme may be partially occluded within the synaptosomes as found in the A to C fractions of this work.

Cystathionase, as noted earlier, is difficult to measure in brain because of the low activity and only the preliminary fractionation could be carried out (Table 16). The distribution of cystathionase is similar to that of cystathionine β-synthase, with 52.5% of the recovered activity of brain being associated with the pellet and only 18.5% of the recovered activity of liver being found in the pellet. Brain cystathionase would appear to have a component bound to or occluded with the particles which does not seem to be present in the liver.

Cysteine dioxygenase has a significant particulate component in both the brain (78.3%) and the liver (51.4%) as found in the preliminary fractionation (Table 17). The major site of cysteine dioxygenase in the subcellular fractions is in the crude mitochondrial fraction (57.1%) (Table 18). Further fractionation of fraction P_2 on the A to C gradient

TABLE 16

PRELIMINARY FRACTIONATION OF CYSTATHIONASE

		-Triton X-100			+Triton X-100		
Tissue	Fraction	Activity (nmole/fraction/hour)	Recovery (%)	Distribution[2] (%)	Activity (nmole/fraction/hour)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	2841			1262		
	Pellet	1396		52.5	548		30.5
	Supernatant	1265		47.5	1249		69.5
	Total	2661	93.6		1797	142.4	
Liver	Homogenate	65960			59535		
	Pellet	9874		18.5	5824		10.3
	Supernatant	43455		81.5	50606		89.7
	Total	53329	80.9		56430	94.8	

[1] The homogenates were prepared from 2.7 g (-Triton X-100) and 2.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of the total recovered activity.

TABLE 17

PRELIMINARY FRACTIONATION OF CYSTEINE DIOXYGENASE

		-Triton X-100			+Triton X-100		
Tissue	Fraction	Activity (nmole/fraction/hour)	Recovery (%)	Distribution[2] (%)	Activity (nmole/fraction/hour)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	7290			6633		
	Pellet	5127		78.3	2499		30.6
	Supernatant	1424		21.7	5670		69.4
	Total	6551	89.8		8169	123.1	
Liver	Homogenate	18710			22062		
	Pellet	7029		51.4	1502		7.8
	Supernatant	6636		48.6	17864		92.2
	Total	13665	73.0		19366	87.8	

[1] The homogenates were prepared from 2.7 g (-Triton X-100) and 2.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of total recovered activity.

TABLE 18

SUBCELLULAR LOCALIZATION OF CYSTEINE DIOXYGENASE

Subcellular Fractions[1]:		Activity (nmole/fraction/hour ± SEM)	Distribution[3] (%)	Relative Specific Activity
Primary Fractions				
(HOM)	Homogenate[2]	8511 ± 1370		
(P$_1$)	Crude Nuclear	1675 ± 286	16.1	0.77
(P$_2$)	Crude Mitochondrial	5952 ± 837	57.1	1.21
(P$_3$)	Microsomal	981 ± 81	9.4	1.86
(S$_3$)	Supernatant	1807 ± 250	17.4	0.73
	(% Recovered (± SEM) from HOM + 134.4 ± 18.0)			
Secondary Fractions				
(P$_2$)	Crude Mitochondrial Layered	5952 ± 837		
(A)	Myelin	1503 ± 113	34.8	1.24
(B)	Synaptosomes	2028 ± 80	47.0	1.01
(C)	Mitochondria	788 ± 119	18.2	0.93
	(% Recovered (± SEM) from P$_2$ = 78.7 ± 9.4)			
(P$_2$W)	Crude Mitochondrial Layered	5431 ± 231		
(O)	Cytoplasm	1962 ± 250	26.3	1.82
(D)	Synaptic Vesicles	849 ± 268	11.3	2.17
(E)	Membranes	684 ± 108	9.1	1.42
(F)		877 ± 250	11.7	1.17
(G)		1066 ± 265	14.2	1.71
(H)	Partially Disrupted Synaptosomes	1019 ± 348	13.6	1.13
(I)	Mitochondria	1038 ± 226	13.8	0.92
	(% Recovered (± SEM) from P$_2$W = 139.2 ± 30.5)			

[1] The data represent 6 fractionations. [2] The homogenate was prepared from an average of 3.8 g of rat brain.

[3] The distribution is calculated as a percent of the total recovered activity.

showed a synaptosomal localization and when P_2 was disrupted the main site of activity was in the cytoplasm, as seen in the results from the 0 to I gradient. Cysteine dioxygenase is apparently primarily occluded within the synaptosomes.

Previous work has implied that there is not a particulate component of cysteine dioxygenase in the liver nor any activity in the brain of rats (Ewetz and Sorbo, 1966; Wainer, 1967). These studies did show that cysteine was oxidized by the mitochondria but apparently not to cysteine sulfinic acid or to any other ninhydrin positive product, although the liver supernatant preparation could convert cysteine to cysteine sulfinic acid. However, recent work has demonstrated a high activity of cysteine dioxygenase in brain, discrepancies with previous studies being explained as due to the use in the enzyme reaction of NADH or NADPH in the earlier work instead of NAD^+ (as also used in the present study) (Yamaguchi et al, 1973). In the present study the production of cysteine sulfinic acid was measured both by reaction with ninhydrin and by incorporation of radioactivity. The same results (in nmoles/mg protein/hour) were obtained by the ninhydrin reaction, 46 for brain homogenate and 38 for the brain pellet; by incorporation of radioactivity, 45 for brain homogenate and 44 for the brain pellet.

Cysteine sulfinic acid decarboxylase has a significant particulate component in brain (58.8%) but not in the liver (0.9%) (Table 19). The activity of brain is concentrated in the crude mitochondrial fraction (P_2), which when further fractionated shows a concentration in the synaptosomal fraction (70.0%) (Table 20). The synaptosomal activity apparently is due to an occluded enzyme because disruption of fraction P_2 followed by fractionation localizes the enzyme in the cytoplasmic

TABLE 19

PRELIMINARY FRACTIONATION OF CYSTEINE SULFINIC ACID DECARBOXYLASE

		_Triton X-100			+Triton X-100		
Tissue	Fraction	Activity (nmole/fraction/hour)	Recovery (%)	Distribution[2] (%)	Activity (nmole/fraction/hour)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	37037			40171		
	Pellet	25298		58.8	0		0
	Supernatant	17710		41.2	42611		100
	Total	43008	116.1		42611	106.1	
Liver	Homogenate	200466			172960		
	Pellet	1871		0.9	0		0
	Supernatant	201624		99.1	166436		100
	Total	203495	100.7		166436	96.2	

[1] The homogenates were prepared from 2.7 g (-Triton X-100) and 2.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of the total recovered activity.

TABLE 20

SUBCELLULAR LOCALIZATION OF CYSTEINE SULFINIC ACID DECARBOXYLASE

Subcellular Fractions[1]:		Activity (nmole/fraction/hour ± SEM)	Distribution[3] (%)	Relative Specific Activity
Primary Fractions				
(HOM)	Homogenate[2]	68362 ± 4407		
(P_1)	Crude Nuclear	9697 ± 1376	14.8	0.56
(P_2)	Crude Mitochondrial	39595 ± 5179	60.6	0.95
(P_3)	Microsomal	1493 ± 164	2.3	0.31
(S_3)	Supernatant	14507 ± 959	22.2	0.61
	(% Recovered (±SEM) from Hom = 97.2 ± 9.3)			
Secondary Fractions				
(P_2)	Crude Mitochondrial Layered	39595 ± 5179		
(A)	Myelin	527 ± 203	3.6	0.06
(B)	Synaptosomes	10148 ± 840	70.0	0.75
(C)	Mitochondria	3814 ± 383	26.4	0.70
	(% Recovered (±SEM) from P_2 = 40.2 ± 5.6)			
(P_2W)	Crude Mitochondrial Layered	7336 ± 440		
(O)	Cytoplasm	2297 ± 366	30.2	1.59
(D)	Synaptic Vesicles	1305 ± 205	17.2	2.45
(E)		798 ± 127	10.5	1.30
(F)	Membranes	919 ± 234	12.1	0.96
(G)		675 ± 94	8.9	0.85
(H)	Partially Disrupted Synaptosomes	757 ± 199	10.0	0.67
(I)	Mitochondria	846 ± 152	11.1	0.57
	(% Recovered (±SEM) from P_2W = 102.4 ± 18.6)			

[1] The data represent 6 fractionations.

[2] The homogenate was prepared from an average of 3.8 g of rat brain.

[3] The distribution is calculated as a percent of the total recovered activity.

fraction of the 0 to I gradient. These results are in agreement with those of Agrawal, Davison and Kaczmarek (1971), who found 45% of the cysteine sulfinic acid decarboxylase activity in the crude mitochondrial fraction. Most of the activity recovered from the crude mitochondrial fraction was apparently localized in the two synaptosomal fractions obtained with the fractionation technique of DeRobertis et al. (1962).

Serine hydroxymethyltransferase

Preliminary fractionation of brain and liver indicated that there were particulate-bound components of serine hydroxymethyltransferase in both tissues (Table 21). This is in agreement with the findings of Nakano, Fujioka and Wada (1968) for rat liver, as well as with the studies of rat brain by Davies and Johnston (1973). Both groups found that serine hydroxymethyltransferase is a mitochondrial enzyme and this has been confirmed by the present work. The subcellular distribution of this enzyme showed 74.4% in the crude mitochondrial fraction (P_2). When fraction P_2 was further fractionated on an A to C gradient, 78.0% of the recovered activity was in the mitochondrial fraction. After disruption of P_2 and separation of the components on the 0 to I gradient, 83.5% of the recovered activity was found in the mitochondrial fraction. Serine hydroxymethyltransferase appears to be a better marker for the mitochondria than the recommended mitochondrial enzyme, fumarase (compare the distributions in Table 9 with those of Table 22). Actually it has been noted that brain fumarase may not be completely mitochondrial; there may be approximately 30% of a soluble component (Shepard and Kalnitsky, 1954; Lowenstein, 1971).

TABLE 21

PRELIMINARY FRACTIONATION OF SERINE HYDROXYMETHYLTRANSFERASE

		-Triton X-100			+Triton X-100		
Tissue	Fraction	Activity (nmole/fraction/hour)	Recovery (%)	Distribution[2] (%)	Activity (nmole/fraction/hour)	Recovery (%)	Distribution (%)
Brain	Homogenate[1]	2849			4575		
	Pellet	2511		88.5	1264		23.0
	Supernatant	327		11.5	4242		77.0
	Total	2838	99.6		5506	120.3	
Liver	Homogenate	181537			212806		
	Pellet	85655		50.0	17102		7.7
	Supernatant	85664		50.0	204707		92.3
	Total	171319	94.4		221809	104.2	

[1] The homogenates were prepared from 2.7 g (-Triton X-100) and 2.8 g (+Triton X-100) of brain and 2.7 g (-Triton X-100) and 3.1 g (+Triton X-100) of liver.

[2] Distribution is calculated as a percent of the total recovered activity.

TABLE 22

SUBCELLULAR LOCALIZATION OF SERINE HYDROXYMETHYLTRANSFERASE

Subcellular Fractions[1]:		Activity (nmole/fraction/hour ± SEM)	Distribution[3] (%)	Relative Specific Activity
Primary Fractions				
(HOM)	Homogenate[2]	5535 ± 136		
(P_1)	Crude Nuclear	887 ± 170	19.0	0.75
(P_2)	Crude Mitochondrial	3482 ± 147	74.4	1.02
(P_3)	Microsomal	63 ± 18	1.4	0.17
(S_3)	Supernatant	245 ± 33	5.2	0.12
	(% Recovered (±SEM) from Hom = 88.3 ± 2.9)			
Secondary Fractions				
(P_2)	Crude Mitochondrial Layered	3482 ± 147		
(A)	Myelin	132 ± 16	6.0	0.16
(B)	Synaptosomes	353 ± 19	16.0	0.28
(C)	Mitochondria	1756 ± 75	78.0	3.10
	(% Recovered (±SEM) from P_2 = 64.2 ± 4.1)			
(P_2W)	Crude Mitochondrial Layered	1413 ± 111		
(O)	Cytoplasm	17 ± 13	2.1	0.06
(D)	Synaptic Vesicles	2 ± 1	0.2	0.01
(E)	Membranes	5 ± 5	0.6	0.03
(F)		1 ± 1	0.1	0.01
(G)		7 ± 4	0.9	0.03
(H)	Partially Disrupted Synaptosomes	103 ± 31	12.5	0.42
(I)	Mitochondria	683 ± 82	83.5	2.29
	(% Recovered (±SEM) from P_2W = 55.5 ± 4.0)			

[1] The data represent 6 fractionations.
[2] The homogenate was prepared from an average of 3.8 g of rat brain.
[3] The distribution is calculated as a percent of the total recovered activity.

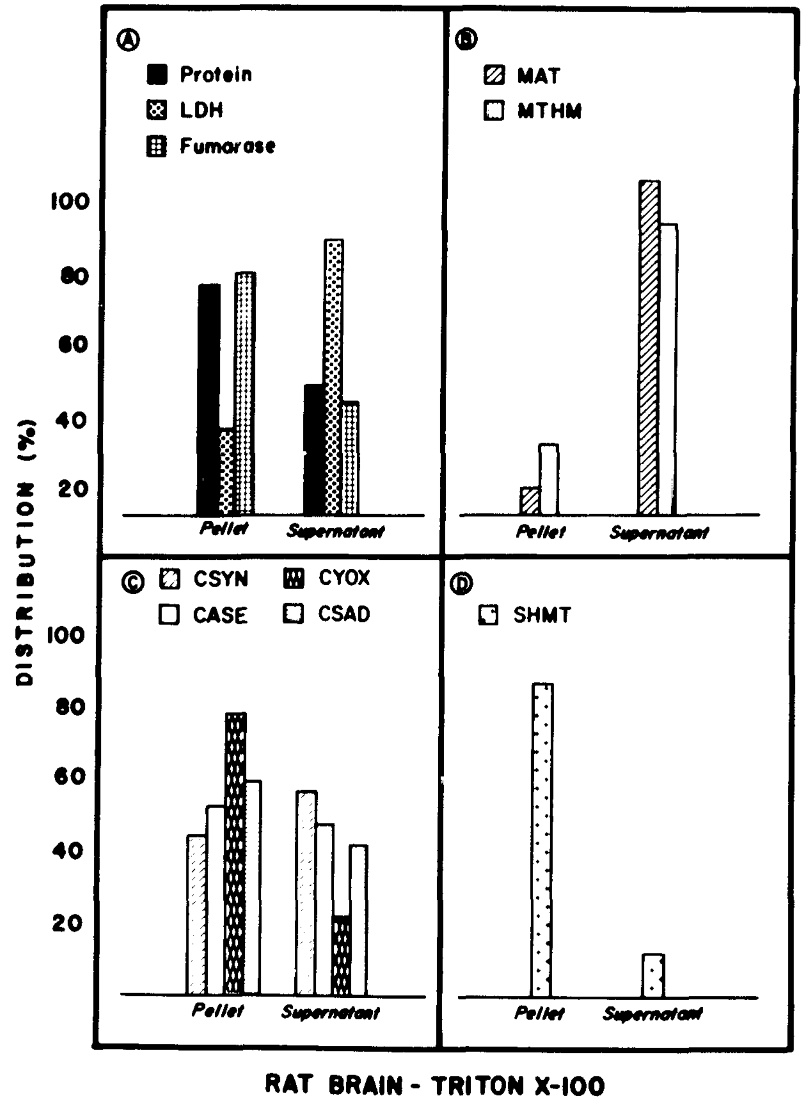
4. DISCUSSION

The localization of the enzymes associated with the metabolism of sulfur amino acids appears to be consistent with a compartmentation in which methylation function is soluble and neurotransmitter function is particulate. Preliminary fractionations of rat brain gave the first indication of a possible compartmentation of the pathways of metabolism associated with the sulfur amino acids (Figure 9). Methionine adenosyltransferase and N^5-methyltetrahydrofolic acid-homocysteine methyltransferase were clearly associated with the soluble component of these crude fractions. The transsulfuration enzymes — cystathionine β-synthase, cystathionase, cysteine dioxygenase and cysteine sulfinic acid decarboxylase — appeared to be approximately half in the pellet and half in the supernatant solution. Serine hydroxymethyltransferase was associated primarily with the pellet.

Fractionation of rat brain into the primary subcellular fractions gave further evidence for the compartmentation of the pathways of metabolism of the sulfur amino acids. Methionine adenosyltransferase and N^5-methyltetrahydrofolic acid-homocysteine methyltransferase were primarily located in the high speed supernatant fraction or cytoplasm (S_3) (Figure 10A). Those enzymes that were associated with the pellet in the preliminary fractionation were found in the crude mitochondrial fraction after primary subcellular fractionation (Figure 10B). The pattern of distribution seen in Figure 10 clearly shows the difference between the localization of the methylation enzymes (fraction S_3) versus the enzymes involved in the production of putative transmitter and modulator substances (fraction P_2).

FIGURE 9

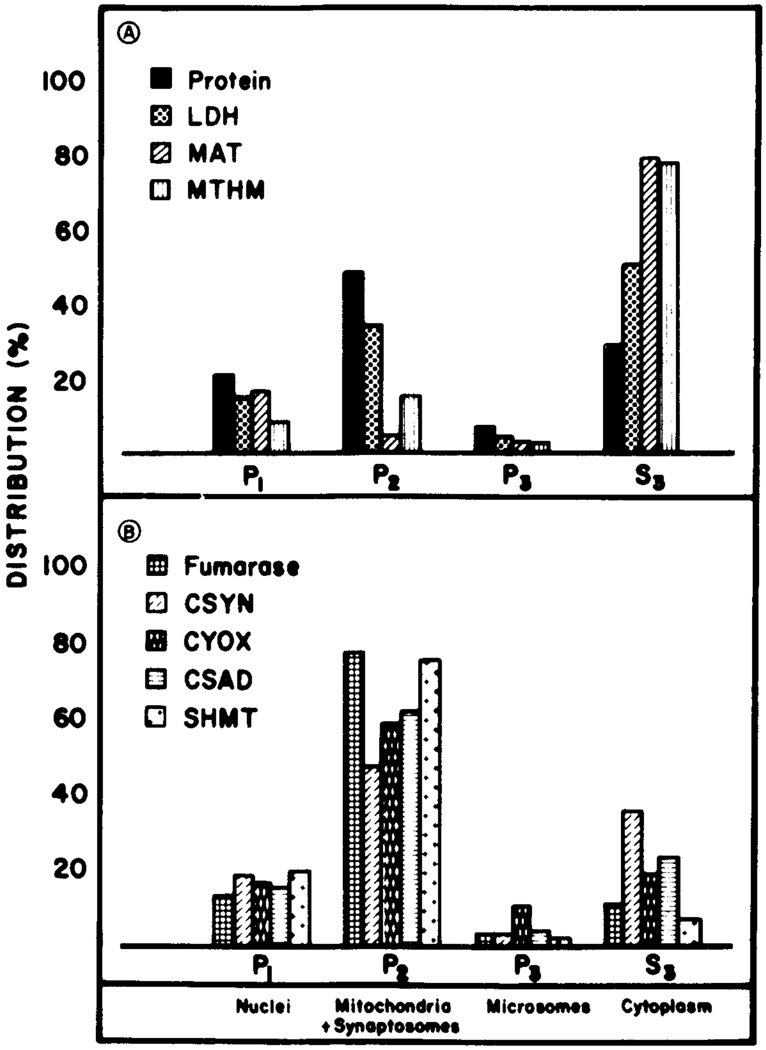
PRELIMINARY FRACTIONATION OF THE SULFUR AMINO ACID PATHWAY IN BRAIN



Abbreviations: LDH, Lactate dehydrogenase; MAT, Methionine adenosyltransferase; MTHM, N^5-methyltetrahydrofolic acid homocysteine methyltransferase; CSYN, cystathionine β-synthase; CASE, cystathionase, CYOX, cysteine dioxygenase; CSAD, cysteine sulfinic acid decarboxylase; SHMT, serine hydroxymethyltransferase.

FIGURE 10

PRIMARY SUBCELLULAR FRACTIONS OF THE SULFUR AMINO ACID PATHWAY



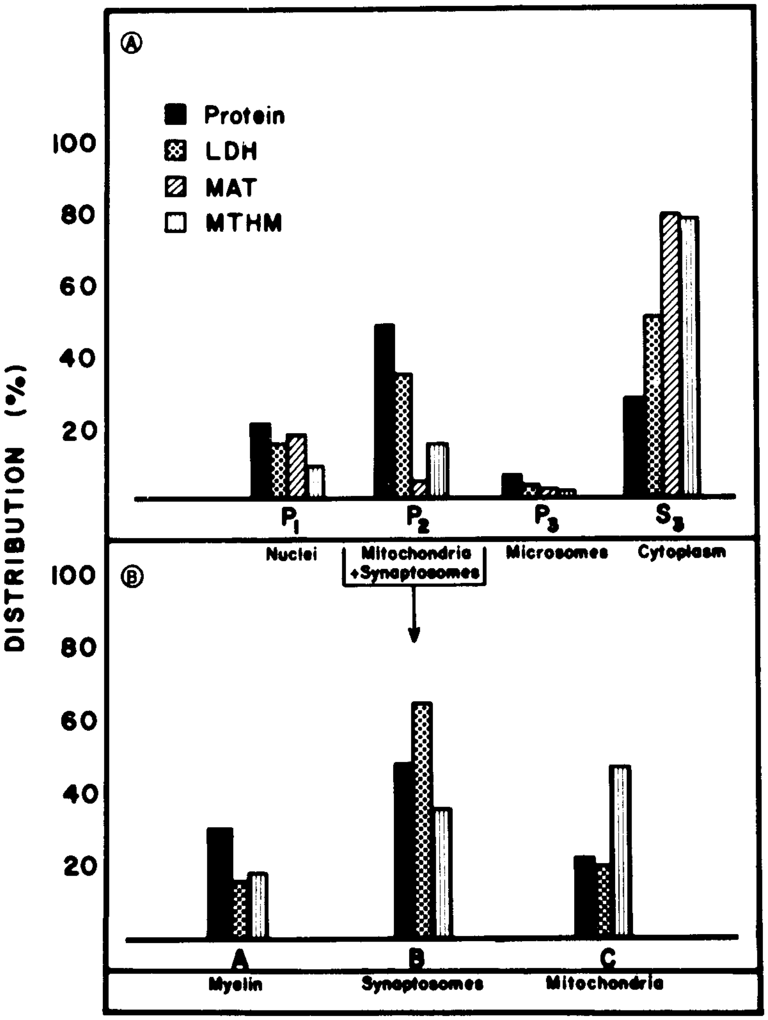
Abbreviations: LDH, Lactate dehydrogenase; MAT, methionine adenosyltransferase; MTHM, N^5-methyltetrahydrofolic acid homocysteine methyltransferase; CSYN, cystathionine β-synthase; CYOX, cysteine dioxygenase; CSAD, cysteine sulfinic acid decarboxylase; SHMT, serine hydroxymethyltransferase.

Measurement of the soluble components of the sulfur amino acid pathway after secondary fractionation of the crude mitochondrial fraction (P_2) without disruption provided little further information (Figure 11). It was not possible to measure methionine adenosyltransferase in the secondary fractions, and the results of the measurements of N^5-methyltetrahydrofolic acid-homocysteine methyltransferase reflected the inherent difficulties in separating into components what was only a very small percentage of the total activity (14.2% in fraction P_2). However, the cytoplasmic marker enzyme, lactate dehydrogenase (of which 34% was present in fraction P_2), was recovered primarily in the synaptosomal fraction (B), as would be expected if it were in the cytoplasm but occluded within the nerve ending. It is relevant to note here that catechol-O-methyltransferase, one of the enzymes that uses the methyl group of S-adenosylmethionine and forms S-adenosylhomocysteine, has been shown to have much the same distribution as lactate dehydrogenase (Broch and Fonnum, 1972). The only step in the methylation cycle that has not been studied in brain at this time is the hydrolysis of S-adenosylhomocysteine to homocysteine. The present hypothesis predicts that this enzyme will also be cytoplasmic in rat brain. S-Adenosylhomocysteine hydrolase is a soluble enzyme in rat liver; although the activity is present in rat brain, the subcellular localization of this enzyme has not been determined (Finkelstein and Harris, 1973).

Analysis of the enzymes associated with the production of putative transmitter and modulator substances in the secondary fractions of undisrupted fraction P_2 (myelin, synaptosomes and mitochondrial) showed

FIGURE 11

THE A TO C FRACTIONS OF THE SOLUBLE COMPONENTS



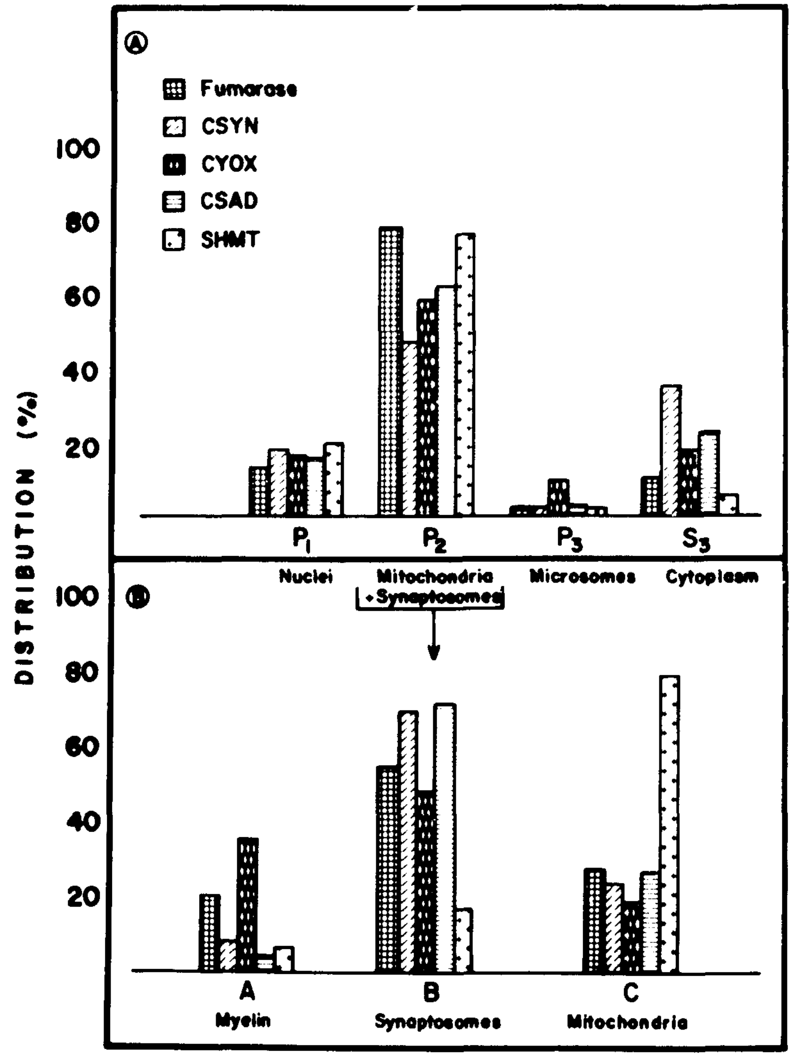
Abbreviations: LDH, Lactate dehydrogenase; MAT methionine adenosyltransferase; MTHM, N^5-methyltetrahydrofolic acid homocysteine methyltransferase.

that most of the recovered activity of cystathionine β-synthase, cysteine dioxygenase and cysteine sulfinic acid decarboxylase was in the synaptosomes (Figure 12). Serine hydroxymethyltransferase activity, in contrast, was primarily mitochondrial (Figure 12). Disruption of the crude mitochondrial fraction (P_2) by hypo-osmotic shock and separation of the organelles provided further information concerning the localization of the enzymes (Figure 13). As expected N^5-methyltetrahydrofolic acid-homocysteine methyltransferase was cytoplasmic. Cysteine dioxygenase and cysteine sulfinic acid decarboxylase were released into the cytoplasm, indicating that they had been occluded within the cytosol of the synaptosomes. Cystathionine β-synthase appeared to have both a cytoplasmic and a mitochondrial component, with residual activity still present in the undisrupted synaptosomes. Serine hydroxymethyltransferase was almost completely recovered in the mitochondrial fraction.

Thus, the enzymes associated with methylation function appear to be entirely soluble or cytoplasmic in character. Their subcellular distribution suggests that it may be derived from the perikaryon or axon of the neuron or possibly from glial cells: comparison of the distribution of lactate dehydrogenase (33.5% occluded within the synaptosomes, *i.e.* in P_2) with methionine adenosyltransferase (4% in P_2) and N^5-methyltetrahydrofolic acid-homocysteine methyltransferase (14.2% in P_2) suggests that very little of the enzymes of methylation is occluded within the nerve ending. It may be inferred, then, that these two enzymes of methylation are derived from the cytosol of either other portions of the neuron or of other cells such as the glial cells. The small portion of the activity of the methylation enzymes associated

FIGURE 12

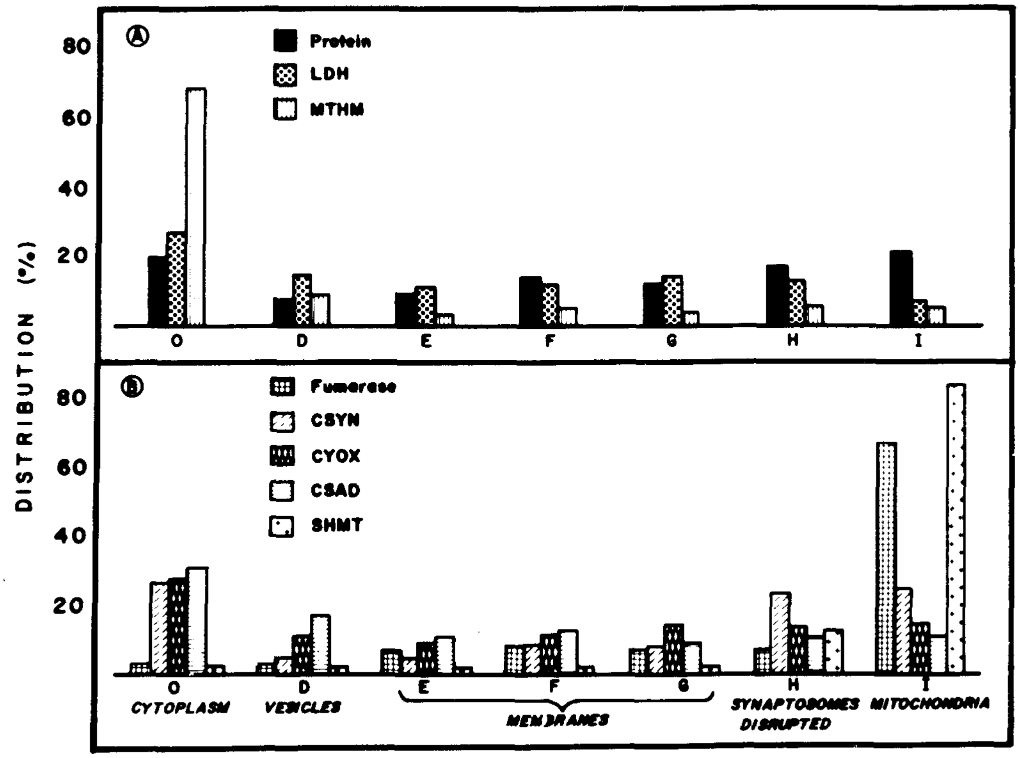
THE A TO C FRACTIONS OF THE PARTICULATE COMPONENTS



Abbreviations: CYSN, cystathionine β-synthase; CYOX, cysteine dioxygenase; CSAD, cysteine sulfinic acid decarboxylase; SHMT, serine hydroxymethyltransferase.

FIGURE 13

THE O TO I FRACTIONS OF THE SULFUR AMINO ACID PATHWAY



Abbreviations: LDH, lactate dehydrogenase; MTHM, N^5-methyltetrahydrofolic acid homocysteine methyltransferase; CSYN, cystathionine β-synthase; CYOX, cysteine dioxygenase; CSAD, cysteine sulfinic acid decarboxylase; SHMT, serine hydroxymethyltransferase.

with particulate fractions may come from undisrupted cells (the crude nuclear fraction, P_1) or from cytoplasm trapped within the synaptosomes (the crude mitochondrial fraction, P_2).

The synaptosomes contain a relatively high concentration of those enzymes of the metabolism of sulfur amino acids that are concerned with reactions that produce putative neurotransmitter compounds. Serine hydroxymethyltransferase, also concerned with the production of a neurotransmitter, glycine, was found to be purely mitochondrial in nature. It is tempting to hypothesize that the pathway of synthesis for this putative neurotransmitter is localized in the mitochondria to separate the metabolic and transmitter pools of glycine, which is probably provided to the central nervous system by this route rather than by transport across the blood-brain barrier (Shank and Aprison, 1970).

A comparison of the localization of serine hydroxymethyltransferase and of fumarase (Figure 12) raises the possibility that serine hydroxymethyltransferase is associated with mitochondria that may originate from the perikaryon or from glial cells. Fumarase, considered a mitochondrial marker, is found primarily in the synaptosomal fraction (53.5% in fraction B) of the secondary fractions of the undisrupted crude mitochondrial fraction (P_2). The synaptosomes contain mitochondria (see Figure 6) and fumarase in the synaptosomal fraction is associated with these mitochondria, for after disruption of the crude mitochondrial fraction (P_2) significant fumarase activity is found only in the mitochondrial fraction (I) (Figure 13). The localization of fumarase demonstrated that there are a substantial number of mitochondria within the synaptosomes. In contrast, serine hydroxymethyltransferase is only minimally associated with the synaptosomal fraction (16.0% in fraction B) from the undisrupted crude mitochondrial fraction but is primarily associated with the mitochondrial fraction (78.0% in fraction C). Thus, serine hydroxymethyltransferase is not associated to any great degree with those mitochondria that are present within the synaptosomes of fraction B, whereas fumarase is associated with both the mitochondria of the synaptosomes and the mitochondria from elsewhere. One possible implication of this localization of serine hydroxymethyltransferase is an association with the mitochondria that derive from sites other than the nerve ending, such as the axon, the perikaryon or the glial cells. It has been suggested that glial cells may be important in the regulation of neuronal excitability by controlling the concentrations of neurotransmitters by means of metabolic and transport mechanisms (Schrier and Thompson, 1974).

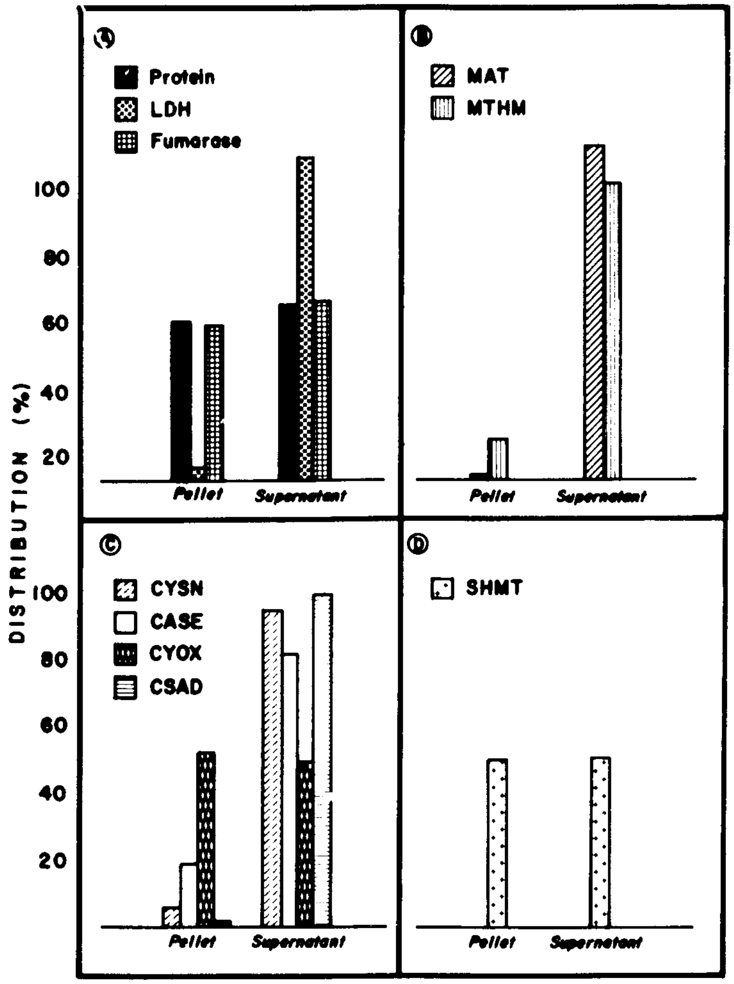
A recently published abstract has indicated that N^5,N^{10}-methylenetetrahydrofolic acid reductase is soluble (Burton and Sallach, 1974). This report is compatible with the working hypothesis presented here if the methylation function that has been attributed to N^5-methyltetrahydrofolic acid is as important as preliminary studies indicate it may be (Banerjee and Snyder, 1973; Laduron, 1973; Laduron, Gommeren and Leysen, 1974). The enzymes that synthesize S-adenosylmethionine and N^5-methyltetrahydrofolic acid, methionine adenosyltransferase and N^5,N^{10}-methylenetetrahydrofolic acid reductase, and the enzymes that utilize these methyl donors, catechol-O-methyltransferase and N^5-methyltetrahydrofolic acid-homocysteine methyltransferase, all appear to be associated with soluble brain fractions.

In addition to the data concerning the subcellular localization of the enzymes of sulfur amino acid metabolism in rat brain, it was shown that these enzymes had different subcellular distribution in rat liver. Preliminary fractionation of rat liver clearly showed that only cysteine oxidase and serine hydroxymethyltransferase had particulate components in this tissue (Figure 14). Cystathionine β-synthase, cystathionase and cysteine sulfinic acid decarboxylase were all associated with the soluble liver fraction. The difference between the brain and liver enzymes further reinforces the hypothesis that in brain these enzymes are responsible not only for amino acid catabolism and interconversion, but also for the synthesis of amino acid neurotransmitters.

A number of investigations into the metabolism of sulfur amino acids in rat brain have failed to take into account the considerable portion of the enzymes that are present in the particulate components. Unfortunately these various studies of cystathionine β-synthase and cystathionase have not been uniform in the procedure used for preparation of rat brain extracts. Most of these investigations used 0.03 M potassium phosphate buffer, pH 6.9, as a medium for preparation of rat brain homogenates (with the exception of Brown and Gordon (1971) who used 0.1 M phosphate buffer, pH 7.5) which when centrifuged at 360,000 g-min or greater would cause all the particulate material, including the microsomes, to be sedimented into a pellet. Centrifugation of less than 360,000 g-min would leave undetermined amounts and types of particulate materials in the supernatant solution. Studies of the activity of cystathionine β-synthase and cystathionase of whole rat brain have been made using centrifugations of 35,000 g-min (Mudd et al., 1965) and 90,000 g-min (Sturman, Rassin and Gaull 1970a) which would have included an indeterminate amount of particulate material in the supernatant. The specific activity of cystathionine β-synthase found

FIGURE 14

PRELIMINARY FRACTIONATION OF THE SULFUR AMINO ACID PATHWAY IN LIVER



RAT LIVER - TRITON X-100

Abbreviations: LDH, lactate dehydrogenase; MAT, methionine adenosyltransferase; MTHM, N^5-methyltetrahydrofolic acid homocysteine methyltransferase; CSYN, cystathionine β-synthase; CASE, cystathioninase; CYOX, cysteine dioxygenase, CSAD, cysteine sulfinic acid decarboxylase, SHMT, serine hydroxymethyltransferase.

in the potassium phosphate homogenate of whole rat brain in the present work (54.8 nmoles/mg protein/hour) is not strikingly different from that of the 90,000 g-min supernatant solution measured with the same analytical procedures in a previous study (65.4 nmoles/mg protein/hour) (Sturman, Rassin and Gaull, 1970a). However, the specific activity of the 360,000 g-min supernatant solution in the present work (10.3 nmoles/mg protein/hour) is very different from the specific activities cited above.

The greatest problem inherent in ignoring the particulate components of these enzymes is the interpretation of data that are concerned with the effects of pyridoxine, the developmental pattern, or the comparison of activity located in different brain regions. It cannot be assumed that effects upon the soluble component of the enzymes are identical to those upon the particulate component. The particulate components of cystathionase and cystathionine β-synthase were not measured in studies of the effect of pyridoxine upon the brain enzymes, using a 480,000 g-min supernatant solution (Brown and Gordon, 1971) or in studies of the postnatal brain development of the enzymes using a 540,000 g-min supernatant solution (Volpe and Laster, 1972). Effects of development or of drug treatment also might be upon the subcellular distribution rather than upon total activity of the enzyme. Histamine, for example, is associated principally with the crude nuclear fraction (P_1) of brain in the 3-day-old neonatal rat (19%), but with the supernatant and crude mitochondrial fractions (44 and 30%, respectively) in the 21-day-old rat brain (Snyder and Taylor, 1972).

Regional studies of the activities of the transsulfuration enzymes in monkey brain have been published (Volpe and Laster, 1970a, 1970b;

Sturman, Rassin and Gaull, 1970b). Particulate components of the enzymes were not measured in these studies, which may indicate that complete activity in any one region of the brain was not obtained. It has been reported that histamine methyltransferase may be distributed quite differently in the cerebellum (74.5% nuclear, 10.4% mitochondrial, 15.1% soluble) than in the cerebral cortex (19.8% nuclear, 41.0% mitochondrial, 39.2% soluble) (Snyder and Taylor, 1972). Since only 15.1% of the histamine methyltransferase is soluble in the cerebellum while 39.2% is soluble in the cerebral cortex, measuring only the soluble component would result in a considerable error if total regional activity were to be compared. This evidence of a particulate histamine methyltransferase is contrary to part of the hypothesis that has been presented in this work; however, if S-adenosylmethionine is a methyl donor for many methyltransferase reactions it might be that some of the methyltransferases are localized in particulate fractions and some in soluble fractions.

The survey of the literature presented in the Introduction implicated some of the sulfur amino acids in brain function, and the experiments presented here have demonstrated how the metabolic and transmitter pools of these amino acids might be separated from one another. Further study in a number of directions is necessary before these compounds can be accepted as neurotransmitters. The present work also requires amplification in order to clarify the role of sulfur amino acids in brain. Other species should be examined to see whether or not subcellular localization is peculiar to the rat or represents a widespread phenomenon. The development of the localization should be studied, especially in light of the histamine methyltransferase studies presented above, in which localization changed during early development. The development of transmitter systems in the brain may provide clues to normal and abnormal function.

The results of the study of serine hydroxymethyltransferase illustrate that subcellular fractionation is not the only technique that should be applied in localization studies. The possible glial localization of this enzyme suggests that separation of glial and neuronal cells, for which techniques are available (Sinha and Rose, 1971), might be a valuable tool in understanding where and how these brain enzymes function. The development of techniques that would provide subcellular preparations homogeneous for a single transmitter would be extremely valuable in the study of neurotransmitters. This has been achieved for acetylcholine by use of the electric organ of _Torpedo_ as a source of synaptic vesicles (reviewed by Whittaker, 1973).

Further investigation of the properties of the enzymes of sulfur amino acid metabolism would be useful as it is possible that their different localization in brain might imply different physical properties. Occluded or particulate bound forms of these enzymes might be different isoenzyme forms of the soluble and liver enzymes. The exact subcellular localization of cystathionase is still only a supposition because of the low activity of this enzyme in brain. The hydrolysis of S-adenosylhomocysteine to homocysteine in brain should be characterized and the reactions which involved S-adenosylmethionine need to be further investigated. The various methyltransferase reactions that use S-adenosylmethionine as a substrate and their possible preference for N^5-methyltetrahydrofolic acid appears to be an important area of research at present. The decarboxylation of S-adenosylmethionine in brain has been studied and found to be primarily a soluble enzymatic step in brain (86.1% of the original activity), with only a small percentage of activity in the crude mitochondrial fraction (1.7%) Schmidt and Cantoni, 1973). The catabolism of taurine and the mechanism of its removal from the synapse is still open to question, particularly in the light of its slow turnover in brain (Sturman, 1973).

Thorough elucidation of the pathway of sulfur amino acid metabolism will probably not become possible until specific blocking agents are available for the various enzymatic steps. Pyridoxine deficiency is one tool that might be used in this fashion, but a number of enzymes in the pathway are dependent upon pyridoxal 5'-phosphate as a cofactor: serine hydroxymethyltransferase, cystathionine β-synthase, cystathionase and cysteine sulfinic acid decarboxylase. It is interesting to note that all these enzymes are involved in the synthesis of putative neurotransmitters. Methotrexate inhibits dihydrofolate reductase and may be useful for studying this pathway if this portion of the folic acid cycle is in any way regulatory for the sulfur amino acid pathway.

5. CONCLUSION

The pattern of subcellular localization of the enzymes of the pathway of sulfur amino acid metabolism may be related to the function of these amino acids in brain. The methylation cycle enzymes, methionine adenosyltransferase and N^5-methyltetrahydrofolic acid-homocysteine methyltransferase, are soluble in rat brain. The transsulfuration enzymes, cystathionase, cystathionine β-synthase, cysteine dioxygenase and cysteine sulfinic acid decarboxylase, have particulate components in addition to the soluble components in rat brain. Serine hydroxymethyltransferase, the major source of glycine in rat brain, is mitochondrial. This suggests that the soluble component of these enzymes is associated with general metabolic functions and the particulate component is associated with the synthesis of neurotransmitters. The particulate component of these transsulfuration enzymes are located within the synaptosomes, which is the site most compatible with their role in transmitter functions. Brain transsulfuration enzymes appear to be different from the liver enzymes and this may be because of their role in separating the transmitter amino acid pool from the general metabolic amino acid pool.

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