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Effect Of Methionine Loading On The Production And Activation Of Methyl Groups In Rat Brain.

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EFFECT OF METHIONINE LOADING ON THE PRODUCTION AND ACTIVATION OF METHYL GROUPS IN RAT BRAIN

by

Frank Charles Benesh

A DISSERTATION

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AICAR	5'-phosphoribosyl-5-aminoimidazole-4-carboxamide
ATP	Adenosine 5'-triphosphate
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CPK	Creatine phosphokinase
CPM	Counts per minute
CSF	Cerebrospinal fluid
DEAE	Diethylaminoethyl
DHF	Dihydrofolic acid
DMPEA	3,4-dimethoxy-β-phenethylamine
DMT	Dimethyltryptamine
DOPA	3,4-dihydroxyphenylalanine
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
FAD,$FADH_2$	Flavin adenine dinucleotide and its reduced form
FIGLU	Formiminoglutamate
GAR	5'-phosphoribosylglycineamide
GSH	Glutathione
HIOMT	Hydroxyindole-O-methyltransferase
LSD	Lysergic acid diethylamide
MAO	Monoamine oxidase
MAT	Methionine adenosyltransferase

MHPG	3-methoxy-4-hydroxy-phenylglycol
NAD,NADH	Nicotinamide adenine dinucleotide and its reduced form
NADP,NADPH	Nicotinamide adenine dinucleotide phosphate and its reduced form
NAS	N-acetylserotonin
$N^5N^{10}CHTHF$	N^5,N^{10}-methenyltetrahydrofolic acid
$N^5N^{10}CH_2THF$	N^5,N^{10}-methylenetetrahydrofolic acid
N^5CH_3THF	N^5-methyltetrahydrofolic acid
$N^{10}CHOTHF$	N^{10}-formyltetrahydrofolic acid
PCA	Perchloric acid
POPOP	1,4-bis-(5-phenyloxazolyl-2)-benzene
PP_i	Inorganic pyrophosphate
PPP_i	Inorganic tripolyphosphate
PPO	2,5-diphenyloxazole
SAH	S-adenosylhomocysteine
SAMe	S-adenosylmethionine
TCA	Trichloroacetic acid
THF	Tetrahydrofolic acid
dTMP	2'-deoxythymidine-5'-monophosphate
Tris	Tris(hydroxymethyl) aminomethane
dUMP	2'-deoxyuridine-5'-monophosphate
VMA	Vanillylmandelic acid

INTRODUCTION

Genetic Factors in Schizophrenia

Many authors have documented what appears to be a familial clustering of psychopathology. Rieder (1973) reviewed the literature dealing with the offspring of schizophrenic parents. He noted a wide spectrum and a high incidence of psychopathology among the offspring. About 45 percent of sibs, parents and children of a schizophrenic are schizoid (schizophrenic-like) or schizophrenic. Children of two schizophrenic parents develop the disease with a frequency of about 66 percent (Heston, 1970). These subjects also are linked with a greater than normal number of apparently nonschizophrenic disorders. Early interpretations ascribed a genetic basis to these findings. These "genetic school" adherents were opposed by those who argued that mental disorders were the result of psychological and environmental experiences. The outcome of the controversy has direct bearing on the establishment of schizophrenia as a biochemical abnormality.

The evidence for a genetic factor in schizophrenia is derived from the different incidence of the disease in pairs of monozygotic and dizygotic twins. Kallman (1958) gave the concordance rate for schizophrenia in monozygotic and

dizygotic pairs as 86.2 and 14.5 percent, respectively. Schizophrenia was diagnosed in sixteen pairs of monozygotic twins, separated at an early age, of which ten were concordant (Slater, 1968). Other studies reported lower rates of concordance for twins. Stabenau (1968) diagnosed schizophrenia in seven pairs of monozygotic twins but reported only three to be concordant. Gottesman and Shields (1966) determined a 42 percent concordance for fraternal twins. Cohen et al. (1972), working with a large but selective population, American veterans, found a concordance rate among the lowest reported for schizophrenic twins, 23.5 and 5.3 percent for monozygotic and dizygotic, respectively. Heston (1973) reviewed the literature and calculated that the concordance rate for schizophrenia in monozygotic twins is about 40 percent. Despite the variations in results from study to study, the data support a genetic contribution to schizophrenic disease. However, with the exceptions of Kallman (1958), Slater (1968), and a few of the earlier studies, the discordance rate for schizophrenia in identical twins is greater than the concordance rate. This implies that factors other than genetic may be operative.

Despite the inferential results of the concordance rate studies, some maintained that social environment and not genes was the disposing factor toward schizophrenia. In an attempt to resolve the problem, the prevalence of schizophrenia among adopted children of schizophrenics and nonschizophrenics was studied (Rosenthal et al., 1968;

Heston, 1966). Thus, persons providing the genetic endowment could be separated from those providing the rearing experience. The studies showed an increased prevalence of schizophrenic-like disorders among adopted offspring of schizophrenics raised by normal parents. Wender et al. (1968) examined the possibility of environmental transmission by studying the biological and adoptive parents of adopted schizophrenics and the biological and adoptive parents of normal offspring. The biological parents of schizophrenics displayed more psychopathology than the adoptive parents. There was no difference between the biological and adoptive parents of normal children. This study was extended by Kety et al. (1968) to include the biological and adoptive relatives of adopted schizophrenics and adopted normal persons. The adoptive families of schizophrenics were no more psychopathologic than the adoptive and biological families of controls. However, biological relatives of adopted schizophrenics did show a greater occurrence of schizophrenic-like disorders. As a final test of social transmission of schizophrenia, Wender et al. (1974) employed the "crossfostering" technique. They studied offspring of normal biological parents reared by schizophrenic parents compared with adopted offspring of schizophrenic biological parents reared by normal adopting parents. The offspring of schizophrenic parents received a schizophrenic diagnosis of severity equal to or greater

than the crossfostered adoptees.

The results of all these studies make a strong case for genetic factors in schizophrenia. Genetic mechanisms which may be operative in a predisposition for schizophrenia have been discussed (Gfeller, 1976).

Biochemical Factors in Schizophrenia

Psychopharmacology had its start in the mid nineteenth century. It was to become an integral part of an attempt fifty years later to establish a scientific basis for mental illness. Jaques-Joseph Moreau (1804-1884) was the first medical man to work systematically with centrally acting compounds. He compiled an extensive amount of information on hashish during his stay in the Orient and published "Du Hachisch et de L'Alienation Mentale" (Moreau, 1845). Another important contributor to psychopharmacology during this period was Louis Lewin (1850-1929). He initiated many studies concerning the side effects of drugs, most notably morphine and mescaline (Lewin, 1931). However, it was Lewin's student, Kurt Beringer, who conducted numerous and detailed experiments with mescaline in man and published "Der Meskalinrausch" (Beringer, 1927). This book is to mescaline what Moreau's book is to hashish" (Holmstedt, 1967).

While experimental psychology was developing, Emil Kraepelin (1856-1926) also became interested in studying the effects of drugs in man. He later turned

entirely to clinical psychiatry which has grown largely on the foundation he built. Kraepelin made observations concerning the "states of dementia quite similar to each other which developed from the most varied initial clinical symptoms" (Kraepelin, 1919). He was influenced by Hecker who in 1871 described a disease beginning in adolescence which progressed to mental deficiency. In 1896, Kraepelin named these conditions "dementia praecox", a term introduced by Morel in 1860. Bleuler (1911) recognized that neither terminal dementia nor precocity is an essential element of the clinical domain. He therefore called dementia praecox "schizophrenia" to emphasize the "splitting" of psychic functions. The fundamental schizophrenic symptoms according to Bleuler were disturbances of association, affectivity or emotion, autism or the desire to escape reality, and ambivalence. Despite the psychological background, Bleuler, like Kraepelin before him, believed schizophrenia to be a physical disease with the delusions and hallucinations as secondary manifestations of "more or less successful attempts at adaptation to the primary disturbances." This prompted others to search for anatomical, biochemical, and physiologial lesions that could account for the disease. Analogy with the action of hallucinogenic drugs sparked a search for endogenous aromatic toxins which were suspected of giving rise to a "sallow yellow skin, profuse perspirations and of offensive odour" (Townsend, 1905). Much of the early literature on research concerned with

schizophrenia has been reviewed by Lewis (1936).

The idea that the phenomenology of schizophrenia results from biochemical abnormalities continued to influence research in this area. Freeman (1931) reported a generalized tendency toward deficient oxidative processes in schizophrenia. Hoskins (1933) studied many cases of schizophrenia with particular emphasis on biochemical and physiological measurements. His main conclusion was a "remarkable degree of variability of the physiologic functions from one test to another". This was often referred to by other investigators. McFarland and Goldstein (1938) review the major findings related to the biochemistry of schizophrenia. Included were a disturbed lipid metabolism, especially hypocholesteremia, and a disordered carbohydrate metabolism. Workers in the 1920's and early 1930's had demonstrated an abnormal sugar tolerance in schizophrenics with a tendency toward hyperglycemia. It was thought that the blood insulin level might be low or that some product of endocrine origin, impeding the effect of insulin, might be overactive. This connection with blood sugar received additional attention when Manfred Sakel, using insulin to induce hypoglycemia in morphine addiction, tried it on schizophrenics (Sakel, 1937). Reporting a high rate of remissions, particularly in cases with an illness of less than 6 months in duration, Sakel stimulated a great deal of research in this area which seemed to peak in the late 1930's. Simultaneous with Sakel's use of insulin,

Meduna began to induce convulsions in chronic schizophrenics by injecting intramuscularly a synthetic soluble camphor preparation, pentamethylenetetrazole, known in the United States as Metrazole (Meduna, 1936). He proceeded on the assumption that since schizophrenia and epilepsy rarely appear together, there must be antagonism between the two and epileptic convulsions might have therapeutic value for schizophrenics. Noting that these treatments often led to severe and irreversible damage to the nervous system, Cerletti and Bini experimented with electric convulsive therapy which eventually displaced insulin and Metrazole (Bini, 1938). Many other forms of somatic therapy were developed during these years including the prefrontal lobotomies first performed in America by Freeman and Watts (Bellak, 1948). However, research in schizophrenia was greatly curtailed during the war years and the work in progress suffered from lack of direction.

Stockings (1940) believed the causative agent in the psychoses to be a toxic amine with properties similar to mescaline. He further observed that mescaline can produce in the normal individual mental changes very similar in nature to those found in psychotic patients. While this was known since Lewin first described the effects of mescaline, Stockings extended his observations by initiating the experimental investigation of psychoses with drugs that mimic them. This was to have a profound effect on the future of psychiatric research although another ten years would

pass before the concept was vigorously pursued. Meanwhile, because of the suggested abnormalities in oxygen consumption rate and carbohydrate metabolism, various forms of endocrine therapy were tried and "adrenal-cortical responsivity" was foremost among the biochemical theories of schizophrenia (Pincus et al., 1949). Of particular interest during this time were the studies of Ashby (1944). Working mainly with carbonic anhydrase, she was one of the first to recognize the importance of enzymes of the brain, their distribution, and relevance to brain function and psychosis. Other investigators took advantage of the accessibility of blood proteins. Richter and Lee (1942a,b) reported raised levels of plasma cholinesterase in mental patients which was later found to be nonspecific and related to stress (Weil-Malherbe and Szara, 1971). Other blood enzymes were implicated in the etiology of schizophrenia during this period without later corroboration.

Several findings, however, were to influence further thought and experimentation. The idea of schizophrenia as an autoimmune disease began with the claim that schizophrenic cerebrospinal fluid (CSF) had proteolytic activity toward cerebral cortex (Abderhalden and Elsaesser, 1944). Several investigators were studying the autoxidation and cyclization of catecholamines to "aminochromes" (Beyer and Shapiro, 1945). These compounds were later claimed to have hallucinogenic properties and were incorporated into the adrenochrome hypothesis of schizophrenia (Hoffer et al., 1954).

Additionally, an increased concentration of histamine in the plasma or serum of schizophrenics was reported (Strengers and Gooszen, 1946). This finding would be referred to many times to account for the low incidence of allergies in schizophrenics. Nevertheless, the overall situation of biochemical research in schizophrenia remained much the same in the 1940's as it had been in the 1930's. "For almost every investigator who finds one biochemical picture of the psychoneuroses there is another investigator who finds that this biochemical picture is not present" (McFarland and Goldstein, 1937). This was also true in subsequent years as the cumulative effect of variables such as unreliability of psychiatric classification, errors of biochemical methods, lack of control of the physical and mental states of patients and lack of adequate control groups continued to hamper biochemical investigation of mental illness.

With this background, Osmond and Smythies (1952) developed the transmethylation hypothesis, a landmark in schizophrenia research. They proposed that O-methylation of either norepinephrine or dopamine could produce a compound with psychotomimetic properties similar to mescaline. Catechol-O-methyltransferase (COMT) had not yet been described. Gaddum and Hameed (1954) observed that d-lysergic acid diethylamide (LSD) inhibited the action of serotonin on rat uterus <u>in vitro</u>. Studies of this antagonism led to the hypothesis, as reviewed by Himwich (1970), that schizophrenia was due to a malfunction of serotonin contain-

ing neurons. The LSD-serotonin interaction has been reviewed (Bradley and Smythies, 1974). Extending the observations of elevated plasma histamine in schizophrenics, Freedman et al. (1956) injected histamine intradermally into schizophrenics and nonschizophrenics. The resulting wheal and degree of erythema was reported to be smaller for schizophrenics.

At this time there was considerable interest in ceruloplasmin, a blood copper binding protein. Akerfeldt (1957) showed that schizophrenic serum was able to oxidize N,N-dimethyl-p-phenylenediamine more readily than normal serum. Leach and Heath (1956) used epinephrine to determine ceruloplasmin activity in normal and schizophrenic serum. Finding a more rapid rate of oxidation in schizophrenic serum and assuming the cyclization of epinephrine to adrenochrome, these workers gave additional credence to the adrenochrome hypothesis put forth in 1954. Fabing (1956) reported on the hallucinogenic effects of bufotenin, the N-methylated product of serotonin. This led to the theory of schizophrenia that N-methylated derivatives of serotonin were abnormally produced (Erspamer, 1959). Gessner (1970) subsequently showed that bufotenin is inactive as an hallucinogen. Axelrod (1961) was the first to demonstrate the enzymatic N-methylation of indoleamines and catecholamines. He showed the formation of N,N-dimethyltryptamine (DMT) from tryptamine, bufotenin from serotonin, and epinine from dopamine. This finding together

with the previous demonstration of O-methylation of catecholamines by catechol-O-methyltransferase (Axelrod and Tomchick, 1958) lent support to the transmethylation hypothesis. Catecholamines could be converted to mescaline-like products *in vivo* and serotonin could give rise to its methylated, hallucinogenic derivatives. Recently, Christian *et al.* (1975) have detected DMT in cerebrospinal fluid of both normals and schizophrenics and in isolated synaptosome preparations from rat brain (Christian *et al.*, 1976).

In addition to the postulation that catecholamines could be converted to adrenochromes, a similar mechanism involving indoleamines was advanced by McIsaac (1961). A ring closure of melatonin would yield 10-methoxyharmalan which is structurally close to the alkaloids, harmine and harmaline, demonstrated to be hallucinogenic in humans by Naranjo (1967). This is of particular interest since the enzyme N^5,N^{10}-methylenetetrahydrofolate reductase in the presence of electron acceptors may act in the reverse direction forming N^5,N^{10}-methylenetetrahydrofolate ($N^5N^{10}CH_2THF$) from N^5-methyltetrahydrofolate (N^5CH_3THF) (Taylor and Hanna, 1975b). Formaldehyde as a dissociation product of $N^5N^{10}CH_2THF$ condenses with indoleamines to form tetrahydro-β-carbolines (Wyatt *et al.*, 1975) which also have a close structural resemblance to harmine and harmaline. The corresponding product with dopamine is

tetrahydroisoquinoline (Laduron and Leysen, 1975). Detection and quantitation of biogenic amines and their metabolites in body tissues occupy much of the effort of current research.

While some investigators devoted their efforts to studying mechanisms by which an endogenous psychotoxin could be produced, others continued to analyze proteins in blood and CSF of schizophrenics. Heath et al. (1957) isolated a protein they called taraxein from schizophrenic serum. Intravenous injection of taraxein into monkeys and humans produced a catatonic state and schizophrenics in remission were more reactive than normal volunteers. Taraxein was described as an α- or β-globulin. After its identification as an antibrain antibody, implicating schizophrenia as an autoimmune disease, taraxein was classed as a γG immunoglobulin. Critics of the autoimmune hypothesis point to the absence of inflammation in schizophrenia which is a characteristic of autoimmune diseases. Also, it is difficult to conceive of a high molecular weight protein being able to cross the blood brain barrier. Frohman et al. (1960) found a difference between plasma of schizophrenics and normals. Their assay involved the incubation of chicken erythrocytes with human plasma and measuring the formation of lactate and pyruvate. An increased lactate to pyruvate ratio was found for schizophrenic plasma and the active factor was an α_2-globulin as determined from fractionation

on diethylaminoethyl (DEAE) cellulose. The alteration in the L/P ratio has been attributed to hemolysis of the chicken erythrocytes (Ryan et al., 1966) and has not been unambiguously demonstrated as specific for schizophrenia (Guchhait et al., 1975). Schiavone and Kaldor (1965) reported increased levels of creatine phosphokinase (CPK) in schizophrenic serum and Meltzer (1969) found elevations of CPK as well as aldolase. Investigations of blood proteins in schizophrenia have been reviewed (Weil-Malherbe and Szara, 1971).

In view of Axelrod's work demonstrating the enzymatic methylation of biogenic amines, Pollin et al. (1961) attempted to increase the concentration of methylated biogenic amines in vivo. They administered methionine which is converted to S-adenosylmethionine (SAMe), the biological methyl donor (Cantoni, 1951), to schizophrenics along with a monoamine oxidase inhibitor to prevent the oxidative destruction of any methylated amines produced. Four out of nine chronic schizophrenics experienced a severe exacerbation of symptoms. This finding has been repeated at least ten times with Antun et al. (1971) demonstrating that L-methionine is sufficient by itself to produce the exacerbation, the monoamine oxidase inhibitor being unnecessary. To date there have been no studies which refute these results. Beaton (1975) has reviewed the relationship of methylation to schizophrenia and the use

of behavioral studies in animals as an investigative approach.

In addition to the work of Pollin et al., the transmethylation hypothesis received further support when Friedhoff and Van Winkle (1962) demonstrated a "pink spot" on paper chromatograms of urine extracts which they identified as 3,4-dimethoxyphenylethylamine (DMPEA). They initially reported that fifteen out of nineteen schizophrenic patients were pink spot positive while fourteen controls were negative. This result is particularly interesting since DMPEA bears a close structural relationship to mescaline. However, dimethylation of catechols in the 3 and 4 position has not been demonstrated in vitro. Subsequent studies to determine the effect of drugs, diet, and chemical isolation technique have cast some doubt on the value of the pink spot. This topic and its relationship to the transmethylation hypothesis have been reviewed (Smythies, 1968).

More recently, evidence has emerged implicating dopamine in schizophrenia. Amphetamine-induced stereotyped behavior, which in humans is very similar to schizophrenic psychoses (Snyder et al., 1974), can be blocked in animals by making a lesion in the dopamine nigrostriatal tract with 6-hydroxydopamine (Fibiger et al., 1973). Antipsychotic drugs such as chlorpromazine will also block stereotyped behavior produced by amphetamine or its agonist, apomorphine. Since d- and l-amphetamine induce psychosis about equally

(Snyder et al., 1974), symmetrical behavior of both isomers for dopamine neuronal uptake and depletion and asymmetrical behavior for norepinephrine would implicate dopamine in the psychosis elicting mechanism. Coyle and Snyder (1969) demonstrated this behavior. Antipsychotics also tend to enhance brain dopamine turnover (Matthysse, 1974). Finally, by observing that antipsychotics increased the levels of methoxylated metabolites of dopamine in brain, Carlsson and Lindqvist (1963) suggested that antipsychotics act by blocking dopamine receptors. These findings point toward the elevation of brain dopamine concentrations in schizophrenics.

Murphy and Wyatt (1972) found a reduced activity of platelet monoamine oxidase in chronic and acute schizophrenic patients. This has been correlated in monozygotic twins, only one of whom was schizophrenic (Wyatt et al., 1973). A reduction in monoamine oxidase would allow dopamine to be overactive and is thus consistent with the dopamine hypothesis. Similar reasoning can be applied to other amines such as phenylethylamine which has properties similar to amphetamine, especially under conditions of reduced monoamine oxidase activity (Sandler and Reynolds, 1976). Wise and Stein (1973) found that brains of schizophrenic patients had a diminished activity of dopamine β-hydroxylase activity. This would also cause dopamine concentrations to increase putting the result in accord with the dopamine

hypothesis. This subject has been reviewed by Bradley and Smythies (1976).

Recognizing that antipsychotics (phenothiazines) vary in degree of producing extrapyramidal side effects, Snyder et al. (1974) speculated that the most potent anticholinergics would yield the fewest extrapyramidal effects. This is based on the reasoning that a dynamic balance between acetylcholine and dopamine exists in the brain. A similar hypothesis for interaction between dopamine and serotonin has been advanced (Green and Grahame-Smith, 1976). Disturbance of the dopamine-serotonin balance is potentially deleterious. Tryptophan, a precursor of serotonin, together with a monoamine oxidase inhibitor has been shown to exacerbate schizophrenic psychoses (Pollin et al., 1961). This could be mediated by an increase in brain serotonin which can also be affected by dietary factors (Wurtman and Fernstrom, 1974). It is well known that 3,4-dihydroxyphenylalanine (L-DOPA) treatment gives rise to psychotic symptoms (Mattysse, 1973). The serotonin/dopamine hypothesis as well as other hypotheses relevant to current psychiatric research have been reviewed by Smythies (1976).

Laduron (1974) reported an exacerbation of schizophrenia by doses of folic acid. This author postulated that an increase in N^5CH_3THF-dependent methylation of biogenic amines might produce schizophrenic disorders. An increase in transmethylation reactions is also generally assumed to

be the mechanism by which methionine loading exacerbates schizophrenic psychoses. However, excess methylation is at variance with the report of Freeman et al. (1975). These authors described a folate responsive homocystinuric patient with schizophrenic-like symptoms. This patient was found to have a deficiency in $N^5N^{10}CH_2THF$ reductase activity which would cause low tissue levels of N^5CH_3THF. Baldessarini (1975a) suggested the possibility that psychosis may be induced by mechanisms peripheral to methylation while Mudd and Freeman (1974) postulated that a decrease in N^5CH_3THF-dependent transmethylation might be responsible for schizophrenic disorders. Since then, N^5CH_3THF-dependent transmethylation has been shown to be based on a biochemical artifact (Taylor and Hanna, 1975b). Decreased levels of N^5CH_3THF, however, may lead to a reduction in SAMe-dependent transmethylation. Remethylation of homocysteine to methionine would be curtailed with a theoretical subsequent decrease in activation of methionine to SAMe. In this light, it is of interest that Andreoli and Maffei (1975) reported SAMe blood levels in acute schizophrenics to be 50 percent of the levels in controls and chronic schizophrenics Matthysse and Baldessarini (1972) also found no difference in SAMe blood levels between controls and chronic schizophrenics. These authors did not include acute schizophrenics in their study.

Methionine Loading

Effect on Tissue Methionine Levels

Van Slyke and Meyer (1913) showed that intravenously injected amino acids in the dog are rapidly removed from the circulation. They also observed that the blood amino acids appeared to be in equilibrium with those in the tissues. These findings prompted other investigators to study, particularly with regard to hepatic function, the tolerance to various individual amino acids, their metabolic fate and biological role. Among the sulfur amino acids, cysteine received most of the early attention (Toennies, 1937). Mueller (1922) observed that a compound containing sulfur, isolated from casein, but not identical to cysteine, was required for the growth of test organisms. This compound was later named methionine in allusion to its structural formula, gamma-methylthiol-alpha-aminobutyric acid. Its synthesis in 1928 paved the way for investigation of its metabolic function.

Kinsell et al. (1950) found that the incorporation of intravenously administered ^{35}S-DL-methionine into plasma proteins and its disappearance from blood occur at a predictable rate in normal individuals. However, significant deviations from the normal were shown for patients with chronic liver disease, Cushing's syndrome or hypoproteinemia. Studying the absorption after feeding of L- and DL-^{14}C-methyl

methionine from the rat alimentary tract, Edwards et al. (1963) also found incorporation of labeled methionine into plasma proteins. The contribution from L-methionine was significantly greater than from DL-methionine. In addition, significantly greater amounts of L-^{14}C-methyl methionine were taken up by brain, liver, heart and lungs as opposed to the DL-mixture. Liver accumulated the label from L- and DL-^{14}C-methyl methionine to a significantly greater extent than the label from DL-^{14}C-alpha methionine. However, alpha and methyl labeled DL methionine accumulated similarly in seventeen other tissues including brain, heart, lungs and spleen.

Daniel and Waisman (1968) studied the effect in rats of seventeen amino acids added to normal diets. L-methionine was found to be the most toxic by causing severe growth depression. A correlation was found between the toxicity of the amino acid and its concentration in the blood. Feeding a diet supplemented with 3 percent of L-methionine, Daniel and Waisman (1969) found 14-and 13-fold increases in rat liver L-methionine levels after 2 and 14 days, respectively. Brain levels of L-methionine were elevated 11-fold after 2 days and 8-fold after 14 days. Acute effects of excess methionine in rats were measured after a single intraperitoneal injection of L-methionine containing 11 mmoles/kg body weight (Daniel and Waisman, 1969). Peak values of liver and brain L-methionine were reached after 4 hours. Liver values were increased by 79-fold over

controls and brain levels were 36 times higher. Similarly, Sanchez and Swendseid (1969) force-fed rats with a normal diet supplemented with 4 percent L-methionine for 3 days. This amino acid was elevated 19-fold in liver, 59-fold in plasma and 33-fold in muscle.

Gaitonde and Richter (1955) injected L-[^{35}S] methionine intraperitoneally into rats. The pattern of uptake of ^{35}S in decreasing order was: liver, kidney, spleen, lung, heart, brain and muscle. While the uptake of ^{35}S into brain was relatively low, there was a rapid incorporation of ^{35}S from the acid-soluble into the protein fraction of brain. In this regard it is interesting to note that after intraperitoneal administration of 20 µg of L-thyroxine daily for five days, L-[^{35}S] methionine injection produced a higher concentration of radioactive sulfur in whole brain and its protein fraction (Schneck et al., 1964). Using the technique of intraperitoneal injection of [^{35}S] methionine, Merei and Gallyas (1964) extended the previous work of Fischer et al. (1956) and demonstrated differing rates of incorporation of [^{35}S] methionine into six regions of grey matter of the CNS. All white matter formed a seventh region. The uptake of [^{35}S] methionine by cells in this region was only one-half to one-fourth of that by cells of the grey matter. The steric configuration of methionine used for these studies was not specified.

Daniel and Waisman (1969) also found that parenteral injection of L-methionine altered the free amino acid pattern of liver and brain tissues. Total free amino acids of liver increased while those in brain decreased. In addition to elevated methionine levels, liver and brain levels of cystathionine and lysine were increased. However, hepatic and brain levels of aspartic acid, threonine, glutamic acid, glycine, alanine and serine were depressed. Fallon et al. (1968) had previously shown that supplementation of the diet with methionine caused hepatic levels of serine, glycine and alanine in rats to decline. This was correlated with a depression of 3-P-glycerate dehydrogenase activity which limits serine production by the phosphorylated pathway (Bridgers, 1970).

Effect on Transmethylation

Cantoni (1951) established that adenosine triphosphate (ATP) and Mg^{++} were essential for transmethylating reactions involving methionine. The "active" methionine was shown to be S-adenosylmethionine (SAMe) (Cantoni, 1953). Once formed, this compound is the methyl donor for a considerable number of transmethylation reactions. The impact of methionine loading on transmethylation therefore depends on the resultant alteration in SAMe levels. It has already been shown that methionine administration, either by diet or injection, causes an increase in blood methionine levels which results in elevated methionine

concentrations in other tissues. To learn whether such increases in tissue methionine can lead to corresponding changes in tissue SAMe concentrations has been the aim of several investigations.

Injecting 100 mg/kg of DL-methionine intraperitoneally into rats, Baldessarini (1966) found liver and brain levels of SAMe to be elevated by 342 and 30 percent, respectively, after 30 minutes. Two hours after the same dose of L-methionine, Rubin et al. (1974) reported rat brain levels of SAMe to be increased by 35 percent. Schatz and Sellinger (1975) found a 100 percent increase in rat brain SAMe levels at 3 hours after injecting 700 mg/kg L-methionine intraperitoneally. Salvatore et al. (1971) showed that rabbit brain SAMe levels were elevated by 47 percent one hour after intraperitoneal injection of methionine (100 mg/kg). Thus, there is good evidence demonstrating that increased availability of the precursor methionine enhances the rate of production of SAMe. To date, a quantitative correlation of tissue increases of SAMe with increases in tissue methionine concentration has not been reported.

There is also evidence that drugs and catechol metabolites deplete SAMe in brain and other tissues by the action of methylating enzymes like COMT (Taylor and Randall, 1975; Baldessarini, 1975b). Administering α-methylDOPA to rats, Lo et al. (1976) found a dose related decline in brain SAMe concentrations. Also a linear relationship was found between the drug dose and the

accumulated metabolites of α-methylDOPA, 3-O-methyl α-methylDOPA and 3-O-methyl α-methyldopamine. Similar results have been reported for the administration of 3,4-dihydroxyphenylalanine and its methylation to 3-O-methylDOPA (Wurtman et al., 1970). However, the more important question is whether the increased availability of the methyl donor, as a result of methionine loading, can increase the rate of methylation of catecholamines and indoleamines.

There is a paucity of information with regard to this problem. In addition, the existing data are often confused by the use of inadequate analytical procedures. In balance, the current evidence has not demonstrated an increase in methylation of biogenic amines with an increase in methyl donor (Baldessarini, 1975b). Thus, Berlet et al. (1965) administering DL-methionine together with the monoamine oxidase inhibitor, tranylcypromine, to male schizophrenic patients did not find an increase in excretion of vanillylmandelic acid (VMA). Kakimoto et al. (1967) did not find increases in metanephrine and normetanephrine in the urine of schizophrenic patients treated with methionine. Antun et al. (1971) reported no significant increase in excretion of 3-methoxy-4-hydroxyphenylglycol (MHPG) or VMA from schizophrenic patients during L-methionine treatment. Sprince et al. (1963) reported similar findings for the indoleamines. They could not detect the presence of N,N-dimethyl derivatives of tryptamine or serotonin in urine

extracts from schizophrenic patients treated with L-methionine and tranylcypromine. Thus, the O- and N-methylated metabolites of biogenic amines do not appear to be significantly elevated in response to methionine loading.

Other Metabolic Effects

Methionine provides protection against the toxicity of bromobenzene, pyridine hydrochloride and nicotinic acid poisoning in rats and the toxicity of chloroform in dogs (Sahyun, 1949). Ershoff (1948) recognized the "similarity of symptoms resulting from a dietary deficiency of methionine with that obtained on the administration of noxious substances requiring methionine for detoxification". These symptoms include massive hepatic necrosis, degeneration in the convoluted tubules of the kidneys, occasional severe chronic convulsions, cessation of growth and eventual death (Sahyun, 1949). Severe liver damage in rats due to methionine deficiency was also demonstrated by Himsworth and Glynn (1944). These pathological disorders are accompanied by fatty livers. Since methionine and choline are capable of preventing fatty infiltration of the liver, these substances are regarded as having lipotropic activity.

In light of this beneficial function of methionine, it has been administered to patients with cirrhosis. Phear *et al.* (1956) administered methionine orally to nine patients who suffered from cirrhosis of the liver and who had previous episodes of impending hepatic coma. Seven showed

neurological deterioration when methionine was given. Five of the patients who responded adversely to methionine were completely protected by chlortetracycline. The authors suggested that bacterial action on methionine leads to production of toxic substances and that methionine supplementation should be abandoned in severe liver disease. Although no definite relationship has been shown between fatty infiltration of the liver and atherosclerosis, rats fed high-fat diets to produce lesions of atherosclerosis also develop fatty livers (Renaud, 1966). This author reported that supplementing a high-fat, low protein diet with methionine markedly prevented the atherosclerotic lesions and the hypercholesteremia induced by such a diet without methionine. However, the bulk of the evidence indicates that a disproportionate amount of methionine in a normal diet has deleterious effects.

Voluntary food intake and growth rate are depressed in young rats receiving excess methionine. Such dietary feeding also produces anatomical alterations in the pancreas, spleen, liver, kidney and small intestine of rats (Klavins and Peacocke, 1964). Hardin and Hove (1951) found that glycine plus arginine in the presence of vitamin E, folacin, and vitamin B_{12} prevented the toxicity of methionine. Arginine and glycine are precursors of guanidinoacetic acid. It was thought that guanidinoacetic acid facilitated methionine degradation by accepting the methyl group from SAMe forming creatine. Benevenga and Harper (1967) reported alleviation,

but not prevention, of methionine induced growth depression in rats by glycine and serine. Glycine was more effective than serine. A metabolic adaptation to high methionine intake was noted in that glycine and serine exerted beneficial effects only after the rats had been eating the diets for several days. Girard-Globa et al. (1972) presented results showing that serine facilitates methionine elimination by forming taurine. In addition, these authors found serine-threonine dehydrase to be stimulated by methionine resulting in a secondary deficiency of threonine. Kimura et al. (1975) also found threonine levels to be reduced below detectable quantities in rat liver after the addition of methionine or cysteine to a protein-free diet. While hepatic fat accumulation did occur under these conditions, it was not eliminated with the addition of threonine to the protein-free, cysteine-supplemented diet.

Hardwick et al. (1970) administered 10 mmole/kg of L-methionine orally to guinea pigs. They reported a condition of fatty liver, hypoglycemia and aminoacidemia followed by hypothermia, profound hypoglycemia and death within 60 hours. These effects were claimed to be mediated by a hepatic ATP deficiency due to its reaction with methionine to form SAMe. The hypoglycemic effects of methionine intoxication could be reversed by the administration of adenine. Hepatic and pancreatic ATP concentrations fell significantly four hours after methionine administration. Choitz and Kurrie (1968) fed an

18 percent casein diet to adult rats supplemented with three percent DL-methionine for periods of two and five weeks. At both time intervals hepatic ATP in the groups fed excess methionine was 20 percent below controls. Intraperitoneal injection of methionine into guinea pigs was also found to fragment hepatic nucleoli (Shinozuka et al., 1971). Administration of adenine 4 hours after the methionine injection reversed the nucleolar lesions. No nucleolar lesions were found after injecting methionine into rats, indicating species differences. However, rat pancreatic acinar cells did develop polyploidy after long term feeding of 2 percent methionine in an 18 percent casein diet (Bourdel et al., 1971). Ploidies of 32 N and higher were observed.

Other general effects of methionine administration include the preferential formation of indoleacetic acid from tryptophan by stimulating the indoleacetic acid pathway and/or inhibiting the nicotinic acid pathway (Sprince, 1970). Oral methionine has been demonstrated to be effective in reducing the severity of liver damage induced by acetaminophen overdose (Crome et al., 1976). L-methionine loading has been reported to be of use in detecting heterozygosity (Fowler et al., 1971) and homozygosity (Hammuri and Scheibenreiter, 1974) for homocystinuria. Plasma amino acid analyses in these tests revealed significant differences in methionine concentration. Ohmori (1975) found homocysteine sulfinic acid in the urine and liver of vitamin B_6 deficient rats

treated orally or intraperitoneally with methionine. This is of particular interest in that the oxidation of homocysteine sulfinic acid yields homocysteic acid. The latter compound has been implicated in the role of precursor of the esterified sulfate of proteoglycans. Enhanced synthesis of these macromolecules by medial and intimal cells can lead to their abnormal deposition in artery walls (McCully, 1971; McCully, 1972). In addition, a number of behavioral studies on rats has shown disruptions due to methionine. Based on an operant conditioning schedule, L-methionine administered subcutaneously produced a reduction in efficient responding (Beaton et al., 1974). Using a technique for time sampling activity, Taylor (1976) reported 100 or 150 mg/kg of L-methionine produced a significant reduction in active behavior which included rearing and walking. In contrast, intravenously administered methionine was found to stimulate spontaneous and locomotor activity (Roussinov et al., 1975).

Relationship to Schizophrenia

Methionine loading experiments involving schizophrenics have been done largely on the basis of the transmethylation hypothesis. Pollin et al. (1961) found that 20 g/70 kg daily of L-methionine together with iproniazid, a monoamine oxidase inhibitor, caused an exacerbation of psychotic symptoms in four out of nine schizophrenic patients. Cohen et al. (1974) reviewed ten studies in which methionine and monoamine oxidase inhibitors were administered to a total

of 107 chronic schizophrenic patients. Of these, 58 percent experienced a "functional psychosis" and 33 percent showed evidence of a functional psychosis without symptoms of organic brain syndrome. These results, together with the previously discussed increases in SAMe in response to methionine loading, provide considerable support for the transmethylation hypothesis. In addition, Israelstam et al. (1970) found significant differences in the oxidative metabolism of the methyl carbon of methionine between acute schizophrenics and normals or schizophrenics in remission. Homocystinuria is often accompanied by hypermethionemia due to absence of cystathionine synthase activity (Gaull et al., 1969). Schizophrenia has been associated with this disorder (Spiro et al., 1965) although the occurrence of psychosis in homocystinuria is only occasional (Freeman et al., 1975). Interestingly, Schatz and Sellinger (1974) demonstrated that the convulsant, L-methionine-D,L-sulphoximine, produced a 35 percent decrease in rat brain SAMe levels in contradistinction to the increase produced by methionine. Heath et al. (1966) reported a diminution of psychotic symptoms in chronic schizophrenics after oral administration of D,L-methionine-D,L-sulphoximine. Despite these findings implicating abnormal methyl metabolism in schizophrenia, definitive proof as to the mechanism producing the functional psychosis after methionine loading remains to be established. Also, the small number of nonschizophrenics studied does not allow a conclusion to be reached as to the

specificity of the reaction to schizophrenia.

Concluding that an increase in the methylation of biogenic amines has not been adequately demonstrated in response to an increase in methyl donor, SAMe, Baldessarini (1975b) suggested that such an increase might occur if the ratio of SAMe:S-adenosylhomocysteine were altered. This is based on the competitive inhibitory action of S-adenosyl-homocysteine on methylating enzymes such as phenylethanol-amine N-methyltransferase and catechol-0-methyltransferase (Deguchi and Barchas, 1971) and indolethylamine-N-methyltransferase (Lin et al., 1973). Another explanation to account for enhanced methylation of biogenic amines stems from the work of Spaide et al. (1967). These investigators demonstrated that L-cysteine loading, plus administration of tranylcypromine, produced behavioral worsening in schizophrenics similar to the effect obtained with L-methionine. This led Spaide et al. (1968) to postulate that free thiol groups from cysteine stimulated an N-methyltransferase to produce psychotomimetic N-methylated indoleamines. Urinary tryptamine levels also increased in response to the cysteine load.

Alternatives to the transmethylation hypothesis to explain the effect of methionine loading have also been proposed. The report by Freeman et al. (1975) on a folate responsive homocystinuric with schizophrenic-like symptoms argues against an increase in methylation as the causative factor in schizophrenia. This patient had a deficiency in

N^5,N^{10}-methylenetetrahydrofolate ($N^5N^{10}CH_2THF$) reductase activity without hypermethioninemia. This led Levi and Waxman (1975) to postulate a diminished methylating capacity, brought about by dietary methionine restriction, altered folate metabolism or a deficient methionine adenosyl-transferase, as the condition necessary to provoke schizophrenic symptoms. Mudd and Freeman (1974) mentioned the possibility that the psychotogenic effect of methionine may be related to the ability of methionine to prevent accumulation of N^5-methyltetrahydrofolate (N^5CH_3THF). This point is pursued further in the folate section of this review.

There is evidence to suggest that toxic metabolites of methionine might be produced by bacterial action (Phear et al., 1956). Homocysteine, the demethylated metabolite of methionine, and its thiolactone have been shown to have convulsant activity and are fatal at doses which rats can tolerate related metabolites of the methionine-cysteine pathway (Sprince, 1970). Toxicity to methionine itself has been demonstrated in rats by correlating excess methionine with growth depression. Explanations by Daniel and Waisman (1968) to account for this effect may also have bearing on schizophrenia. These authors theorized that a disruption of the normal balance of free amino acid pools due to excess methionine may prevent normal protein or macromolecule synthesis. They later gained support for their contention by quantitating the alteration in free amino

acid levels in brain as a result of methionine administration (Daniel and Waisman, 1969). The net effect of methionine was to decrease the total free amino acids of brain. More recently, Tudball and Griffiths (1976) have also shown a decrease in the concentration of free amino acids of rabbit brain in response to high plasma homocysteine or high plasma homocysteine and methionine. This condition was shown to have an inhibitory effect on cerebral protein synthesis. Such alterations in brain free amino acids are not surprising since amino acids of similar structure compete for entry into brain from the blood by a common transport system (Blasberg and Lajtha, 1966). Methionine competes with the other large neutral amino acids, phenylalanine, tyrosine, tryptophan, leucine, isoleucine and valine. Tudball and Griffiths (1976) also found that elevated plasma levels of homocysteine and methionine cause dramatic declines in DOPA, dopamine and norepineprine. No difference between the activities of tyrosine hydroxylase, DOPA decarboxylase or dopamine β-hydroxylase was observed for normal and treated rabbits.

Apart from the effects of altered brain amino acid levels by excess methionine, side chains of amino acids in proteins can be methylated. The biological significance of histone methylation and methylation of other proteins is not clear. However, by maintaining differential concentrations of ions between cellular structures, methylation of proteins may be involved in regulating brain electrical activity

(Paik and Kim, 1969). Similar phenomena to those discussed above could be operational in schizophrenics under the influence of methionine loading.

Methionine, Methylation and Folate Metabolism

The One Carbon Pathway

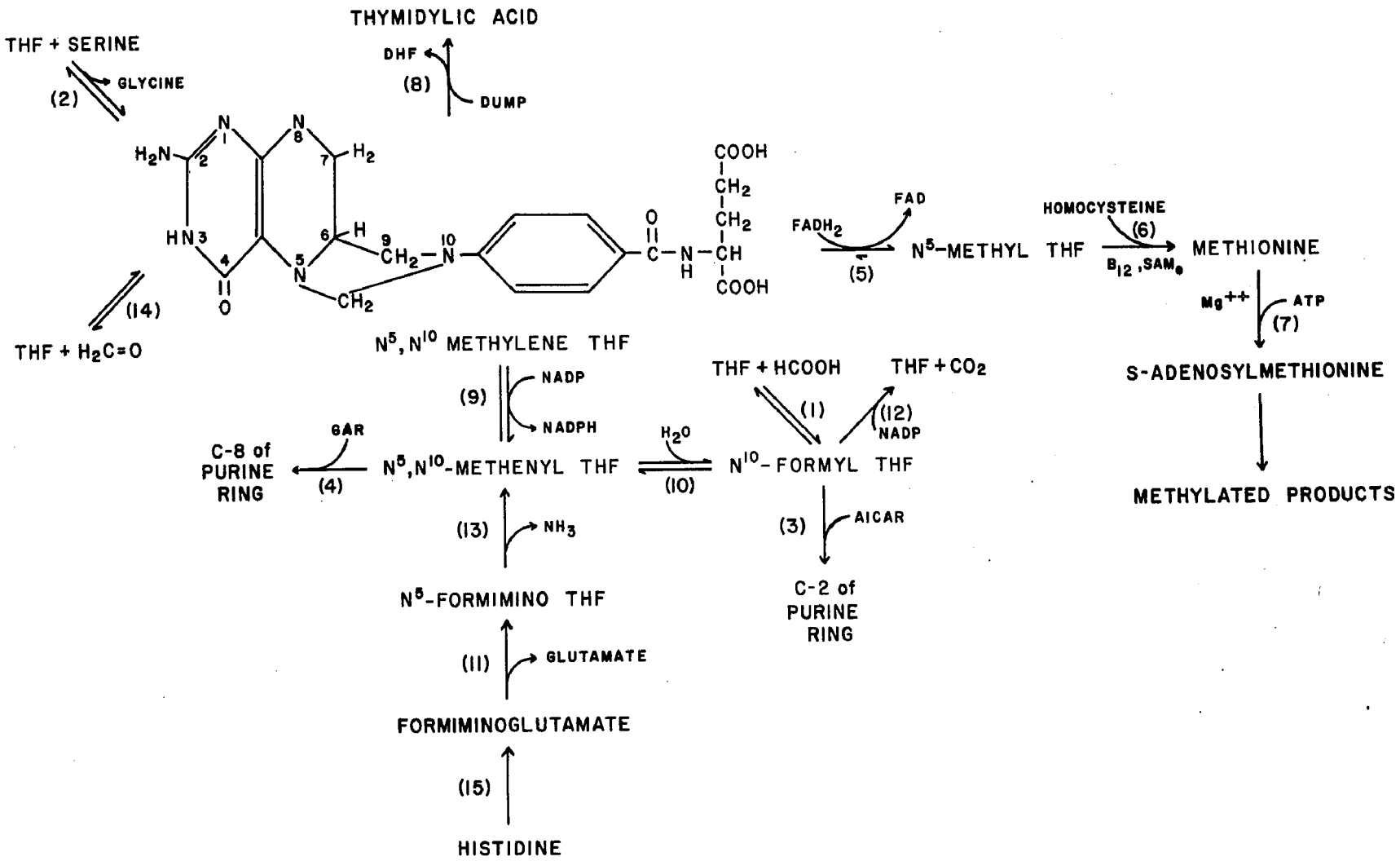
The bicyclic nitrogenous ring system named pteridine was first found to be common to pigments isolated from butterfly wings. Later, another pteridine, folic acid, was demonstrated to be an essential nutrient in primates, chickens and bacteria (Blakely, 1969b). Biologically active folates are derived from tetrahydrofolic acid (THF) which differs from folic acid by being reduced at positions five to eight of the pteridine ring. Figure 1 shows the approved numbering system for pteridines. In addition to the pteridine moiety THF consists of a central p-aminobenzoate group and from one to nine glutamyl residues in gamma-carboxyl linkage. Folates having more than one glutamyl residue are referred to as polyglutamates. The one carbon pool includes THF derivatives bearing substituents at the N^5 or N^{10} position or in a bridge between these positions. These substituents are methyl, methylene, methenyl, formyl, and formimino.

Of considerable functional importance in neuronal metabolism, cerebrospinal fluid (CSF) folate levels are at least three times higher than those in serum (Herbert, 1964). Noronha and Aboobaker (1963) showed that the main derivative of folate in human blood is N^5-methyltetrahydrofolic acid

Figure 1. Pathway of Folate Metabolism

THF represents 5,6,7,8-tetrahydrofolate, DHF dihydrofolate, dUPM deoxyuridine 5' phosphate, NADP nicotinamide adenine dinucleotide phosphate, NADPH reduced NADP, B_{12} methylcobalamin, AICAR 5'-phosphoribosyl-5-aminoimidazole-4-carboxamide, GAR 5'-phosphoribosylglycinamide.

Of the enzymes (1) represents 10-formyl THF synthetase, (2) serine hydroxymethyltransferase, (3) AICAR transformylase, (4) GAR transformylase, (5) N^5,N^{10}-methylene THF reductase, (6) N^5-methyl THF:homocysteine transmethylase, (7) methionine adenosyltransferase, (8) dihydrofolate reductase, (9) N^5,N^{10}-methylene THF dehydrogenase, (10) N^5,N^{10}-methenyl THF cyclohydrolase, (11) formiminoglutamic acid:THF formiminotransferase (12) N^{10}-formyl THF dehydrogenase and (13) N^5 formimino THF cyclodeaminase.


THYMIDYLIC ACID
THF + SERINE
GLYCINE
(2)
DHF
(8)
DUMP
FAD
FADH2
(5)
N5-METHYL THF
HOMOCYSTEINE
(6)
B12 ,SAMe
METHIONINE
Mg++
ATP
(7)
S-ADENOSYLMETHIONINE
METHYLATED PRODUCTS
(14)
THF + H2C=O
N5, N10 METHYLENE THF
(9)
NADP
NADPH
THF + HCOOH
THF + CO2
(1)
(12)
NADP
GAR
C-8 of PURINE RING
(4)
N5,N10-METHENYL THF
H2O
(10)
N10- FORMYL THF
(3)
AICAR
C-2 of PURINE RING
(13)
NH3
N5-FORMIMINO THF
(11)
GLUTAMATE
FORMIMINOGLUTAMATE
(15)
HISTIDINE

(N^5CH_3THF). Approximately 10 percent of the total folate in blood is in plasma, the remainder being associated with the cellular components. Plasma N^5CH_3THF is mainly in the monoglutamate form while that present in the cells is largely polyglutamate. In rats, plasma folate is also in the N^5CH_3THF monoglutamate form while findings of 90-95 percent N^5CH_3THF polyglutamate, mainly penta- and hexaglutamate, in red blood cells have been reported (Bird et al., 1965; Shin et al., 1974). Of the total folate in rat liver, Bird et al. (1965) reported 67 percent to be N^5CH_3THF and 75 percent to be polyglutamates. Shin et al. (1972) found 85-90 percent of rat liver folates to be reduced forms of pentaglutamates, predominantly N^5CH_3THF. In rat brain, at least 75 percent of the endogenous folates are polyglutamates. However, N^5CH_3THF constitutes only 18 percent of the total with THF (60%) being the main form (Brody et al., 1976). Although the folate coenzymes of rat liver are over 90 percent cytoplasmic (Wang et al., 1967), in Swiss mouse brain they are about evenly divided between the soluble and particulate fractions with about 40 percent in the N^5-methyl form in the soluble fraction (McClain et al., 1975). Further differences in folate content occur in cell organelles. Wang et al. (1967) found not more than 30 percent of rat liver mitochondrial extracts to be represented by methylated polyglutamate forms of THF. The major constituent was found to be N^{10}-formyltetrahydrofolic acid ($N^{10}CHOTHF$). McClain et al. (1975) found

mitochondria in Swiss mouse brain to contain the N^5CH_3THF polyglutamate derivatives almost exclusively. It is interesting to note that the highest levels of N^5CH_3THF in mammalian brain have been reported to occur in regions rich in endogenous indoleamines (Korevaar et al., 1973).

Functionally, folate derivatives are utilized (1) as a source of carbons 2 and 8 in purine biosynthesis; (2) in the formation of thymidine-5'-phosphate (dTMP); (3) in the synthesis of the methyl group of methionine which undergoes activation to the biological methyl donor S-adenosyl-methionine (SAMe). In addition, reduced folate derivatives are the source of the one-carbon cofactor required to form N-formyl-methionine, the starter of protein biosynthesis (Clark and Marcker, 1965). Some polyglutamate chain lengths have been shown to have the same specificity for folate enzymes as the monoglutamate form (Cheng et al., 1975), whereas a difference in reactivity with chain length has also been demonstrated (Guest et al., 1964). Polyglutamates have been suggested to promote cellular folate retention (McBurney and Whitmore, 1974).

It is well established that anticonvulsant therapy may lead to megaloblastic anemia. This is considered to be the result of folate deficiency which also develops into folate-responsive schizophrenic-like syndromes in some epileptics (Reynolds et al., 1966). While it was formerly believed that vitamin B_{12} deficiency and not folate deficiency results in neurological disorders (Blakely,

1969a), recent evidence indicates that the latter can also lead to neurological disease (Reynolds et al., 1973). Furthermore, there are reports of neurological disorders responsive to folic acid therapy (Botez et al., 1976). Folic acid deficiency induced in the neonatal period in rats causes a delay in maturation of the electrical activity of the brain (Arakawa et al., 1969). That folate-dependent one-carbon metabolism participates in early postnatal brain development is evidenced by alterations in folate-dependent enzyme activity in new born mouse and rat brain (Bridgers, 1968; Ordonez and Villarroel, 1976). The importance of folic acid to the development of the nervous system is exemplified by the discovery of inborn errors of folate metabolism resulting in malabsorption of folate (Lanzkowsky, 1970) or specific folate enzyme deficiency such as formiminoglutamic acid:THF formimino-transferase (Arakawa, 1970). Patients with these disorders have been reported to be mentally retarded, ranging from mild to severe, with or without other neurological abnormalities.

As shown in Figure 1, the one-carbon bearing derivatives of THF can arise by reaction of free formic acid with THF to form N^{10}CHOTHF (Reaction 1) or the non-enzymatic reaction of free formaldehyde with THF (Reaction 14) to form N^5,N^{10}-methylenetetrahydrofolic acid ($N^5N^{10}CH_2$THF). Formiminoglutamate (FIGLU), a degradation product of histidine (Reaction 15), can react with THF (Reaction 11) to

form N^5-formiminotetrahydrofolic acid. However, the most important input to this pool occurs through transfer of the β-carbon of serine to THF (Reaction 2) to form $N^5N^{10}CH_2THF$ (Mudd and Cantoni, 1964). Reactions of lesser biological and quantitative importance than those mentioned can also contribute one-carbon units to the pool of reduced folates. These one-carbon derivatives can then act as donors of one-carbon units in essentially irreversible reactions (Reactions 3, 4, 6 and 8), thus constituting the drain on these units from the pool. The complexity of these relationships is increased by the enzymatic interconversion of the one-carbon derivatives as illustrated by reactions 9 and 10.

De Novo Synthesis of the Methionine Methyl Group and its Activation

The ability to synthesize labile methyl groups was first recognized by du Vigneaud et al. (1939). They found that homocysteine in place of methionine in the diet would permit rat growth provided a source of N-methyl compounds such as betaine or choline was also present. Bennett et al., (1951) then demonstrated that rats which had ceased to grow on a "labile methyl"-free diet containing homocysteine would resume growth if both folic acid and vitamin B_{12} were added simultaneously to the diet. Arnstein and Neuberger (1953) showed that rats fed diets devoid of preformed methyl groups but containing 3-^{14}C serine and vitamin B_{12} incorporated 70 percent of the radioactivity of the β-carbon

of serine into the methyl group of visceral methionine. Thus, the connections among methionine synthesis, folic acid, and vitamin B_{12} were discerned.

Using cell-free systems, Berg (1953) demonstrated the incorporation of labeled formate in extracts of pigeon liver into the methyl group of methionine in the presence of homocysteine. Cross and Woods (1954) showed the de novo synthesis of methionine in extracts derived from Escherichia coli incubated with serine and homocysteine. Working with a mutant strain of E. coli requiring either methionine or cyanocobalamin (B_{12}) for growth, Helleiner et al. (1958) provided a simplification of the requirements for methionine methyl synthesis. They found N^5,N^{10}-methylene THF would replace other precursors of methionine methyl in the presence of homocysteine, ATP, NADH, and low concentrations of cyanocobalamin.

At about the same time, Donaldson and Keresztesy (1959) isolated an unknown THF derivative, which they named prefolic A, from unautolyzed horse liver. In the presence of a suitable electron acceptor (menadione was later shown to provide the best results (Donaldson and Keresztesy, 1962)), prefolic A was converted to THF by an FAD-linked enzyme system. The THF was subsequently formylated to N^5-formyl THF (citrovorum factor). Hatch et al. (1961) separated the methionine-methyl synthesizing system into three partially purified enzyme fractions. One of these was serine hydroxymethyltransferase (Reaction 2, Figure 1) and

another was found to have vitamin B_{12} as a prosthetic group. Absolute requirements for methionine biosynthesis were demonstrated for serine, homocysteine, pyridoxal-phosphate, THF, NADH, FAD and ATP. Larrabee and Buchanan (1961) then reported the accumulation of a methyl folate derivative when an enzyme preparation from E. coli was incubated with $N^5N^{10}CH_2THF$ and a source of reducing power. They identified the methyl derivative as N^5CH_3THF. Subsequent purification of prefolic A by Donaldson and Keresztesy (1962) led to the identification of this folate derivative as N^5CH_3THF. Finally, Sakami and Ukstins (1961) synthesized N^5CH_3THF chemically and found the derivative to be utilized as a methyl donor to form methionine in pig liver.

The evidence then indicates that the reductive reaction in the sequence that leads to the methionine methyl is as follows (Reaction 5, figure 1):

$$N^5,N^{10}CH_2THF + FADH_2 \longrightarrow N^5CH_3THF + FAD \quad (1)$$

The enzyme effecting the reduction is referred to as N^5,N^{10}-methylene THF reductase (5-methyltetrahydrofolate-NADP oxidoreductase, EC 1.1.1.68) and it is present in microorganisms and animals. Katzen and Buchanan (1965), working with an enzyme purified from E. coli, reported an equilibrium constant for the reaction as written of approximately 2000 to 3000. Wang et al. (1967) demonstrated $N^5N^{10}CH_2THF$ reductase activity to be present in the cytoplasm of rat liver, but not in the mitochondria. Confirming this result for rat liver, Burton and Sallach

(1975) additionally reported the enzyme to be localized in the soluble fraction of rat brain with less than three percent in the mitochondria.

The final step in methionine biosynthesis is the transfer of the methyl group of N^5CH_3THF to the acceptor, homocysteine. Sakami and Ukstins (1961) first demonstrated the reaction in pig liver using purified methyl-THF prepared by the reduction of $N^5N^{10}CH_2THF$ with potassium borohydride. Working with a purified B_{12}-enzyme derived from E. coli, Larrabee et al. (1963) determined that reduced folate in the form of N^5CH_3THF would methylate homocysteine in the presence of ATP, Mg^{++}, FAD and NADH. Weissbach et al. (1963) compared the biosynthesis of methionine in cell-free systems from animal liver and E. coli using N^5-$^{14}CH_3THF$. They demonstrated the direct transfer of the methyl group to homocysteine in all systems examined.

Studies on the enzymatic synthesis of the methyl group of methionine in microorganisms (Guest et al., 1962a; Guest et al., 1964) revealed the existence of two separate systems for methylating homocysteine. One of these systems requires only Mg^{++} as a cofactor, but uses only the triglutamate folate form as substrate.

$$N^5CH_3THF \text{ triglutamate} + \text{homocysteine} \xrightarrow{Mg^{++}} \text{THF triglutamate} + \text{methionine} \quad (2)$$

The second system requires vitamin B_{12}, a reducing system and SAMe. It utilizes either the mono- or triglutamate

forms of N^5CH_3THF:

N^5CH_3THF mono- or triglutamate + homocysteine

$$\xrightarrow[FADH_2]{SAMe \quad B_{12}} \qquad (3)$$

THF mono- or triglutamate + methionine

Loughlin et al. (1964) isolated this methyltransferase from pig liver and demonstrated the similarity in respect to substrate specificity and requirements for cofactors to the B_{12} requiring system of E. coli (Equation 3). The enzyme (Reaction 6, Figure 1) was named N^5CH_3THF:homocysteine transmethylase (5-methyltetrahydropteroyl-L-glutamate:L-homocysteine 5-methyltransferase, EC 2.1.1.13). The enzyme has been found to be localized in the soluble fraction of rat liver and brain (Burton and Sallach, 1975; Rassin and Gaull, 1975). The enzyme is widely distributed in proliferating normal and cancerous tissues. Peytremann et al. (1975) found the specific activity of the enzyme to be significantly higher in chronic leukemic lymphocytes than in normal lymphocytes or normal granulocytes.

Weissbach et al. (1963) observed that the N^5CH_3THF: homocysteine transmethylase was stimulated aerobically by cyano-B_{12} and related cobamide compounds. This was attributed to the Peel effect, the ability of B_{12} derivatives to remove oxygen and maintain a reducing atmosphere for the enzyme during incubation (Peel, 1962). However, Guest et al. (1962b) had shown that in the absence of a reducing system, methylcobalamin was the most effective analogue of

vitamin B_{12} promoting *in vitro* synthesis of methionine. This analogue consists of a methyl group linked to cobalt in place of the 5'-deoxyadenosine group. Thus, a key role for the methyl-B_{12} prosthetic group was implicated for the transmethylation reaction.

Current data indicate that the function of $FADH_2$ is to reduce the trivalent cobalt to its divalent form. Divalent cobalt then distributes between the trivalent and monovalent states. Strong methyl donors such as SAMe can trap the monovalent cobalt as methylcobalamin. The methylcobalamin enzyme then donates the methyl group from cobalamin to homocysteine. After the initial "priming" with SAMe, the regenerated monovalent cobalt can then accept a methyl group from the weaker donor N^5CH_3THF. The mechanism of methylation of the thiol group of homocysteine has been thoroughly reviewed by Rüdiger and Jaenicke (1973).

In addition to the role of SAMe in the transmethylation of homocysteine to methionine, this compound transfers its methyl group to a variety of methyl acceptors. Thus, it is referred to as the biological methyl donor (Mudd and Cantoni, 1964). Another function of SAMe following decarboxylation is the donation of its propylamine side chain to putrescine in the biosynthesis of spermidine (Lombardini and Talalay, 1971).

The enzymatic synthesis of SAMe has been investigated by Cantoni and Durrell (1957) using a partially purified enzyme derived from rabbit liver. In addition to methionine,

ATP and Mg^{++} were found to be essential. ATP serves as donor of the adenosine moiety in SAMe and also as the energy source for the reaction. Similar results were later found by Mudd and Cantoni (1958) for an enzyme derived from baker's yeast. The reaction is as follows:

$$\text{L-methionine} + \text{ATP} \xrightarrow[Mg^{++}]{} \text{SAMe} + \text{PPi} + \text{Pi} \quad (4)$$

The enzyme catalyzing the reaction (Reaction 7, Figure 1) is named methionine adenosyltransferase (ATP:L-methionine S-adenosyltransferase, EC 2.5.1.6). The mechanism of the reaction is unusual in that it involves a complete dephosphorylation of ATP. The innermost two phosphate groups of ATP are released as pyrophosphate and the terminal group appears as inorganic phosphate. There is evidence that tripolyphosphate (PPPi) is an obligatory intermediate in the reaction and the enzyme possesses tripolyphosphatase activity (Mudd, 1962). PPPi is a powerful inhibitor of the enzyme and it is competitive with respect to ATP at constant methionine concentration (Greene, 1969). Chou and Talalay (1972) have shown that the enzyme is under biphasic regulatory control with SAMe displaying stimulatory and inhibitory effects at low and high concentrations, respectively.

The methionine activating enzymes are widely distributed in nature (Tabor and Tabor, 1974; Cohn et al., 1972). Rassin and Gaull (1975) demonstrated the enzyme to be almost exclusively in the soluble fractions of rat liver and brain. In a survey of the enzyme activity in various

mammalian tissues, Mudd et al. (1965) found in the rat that the highest activity was in liver with the brain exhibiting approximately one-tenth of the liver activity (rat liver, 34 nmoles/mg prot/hr; rat brain, 3.3 nmoles/mg prot/hr). Interestingly, Sternowsky et al. (1976) found the methionine adenosyltransferase activity to increase in fetal lung of the human and monkey coincident with the time each acquires the capacity to survive. This leads to the attractive speculation that the enzyme may play a key role in the synthesis of phosphatidyl choline. This phospholipid is regarded as being of importance by some investigators in allowing survival after premature birth (Gluck et al., 1972).

Inborn errors of metabolism have been associated with each of the three enzymes discussed. Of three patients reported to have a deficiency in $N^5N^{10}CH_2THF$ reductase, all manifested homocystinuria, some degree of neurologic abnormality and low serum folate. One patient described by Freeman et al. (1975) manifested schizophrenic delusions and other psychotic symptoms which cleared while she was treated with oral folic acid and pyridoxine. Arakawa et al. (1967) described a Japanese child with mental retardation, megaloblastic anemia and elevated serum folate, but without homocystinuria. The hepatic N^5CH_3THF:homocysteine transmethylase activity was 36 percent of the control value and was not correctable with vitamin B_{12} administration. Mudd et al. (1970) described a patient with vitamin

B_{12} deficiency resulting in low N^5CH_3THF:homocysteine transmethylase activity. This patient was homocystinuric and homocystinemic. Gaull and Tallan (1974) described an infant with low methionine adenosyltransferase activity. Although this patient had hypermethioninemia, the physical and mental development were apparently normal. The enzymatic abnormality was confirmed by Finklestein et al. (1975a).

Regulation of One-Carbon Metabolism

Much that has been learned concerning regulation of folate metabolism has come from biochemical observations of symptoms which accompany a deficiency of either folate or vitamin B_{12}. These include elevated urinary excretion of formiminoglutamic acid (FIGLU), aminoimidazolecarboxamide (AICA) and formate. In cobalamin and folate deficiency, FIGLU is excreted in excess in the urine after histidine loading. The high urinary excretions are restored to normal by methionine supplementation. The accumulation of N^5CH_3THF in vitamin B_{12} deficiency was observed by Herbert and Zalusky (1961) in humans and by Noronha and Silverman (1962) in rats. This accumulation is also restored to normal by administration of methionine. To account for these observations, the controversial "methyl trap" hypothesis was advanced (Noronha and Silverman, 1962). This theory holds that in cyanocobalamin deficiency, the activity of N^5CH_3THF:homocysteine transmethylase is greatly diminished. Thus, N^5CH_3THF accumulates in body tissues at the

expense of other folate derivatives which produces a megaloblastic transition (Blakely, 1969a). A contrary view was given by Tisman et al. (1975).

Buehring et al. (1972) and Shin et al. (1975) found in perfused rat liver that methionine caused a redistribution of THF derivatives by increasing the proportions of N^{10}CHOTHF and free THF relative to N^5CH_3THF. The explanation for this redistribution is the conversion of methionine to SAMe which acts as a potent inhibitor of the $N^5N^{10}CH_2$THF reductase (Kutzbach and Stokstad, 1967, Kutzbach and Stokstad, 1971a). This effect of SAMe is partially reversed by S-adenosylhomocysteine (SAH). Thus, methionine supplementation decreases N^5CH_3THF levels. The resultant increase in other folate derivatives allows the metabolism of FIGLU, AICA and formate so that their levels decline. This effect of methionine, however, does not hold true for all tissues (Shin et al., 1975).

The "methyl trap" theory also provides an explanation for the observed decrease in hepatic folate levels which accompany vitamin B_{12} deficiency. Since the "methyl trap" causes an effective decrease in free THF, the synthesis of polyglutamates would be restricted. Polyglutamates can be formed from THF, but not from other reduced coenzymes of folate or folic acid itself (Shin et al., 1975).
It has been suggested that polyglutamates provide a cellular means of retaining folate (McBurney and Whitmore, 1974). Therefore, a defective conversion of reduced folates to

polyglutamate forms is potentially responsible for the low hepatic folate levels seen in vitamin B_{12} deficiency. Methionine administration, however, functions to reverse the decreased hepatic folate levels. For this effect of methionine to support the theory of Shin et al. (1975), tissue levels of THF should become elevated in response to methionine. In this regard, Krebs et al. (1976), working with isolated perfused rat liver and suspensions of isolated rat hepatocytes, found that methionine enhanced the conversion of formate to CO_2. This finding indicates that the major contribution to THF formation in the presence of methionine is by reaction 12 (Figure 1) catalyzed by N^{10}-formyltetrahydrofolate dehydrogenase. The activity of this enzyme is enhanced by virtue of the higher substrate (N^{10}CHOTHF) levels due to SAMe inhibition of the $N^5N^{10}CH_2$THF reductase. In the presence of NADP, no formation of formate from N^{10}CHOTHF was found in liver extracts (Kutzbach and Stokstad, 1971b).

Folate metabolism may be regulated by the uptake of folate by cells and by the formation of polyglutamates from monoglutamates. In addition, different folate enzymes are regulated by folate derivatives themselves and other important intermediates in the pathway. Studies have been conducted on enzymes derived from mammalian tissue, cells grown in culture and bacterial extracts. In much of this work, a clear distinction has not been made as to the nature of apparent changes in enzyme activity. Thus, induction

or repression of enzyme activity is sometimes discussed but proof of those specific changes is lacking (Katzen and Buchanan, 1965). The regulation of folate-dependent enzymes has been reviewed by Silber and Mansouri (1971) with particular emphasis on the repression and induction of enzyme activity in bacterial systems. Rosenblatt and Erbe (1973) found a reciprocal pattern of folate enzyme activity for human diploid skin fibroblasts in cell culture. During the log phase of growth, enzymes involved in nucleotide synthesis increase 2-20 fold. Activities of $N^5N^{10}CH_2THF$ reductase and N^5CH_3THF:homocysteine transmethylase decrease 3-4 fold during this period. Working with cultured Chinese hamster ovary cells, Taylor and Hanna (1975a) found that high concentrations of either folate or methionine in the medium elevate the B_{12} holoenzyme. This increase was inhibited by cycloheximide. Cells in culture have also been noted to follow a daily pattern in alteration of folate-dependent enzyme activity (Barbiroli et al., 1975).

Folate regulation is affected differently in various tissues of the same species as well as at different ages of the same tissue (Gaull et al., 1973). For example, brain tissue of mammalian species lacks dihydrofolate reductase activity (Makulu et al., 1973). McClain et al. (1975) could not detect formiminoglutamic acid:THF formiminotransferase in Swiss mouse brain. Rowe and Lewis (1973) demonstrated that dihydrofolate reductase,

$N^5N^{10}CH_2THF$ dehydrogenase, and $N^5N^{10}CH_2THF$ cyclohydrolase from bovine liver are inhibited by various folate derivatives. The cyclohydrolase is particularly susceptible to inhibition by $N^5N^{10}CH_2THF$. Schirch and Ropp (1967) demonstrated that serine hydroxymethyltransferase from rabbit liver is competitively inhibited by N^5CH_3THF and $N^5CHOTHF$. More recently, positive cooperativity with THF for this enzyme has been demonstrated in pig kidney and mouse kidney and liver (Kumar et al., 1976). Interestingly, the enzyme from kidney and liver of mice inoculated with L1210 tumor cells did not exhibit the cooperative interactions.

Tabor and Wyngarden (1959) have shown that formimino-tetrahydrofolate cyclodeaminase from pig liver is inhibited by THF. THF also inhibits the $N^{10}CHOTHF$ dehydrogenase (Kutzbach and Stokstad, 1971b). Methionine supplementation has been reported to decrease the activity of N^5CH_3THF: homocysteine transmethylase in chicken liver (Dickerman et al., 1964) and rat liver (Kutzbach et al., 1967). The transmethylase in both studies was significantly lower in activity under dietary conditions of vitamin B_{12} deficiency.

The methionine adenosyltransferase is regulated by a complex mechanism as alluded to previously (Chou and Talalay, 1972). Finklestein et al. (1971a) have demonstrated negative cooperativity for this enzyme from the liver of a patient with hypermethioninemia. In contrast, Lombardini

et al. (1973) showed that the concentration-activity curve for methionine is indicative of positive cooperativity in the low substrate range of 7-175 μM. Tallan and Cohen (1976) have also obtained results which support the existence of positive cooperativity. Cycloleucine which suppresses the growth of a number of tumors and microorganisms was found to inhibit this enzyme in competition with L-methionine (Lombardini and Talalay, 1973). The inhibition was demonstrated in normal rat tissues and in transplantable neoplasms.

A result of SAMe-dependent methylation is the production of homocysteine. This apparently toxic metabolite is central to folate metabolism, methylation and the transsulfuration pathway. Homocysteine can be either methylated to form methionine by the N^5CH_3THF:homocysteine transmethylase or condensed with serine by means of cystathionine β-synthase to form cystathionine. The apparent Km of the former enzyme for homocysteine is on the order of 10^{-5} M, whereas for the latter enzyme it is of the order of 10^{-3} M (Finklestein, 1971). Finklestein and Mudd (1967) have shown that both the methionine adenosyltransferase and cystathionine β-synthase are inhibited in vitro by L-cystine. Cystathionine β-synthase is also activated by SAMe (Finklestein et al., 1975b). This latter result, along with the inhibition of the $N^5N^{10}CH_2THF$ reductase by SAMe, indicates that excess homocysteine will be metabolized by means of the transsulfuration pathway.

An alternative enzyme for remethylation of homocysteine to methionine is betaine:homocysteine methyltransferase. This enzyme was shown to be less active than N^5CH_3THF:homocysteine transmethylase in human fetal liver and brain (Gaull et al., 1973). These authors also showed the betaine:homocysteine methyltransferase to have approximately one-third the activity of the N^5CH_3THF:homocysteine transmethylase in mature human brain. This result may vary with diet in light of the reciprocal action demonstrated by Finklestein et al. (1971) between these two enzymes. Hepatic activity of N^5CH_3THF:homocysteine transmethylase in rats increases with methionine deprivation or a low protein diet while the betaine:homocysteine methyltransferase activity decreases. The converse is true on a high protein diet or methionine supplementation. Finklestein et al. (1972) later showed that L-cysteine and L-cystine inhibit the betaine:homocysteine methyltransferase.

STATEMENT OF THE PROBLEM

Several lines of evidence support the concept that the schizophrenias are disease states with underlying biochemical abnormalities. Genetic studies support this conclusion as do investigations into the mode of action of psychotropic and antipsychotic drugs. These drugs are thought to mediate their effects by altering the functional activity of synaptic neurotransmitters. Thus, many hypotheses regarding the biochemical defect in the schizophrenias were put forth implicating abnormalities in brain levels of biogenic amines or their metabolism. One of these was the transmethylation hypothesis which proposed that O-methylation of norepinephrine or dopamine might produce a compound with psychotomimetic properties similar to mescaline. The experiments reporting exacerbation of schizophrenic psychoses with methionine administration support this hypothesis. The behavioral disrupting effects of methionine in animals give further support to the transmethylation hypothesis. However, methionine has many biochemical functions which are susceptible to perturbation by methionine loading. These effects might also contribute to the methionine induced psychotic exacerbations in schizophrenics.

One such function of methionine and its metabolites is the regulation of the enzymes and coenzymes of the

one-carbon pathway. The importance of the relationship between folic acid metabolism and methylation is emphasized by this regulation. In addition to the ingestion of preformed methyl groups, the methyl group of methionine can be synthesized de novo by enzymes in the one-carbon pathway. A patient with a reduction in the rate of this synthesis due to enzyme deficiency has been associated with schizophrenic-like symptoms. This suggests that a decrease in methylating capacity might be the predisposing factor toward at least one type of schizophrenia. Folate deficiency and inborn errors of folate metabolism have also been reported to result in mental and neurological disorders.

The general problem is to determine the mechanism by which methionine loading exacerbates schizophrenic psychoses and why reduced methylating capacity can produce a similar symptomatology. The research undertaken was designed to investigate the common ground between folate metabolism and methylation. The approach was to study the effect of methionine loading on one-carbon pathway enzymes involved in the de novo synthesis of methyl groups and their activation. Specifically, the objectives of the research were: (1) To determine the extent to which the in vivo availability of methionine, or possibly its metabolites, regulates the de novo synthesis of methyl groups and their activation in mammalian brain. (2) To determine the effect that chronic administration of methionine has on levels of the biological methyl donor, SAMe, in brain

and thus to assess its influence on the transmethylation hypothesis. (3) To determine the extent to which the methionine behavioral disruption in rats might be mediated through alterations of one-carbon metabolism. (4) To provide insight as to the types of future experimentation which may further delineate the behavior disrupting effects of methionine.

MATERIALS AND METHODS

Purchased Materials

N^5-(^{14}C-methyl)-methyltetrahydrofolate (barium salt; 57 μCi/μmole), ^{14}C-formaldehyde (17 μCi/μmole) and Triton-X-100 were obtained from Amersham Searle Corporation, Arlington Heights, Ill. Serotonin creatinine sulfate, 5[1,2-^{3}H(N)] (27.6 Ci/mmole), L-(^{14}C-methyl)-methionine (53 μCi/μmole), L(^{3}H-methyl)-methionine (14.6 Ci/mmole), and S-(^{14}C-methyl)-adenosyl-L-methionine (64 μCi/μmole) were obtained from New England Nuclear Corporation, Boston, Mass. (see Appendix, Nomenclature of Radioactive Compounds). DL-N^5-methyl-tetrahydrofolate (barium salt; approximate purity, 90%), tetrahydrofolic acid, L-methionine, FAD, SAMe, homocysteine thiolactone, menadione, dimedon (5,5-dimethyl-1,3-cyclohexanedione), and N-acetylserotonin were obtained in the highest available purity from Sigma Chemical Company, St. Louis, Mo. Triethylamine was purchased from Aldrich Chemical Company, Milwaukee, Wis. 2,5-Diphenyl-oxazole (PPO) was obtained from ICN Pharmaceuticals Inc., Cleveland, Ohio. 1,4-bis-[2-(4-methyl)-5-phenyloxazolyl)]-Benzene (Dimethyl POPOP) was obtained from Packard Instrument Company, Inc., Downers Grove, Ill. All other chemicals were reagent grade. Bovine pineal glands were

either purchased (Pel Freez Biologicals, Inc., Rogers, Ark.; Canada Packers, Ltd., Toronto) or obtained locally.

Prepared Materials

Solubilization of N^5-Methyltetrahydrofolate

A. Cold N^5-methyltetrahydrofolate

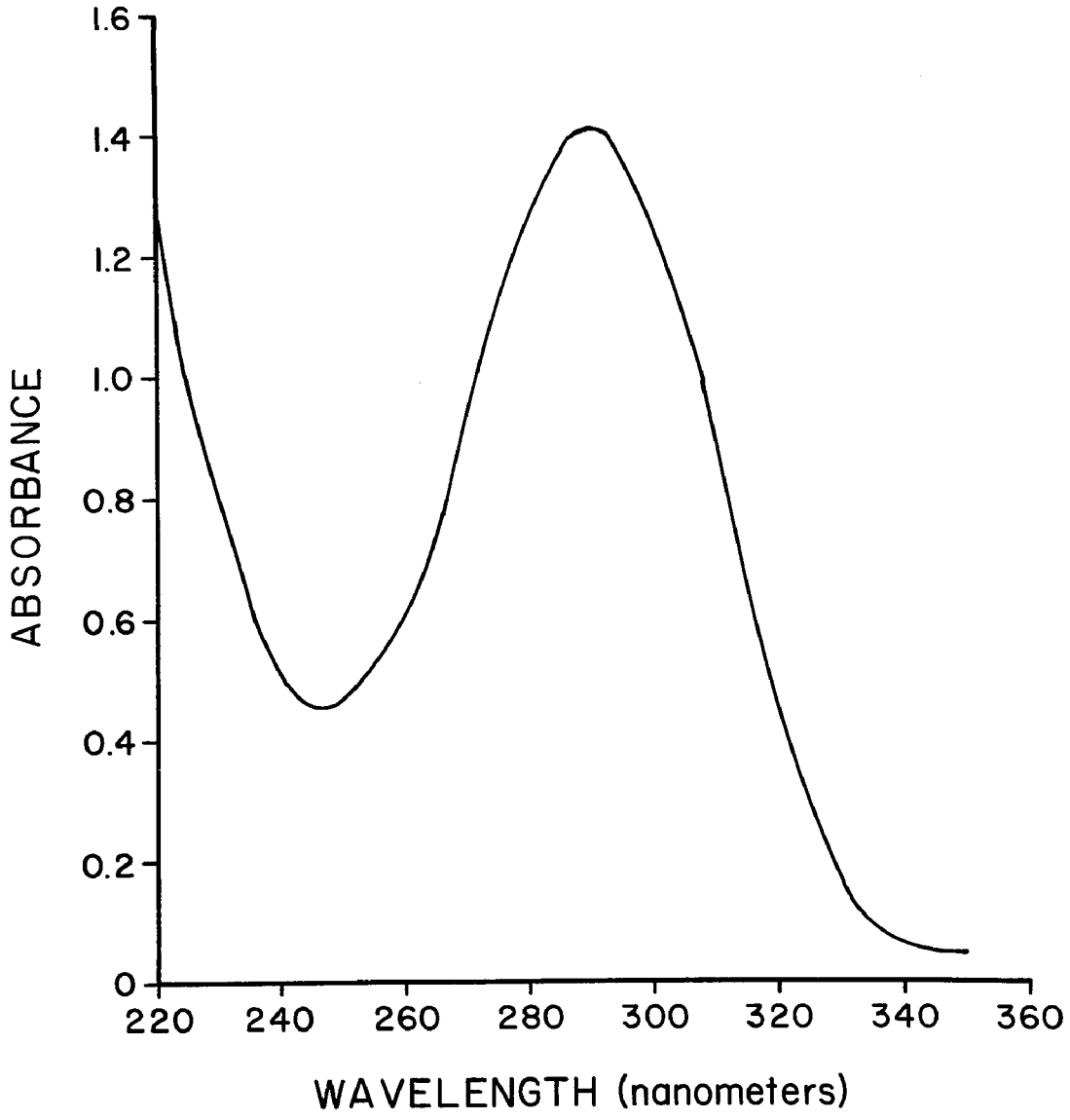
One hundred milligrams of DL-N^5-methyltetrahydrofolic acid (barium salt) were suspended in 10 ml of ice-cold 0.5 M sodium phosphate buffer, pH 7.0, for 15 minutes in the dark with occasional agitation. After separating the barium phosphate precipitate by centrifugation for two minutes in a clinical centrifuge, the supernatant was aspirated into a tube on ice. The pellet was washed twice by suspending it in 7.5 ml of buffer for five minutes. The supernatants were aspirated and pooled after centrifugation. The concentration of N^5CH_3THF in the supernatant was determined by measuring the absorbance in a Beckman Acta C III Spectrophotometer and dividing by the molar extinction coefficient (ε_{290} N^5CH_3THF = 31.7×10^3 M^{-1} cm^{-1} at pH 7.0) (Gupta and Huennekens, 1967). The absorption spectrum of N^5CH_3THF solubilized according to this procedure is shown in Figure 2. An equal mole content of sodium ascorbate as an antioxidant was added to the N^5CH_3THF solution.

B. N^5-(^{14}C-methyl)-methyltetrahydrofolate

Fifty microcuries of N^5-(^{14}C-methyl)-methyltetrahydrofolic acid (barium salt) were suspended in 3.0 ml of ice-cold 0.05 M sodium phosphate buffer, pH 7.0, for 15 minutes

Figure 2. Absorption Spectrum of N^5-Methyltetrahydrofolic Acid.

N^5-methyltetrahydrofolic acid was prepared as described in the text. The spectrum was recorded using a one cm path length curvette. The sample solution represents a 1:120 dilution of the stock solution as prepared. ε_{290} N^5CH_3THF = 31.7 x 10^3 M^{-1} cm^{-1}.


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
220
240
260
280
300
320
340
360
WAVELENGTH (nanometers)

in the dark with occasional stirring. After centrifuging for two minutes at high speed in a clinical centrifuge, the supernatant was aspirated into a tube on ice. The pellet was washed twice by suspending it in 1.0 ml of buffer for five minutes. The supernatants were aspirated and pooled after centrifugation. Less than 0.01 percent of the radioactive material was lost by this procedure. Fifty milligrams of sodium ascorbate as an antioxidant were added to the combined supernatants.

Hydroxyindole-O-Methyltransferase

A. Partial purification

Hydroxyindole-O-methyltransferase (HIOMT) was partially purified from fresh or frozen beef pineal glands by a modification of the method of Axelrod and Weissbach (1961). Pineal glands were homogenized in five volumes of cold isotonic KCl in a Waring blender for 30 seconds. The homogenate was centrifuged at 80,000 g in a Beckman Model L3-50 ultracentrifuge (T40 rotor) for 30 minutes. The supernatant was aspirated and adjusted to pH 5.2 with 1.0 M acetic acid, immersed in a water bath at 48°C for three minutes, cooled to room temperature, readjusted to pH 6.5 with 0.5 M NaOH, and centrifuged at 8000 g for ten minutes. This supernatant was aspirated and brought to 45 percent saturation with saturated ammonium sulfate (adjusted to pH 8.0) and again centrifuged at 8000 g for ten minutes. After aspiration this supernatant was brought to 68 percent saturation with saturated ammonium

sulfate, pH 8.0. Each ammonium sulfate addition was made during a 30-minute period with gentle stirring of the solution maintained in ice. After a final centrifugation at 8000 g for ten minutes, the supernatant was discarded and the pellet dissolved in one volume of ice-cold isotonic KCl. This solution was poured into dialysis tubing which had been soaked overnight in cold 10 mM ethylenediaminetetraacetate (EDTA) and then thoroughly rinsed. Dialysis was carried out overnight at 4°C against 50 volumes of 2.5 mM sodium phosphate buffer, pH 6.5, with six changes. The dialysate was diluted to three volumes of the starting pineal weight with the buffer. Aliquots of this solution were added directly to the assay incubations. The preparation was stored at -70°C.

B. HIOMT Assay

To determine the activity of hydroxyindole-O-methyl-transferase (EC 2.1.1.4) in the partially purified preparation, the enzyme was assayed by a modification of the method of Axelrod and Weissbach (1961). The enzyme catalyzes the following reaction:

$$\text{N-acetylserotonin} + (^{14}\text{C})\text{SAMe} \longrightarrow (^{14}\text{C-}\underline{\text{methyl}})\text{melatonin} + \text{S-adenosylhomocysteine} \quad (1)$$

The (^{14}C-methyl)melatonin is separated from the (^{14}C)SAMe by extraction into chloroform. Solutions required for the assay include the following:

1. Phosphate buffer, pH 6.7, was prepared by dissolving 0.067 mole NaH_2PO_4 in about 800 ml water and adjusting the

pH to 6.7 with NaOH. 50 µmoles of $NaHCO_3$ were then added and the solution made up to 1.0 liter with water.

2. NaOH, 1.0 N, saturated with NaCl was prepared by dissolving 40 grams of NaOH and approximately 350 grams of NaCl in 1.0 liter of water.

Incubations were prepared in duplicate in 15 milliliter glass tubes on ice. A final incubation volume of 0.5 ml was obtained by adding to each tube (1) 250 µl phosphate buffer (solution 1), (2) 100 µl of 0.41 mM N-acetylserotonin, (3) 25 µl of 271 µM (^{14}C)SAMe (57.8 µCi/µmole), (4) 75 µl water, and (5) 50 µl of partially purified HIOMT preparation to start the reaction. Backgrounds were determined by substitution of 50 µl of boiled HIOMT preparation for the active enzyme.

All tubes were incubated in a 37°C water bath with shaking for 30 minutes. The reaction was terminated by adding 2.0 ml of 1.0 N NaOH saturated with NaCl (solution 2). The reaction product (^{14}C-methyl)melatonin was extracted into 7.0 ml of chloroform. After Vortexing, the aqueous layer was aspirated and washed three times with 2.0 ml of 1.0 N NaOH saturated with NaCl. A 5.0 ml aliquot of the chloroform extract was pipetted into scintillation vials, evaporated to dryness and ten milliliters of scintillation fluid were added to each vial. The samples were counted for ^{14}C in a Packard Tri-Carb 3375 Liquid Scintillation Spectrometer.

HIOMT activity in the pineal homogenate prepared in five volumes of isotonic KCl was compared with the enzyme activity in the partially purified preparation. Both stock solutions of enzyme used for these experiments were adjusted to the same volume. Figures 3 through 5 show that approximately 43 percent of the activity in the crude pineal homogenate is recovered in the partially purified preparation. Figure 3 shows the response in enzyme activity with variable incubation time. The enzyme activity of the crude pineal homogenate is linear with time to 80 minutes. The partially purified enzyme is linear in activity to approximately 50 minutes. The enzyme activity in the crude homogenate was compared with that in the partially purified preparation at different incubation enzyme concentrations (Figure 4). This was done by varying the volume of enzyme stock solution added to the incubation and compensating with the volume of water added. Despite an apparent lack of linearity at low enzyme concentrations, both enzyme sources are linear at higher enzyme concentrations to at least 100 µl added to the incubation. Figure 5 shows that both enzyme preparations display similar saturation behavior with respect to SAMe. The concentration of SAMe was varied by adding different volumes of a stock SAMe solution to the incubation and adjusting the volume of water added to maintain a constant incubation volume. Under the incubation conditions of 82 µM N-acetylserotonin and 13.5 µM SAMe, each 100 µl of the partially purified preparation of HIOMT

Figure 3. Hydroxyindole-O-Methyltransferase Activity as a Function of Time.

●——● Partially purified pineal enzyme as described in the text. o——o Pineal homogenate in five volumes of isotonic KCl used directly. Both enzyme stock solutions were of the same volume. The concentration of S-adenosylmethionine and N-acetylserotonin in the incubation were 13.5 μM and 82 μM, respectively. See text for assay procedure.

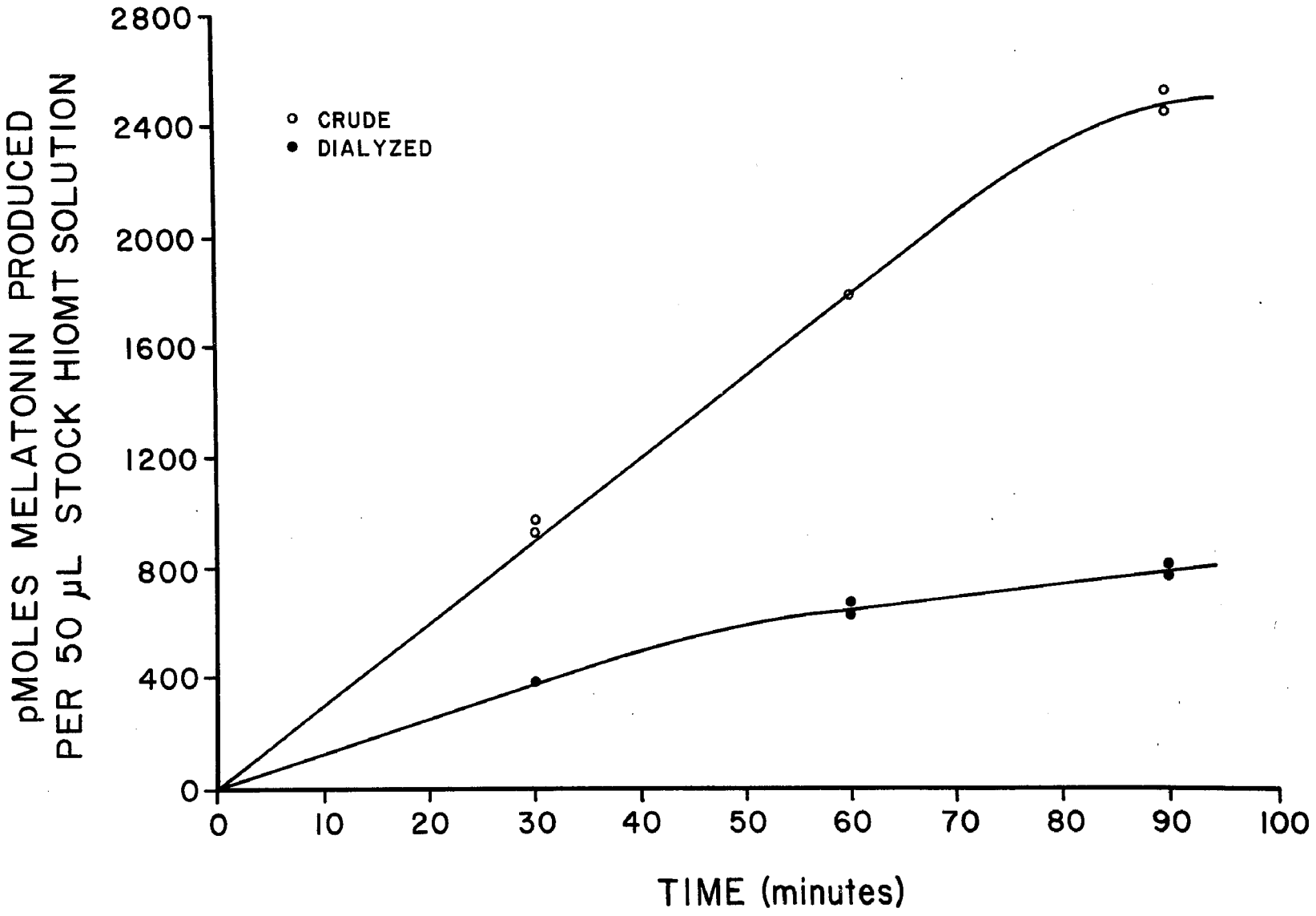

pMOLES MELATONIN PRODUCED
PER 50 µL STOCK HIOMT SOLUTION
2800
2400
2000
1600
1200
800
400
0
CRUDE
DIALYZED
0
10
20
30
40
50
60
70
80
90
100
TIME (minutes)

Figure 4. Hydroxyindole-O-Methyltransferase Activity as a Function of Enzyme Concentration.

▲——▲ Partially purified pineal enzyme as described in the text. o——o Pineal homogenate in five volumes of isotonic KCl used directly. Both enzyme stock solutions were of the same volume. The concentrations of S-adenosyl-methionine and N-acetylserotonin in the incubation were 13.5 μM and 82 μM, respectively. Incubation time was 30 minutes. See text for assay procedure.

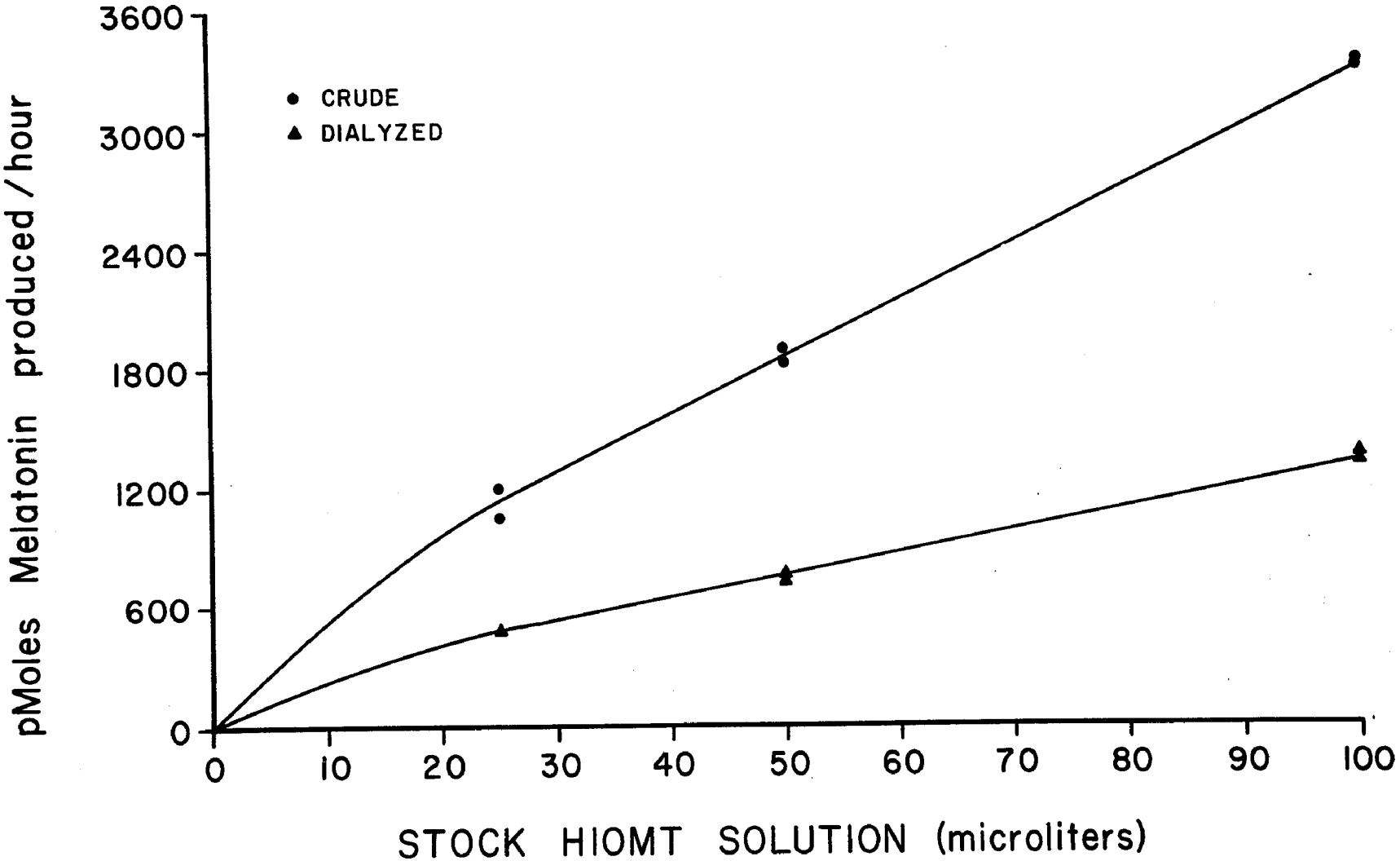
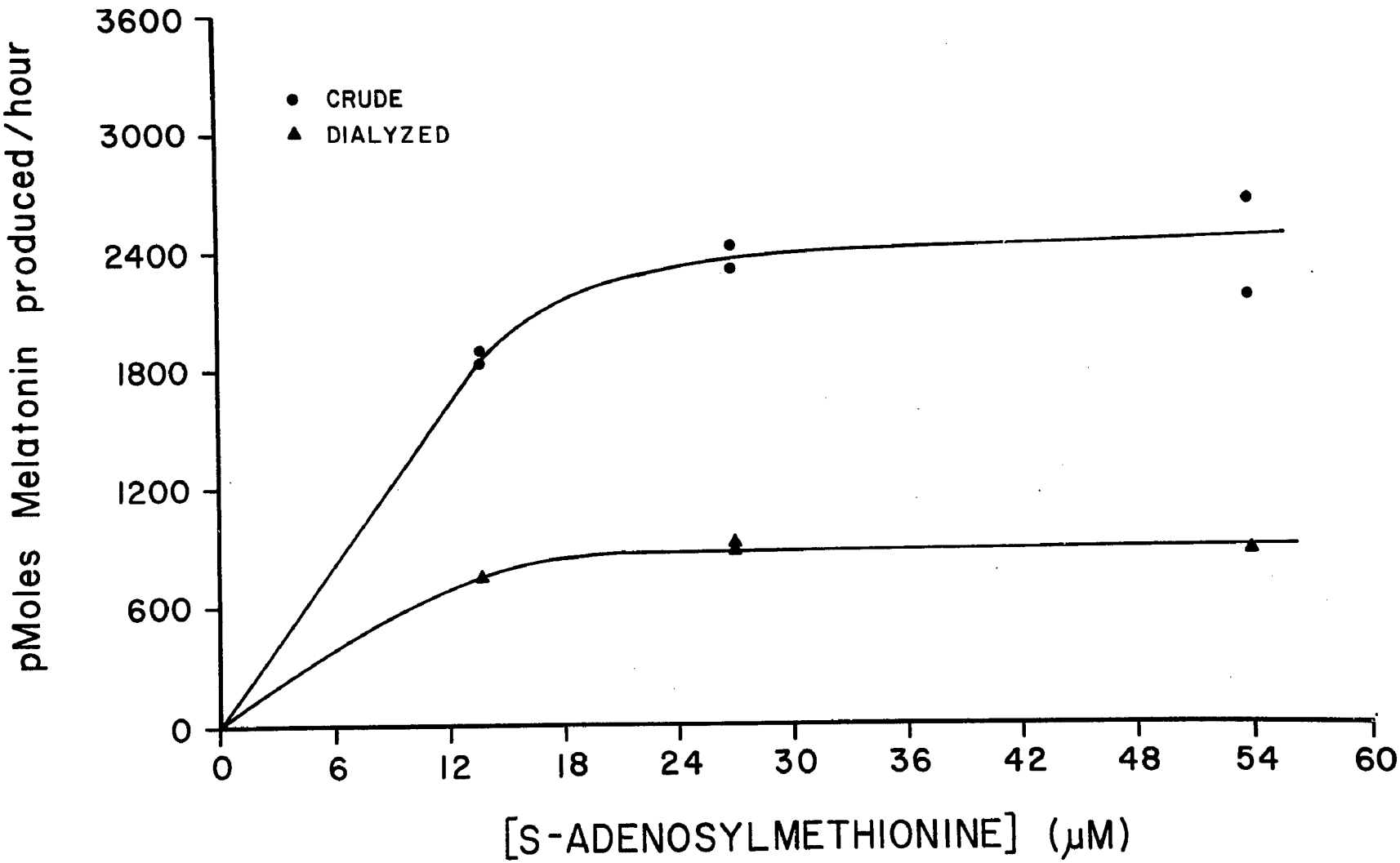

3600
3000
2400
1800
1200
600
0
pMoles Melatonin produced / hour
CRUDE
DIALYZED
0
10
20
30
40
50
60
70
80
90
100
STOCK HIOMT SOLUTION (microliters)

Figure 5. Hydroxyindole-O-Methyltransferase Activity as a Function of S-Adenosylmethionine Concentration.

▲——▲ Partially purified pineal enzyme as described in the text. o——o Pineal homogenate in five volumes of isotonic KCl used directly. Both enzyme stock solutions were of the same volume. The concentration of N-acetyl-serotonin in the incubation was 82 µM. 50 µl of enzyme preparation was added to the incubation. Incubation time was 30 minutes. See text for assay procedure.


3600
3000
2400
1800
1200
600
0
pMoles Melatonin produced/hour
CRUDE
DIALYZED
0
6
12
18
24
30
36
42
48
54
60
[S-ADENOSYLMETHIONINE] (μM)

contained approximately 1.5 units of activity (a unit of activity is defined as one nmole of melatonin produced per hour).

N-(1,2-^{3}H(N))-Acetylserotonin

N-(1,2-^{3}H(N))-Acetylserotonin was prepared by a modification of the method of Kopin et al. (1961). In a 15 ml test tube, 5.0 mg cold serotonin creatinine sulfate was dissolved in 0.3 ml boiled, degassed water to which was added five 500 microcuries of serotonin creatinine sulfate, 5(1,2-^{3}H(N)) (27.6 Ci/mmole) in 0.5 ml two percent ethanol. The tube was fitted with a rubber stopper and flushed with nitrogen for ten minutes. The stopper was then removed, 0.2 ml of triethylamine was rapidly pipetted into the tube, and the stopper was replaced as quickly as possible. The tube was Vortexed and flushed with nitrogen an additional five minutes. The stopper was again removed for addition of two microliters of acetic anhydride and flushing with nitrogen was immediately resumed for one hour. Final reaction concentrations of serotonin creatinine sulfate, triethylamine, and acetic anhydride were 12.9 mM, 1.4 M, and 53 mM, respectively.

The stopper was then removed and 5.0 ml of chloroform were added to the tube to extract any N,O-diacetylated product formed. Afer Vortexing for one minute, the aqueous layer was aspirated off and distributed along a narrow 15 cm band, 12 cm from the short edge of 23 x 60 cm Whatman 3MM filter paper. The filter paper had been previously

equilibrated with eight percent HCl:one percent acetic acid, then washed copiously with deionized water and dried. Along the starting line, near the long edge of the paper, 25 µl of a one percent solution of N-acetylserotonin (NAS) were spotted. Between this spot and the 15 cm band along which the reactive mixture was distributed, 25 µl of a one percent solution of serotonin creatinine sulfate were spotted. Solution application was under a stream of hot air. After drying, the filter paper was developed for five hours by descending chromatography in eight percent HCl:one percent acetic acid. The product was visualized by fluorescence and identified by the difference in migration rates of N-acetylserotonin (R_f = 0.60) and serotonin (R_f = 0.43) in the solvent system. The band corresponding to N-(1,2-^{3}H(N))-acetyl-serotonin was cut out and eluted with water. The product (38.6 mCi/mmole, 11 µCi/ml) was stored at -70°C.

Animals

Adult male Long Evans hooded rats weighing 240-260 g were purchased from Southern Animal Farms, Prattville, Ala. The rats were housed one per cage and maintained on Wayne Lab-Blox (Allied Mills, Inc., Chicago) and water ad libitum. The animal quarters were illuminated from 6 A.M. to 6 P.M. daily. Each experimental group contained eight rats equally divided at random into methionine and control groups. The former were injected subcutaneously between 10 A.M. and noon daily for seven weeks with 250 mg L-methionine per kg body

weight. The injection solution was prepared fresh daily by dissolving one gram of L-methionine in 20 ml de-ionized water with gentle heating and stirring. The pH was brought to 7.0 by adding 0.5 M NaOH. Control animals received 5.0 ml/kg of 0.9% NaCl solution.

Tissue Preparation

The rats were sacrificed by decapitation one hour after the final injection. Each group of animals was killed at the same hour of the day to minimize the effect of possible daily variations on the fate of the injected methionine. The brain and liver were excised, weighed and homogenized in 10 volumes of ice cold 0.32 M sucrose using ground glass hand homogenizers (brain) or a Sorvall Omni-Mixer tissue homogenizer (liver). This procedure was completed within five minutes. The homogenates were then centrifuged at 4°C (Sorvall RC2-B refrigerated high speed centrifuge with SS-34 rotor) at 1000 g for 10 minutes. The supernatants (postnuclear homogenate) were aspirated to clean tubes on ice. Aliquots were withdrawn for assays to be performed the same day while the remaining portions were frozen immediately. The N^5,N^{10}-methylenetetrahydrofolate ($N^5N^{10}CH_2THF$) reductase and S-adenosylmethionine (SAMe) assays were always begun immediately after preparation of tissue the first day. The methionine adenosyltransferase (MAT) assay was begun the second day approximately 24 hours after sacrificing the animals. The N^5-methyltetrahydro-

folate (N^5CH_3THF):homocysteine transmethylase assay was performed the third day. The methionine assay was performed within one month after sacrificing the animals.

N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase Assay

N^5,N^{10}-methylenetetrahydrofolic acid reductase (N^5-methyltetrahydrofolate-NAD oxidoreductase, EC 1.1.1.68) activity was measured by a modification of the method of Donaldson and Keresztesy (1959). The assay was performed by reversing the physiological direction of the reaction using menadione as an electron acceptor. The reverse direction is shown in equation (2).

$$N^5\text{-}^{14}CH_3THF + (\text{Enzyme})\text{-}FAD \longrightarrow (\text{Enzyme})\text{-}FADH_2 + N^5N^{10}\text{-}^{14}CH_2THF \quad (2)$$

The electron acceptor functions to disturb the physiological equilibrium by reoxidizing the flavin according to equation (3).

$$(\text{Enzyme})\text{-}FADH_2 + \text{Menadione} \longrightarrow (\text{Enzyme})\text{-}FAD + \text{Menadione-}H_2 \quad (3)$$

The $N^5N^{10}\text{-}^{14}CH_2THF$ formed dissociates easily according to equation (4).

$$N^5N^{10}\text{-}^{14}CH_2THF \longrightarrow THF + H^{14}CHO \quad (4)$$

In the presence of 1000-fold excess cold formaldehyde, 99.9 percent of the ^{14}C label is present as free $H^{14}CHO$ which can be isolated as the dimedon adduct (Taylor and Weissbach, 1965) by extraction into an organic solvent.

Solutions

A. Reductase buffer, 0.2 M, pH 6.6, was prepared by dissolving 1.72 g monobasic sodium phosphate (NaH_2PO_4 H_2O), 1.06 g dibasic sodium phosphate (Na_2HPO_4), 700 mg sodium ascorbate, and 0.40 ml 12.3 M formaldehyde in water to a final volume of 100 ml.

B. FAD-menadione solution was prepared by combining one volume each of 1.74 mM menadione and 36 µM FAD with eight volumes of water. Menadione is not soluble in water. To make the 1.74 mM stock solution, menadione was first dissolved in one-tenth volume of methanol and brought to volume with water. Menadione crystallizes from this solution with storage in the cold and must be heated before use to effect dissolution.

C. Dimedon solution was prepared by suspending 0.42 g dimedon in water to a final volume of five milliliters. The dimedon was solubilized by adding five milliliters of ethanol followed by Vortexing with gentle heating.

D. Sodium acetate, 0.4 M, was adjusted to pH 4.4 with glacial acetic acid.

Assay Procedure

Incubations were prepared in quadruplicate in seven milliliter glass tubes on ice. An incubation volume of 0.675 ml was obtained by adding to each tube (1) 100 µl reductase buffer (solution A), (2) 400 µl water, (3) 25 µl N^5-(^{14}C-methyl)-methyltetrahydrofolate (57 µCi/µmole),

(4) 100 µl postnuclear homogenate from liver or brain, and (5) 50 µl FAD-menadione (solution B) to start the reaction. The incubation solution contained (1) 30 mM sodium phosphate, pH 6.6, (2) 5.2 mM sodium ascorbate, (3) 7.4 mM formaldehyde, (4) 1.62 µM N^5-(^{14}C-methyl)-methyltetrahydrofolate, (5) 0.26 µM FAD, (6) 12.8 µM menadione, (7) 0.47 mM sucrose, and (8) 0.80-1.40 mg protein (liver) or 300-800 µg protein (brain). Formaldehyde recoveries were determined by substitution of H^{14}CHO (17 mCi/mmole) for N^5-(^{14}C-methyl)-methyltetrahydrofolate to a final concentration of 0.08 µM. Recoveries ranged from 57-60 percent for the added ^{14}C-formaldehyde. Backgrounds were determined by substitution of 50 µl water for the FAD-menadione solution.

The tubes were incubated in a 37°C water bath with shaking for 30 minutes. The reaction was stopped by adding 10 µl of 0.4 M sodium acetate, pH 4.4 (solution D), followed immediately by the addition of 50 µl of the dimedon solution (solution C). The tubes were placed in a boiling water bath for five minutes to form the ^{14}C-formaldehyde-dimedon adduct. After cooling, two milliliters of toluene were added to each tube followed by Vortexing for 30 seconds. After centrifugation, one milliliter of each toluene layer was pipetted into a scintillation vial, 10 ml of scintillation fluid added to each vial, and the vials were assayed for ^{14}C activity using a Packard Tri-Carb 3375 Liquid Scintillation Spectrometer.

Reductase activity as a function of enzyme concentration was determined by varying the volume of postnuclear homogenate added to the incubation reaction and compensating with the amount of water added to maintain a constant incubation volume. Linearity of enzyme activity with time was determined by varying the incubation time from 15 minutes to 2 hours. Saturation of the enzyme with respect to N^5-methyltetrahydrofolate was studied by preparing serial dilutions of cold N^5-methyltetrahydrofolate and adding appropriate volumes to the incubation mixture to cover a range of N^5-methyltetrahydrofolate concentration up to 200 μM. The incubation volume was again maintained constant by adjusting the amount of water added. Lineweaver-Burk plots were generated by plotting the reciprocal of the calculated reaction velocity as a function of the reciprocal of the incubation N^5-methyltetrahydrofolate concentration.

N^5-Methyltetrahydrofolic Acid:Homocysteine Transmethylase Assay

N^5-methyltetrahydrofolic acid:homocysteine transmethylase (5-methyltetrahydropteroyl-L-glutamate:L-homocysteine 5-methyltransferase, EC 2.1.1.13) was assayed by a modification of the method of Draper *et al.* (1972). The assay depends on the transfer of the labeled methyl group from N^5-(^{14}C-*methyl*)-methyltetrahydrofolate to homocysteine forming (^{14}C-*methyl*)-methionine as shown in equation 5.

$$N^5\text{-}^{14}CH_3THF + \text{Homocysteine} \longrightarrow$$

$$(^{14}C\text{-Methyl})\text{-Methionine} + THF \quad (5)$$

The (^{14}C-methyl)-methionine is separated from the N^5-(^{14}C-methyl)-methyltetrahydrofolate by thin layer chromatography.

Solutions

A. Potassium phosphate buffer, 0.2 M, pH 7.4, was prepared by dissolving 0.52 g monobasic potassium phosphate (KH_2PO_4) and 2.82 g dibasic potassium phosphate (K_2HPO_4) in water to a final volume of 100 ml.

B. $FADH_2$, 3.1 mM, was prepared by bubbling H_2 for 60 minutes through 3.1 mM FAD in a stoppered tube containing 25 µmoles of PtO_2.

C. L-Homocysteine, 21.6 mM, was prepared fresh daily by base hydrolysis of L-homocysteine thiolactone. 10 mg of L-homocysteine thiolactone were dissolved in 250 µl of 0.3 M NaOH and allowed to stand at room temperature for three hours. After neutralizing with 250 µl of 0.3 M HCl, the solution was made up to 3.0 ml with buffer (solution A).

D. L-methionine, 6.7 mM, was prepared in four percent Triton X-100.

Assay Procedure

Incubations were prepared in quadruplicate in seven milliliter glass tubes on ice. An incubation volume of 445 µl was obtained by adding to each tube (1) 100 µl potassium phosphate buffer, (2) 10 µl of 4.6 mM SAMe,

(3) 25 µl N^5-(^{14}C-methyl)-methyltetrahydrofolate (57 µCi/µmole), and (4) 250 µl postnuclear homogenate from liver or brain. The tubes were then fitted with rubber stoppers and flushed with ultrapure hydrogen for 60 minutes. With the rubber stopper still inserted, the flushing system was disconnected. Into each tube by means of 50 µl Hamilton syringes were injected (5) 10 µl of 3.1 mM $FADH_2$ (solution B) and (6) 50 µl of 21.6 mM L-homocysteine (solution C). The incubation solution contained (1) 45 mM potassium phosphate buffer, pH 7.4, (2) 0.1 mM SAMe, (3) 3 µM N^5-(^{14}C-methyl)-methyltetrahydrofolate, (4) 70 µM $FADH_2$, (5) 2.43 mM L-homocysteine, (6) 2.0-3.5 mg protein (liver) or 1.0-2.0 mg protein (brain), and (7) 180 mM sucrose. Methionine recoveries were determined by substitution of (^{14}C-methyl)-methionine (53 µCi/µmole) for N^5-(^{14}C-methyl)-methyltetrahydrofolate to a final concentration of 0.05 µM. Recoveries varied in the range of 69±6 percent. Backgrounds were obtained by substituting a boiled (10 minutes) preparation of the tissue homogenate for the active enzyme.

The tubes were incubated in a 37°C water bath with shaking for 90 minutes. The reaction was stopped by removing the rubber stopper from the tubes, adding 25 µl of 6.7 mM L-methionine in four percent Triton-X-100 (solution D) and immediately immersing the tubes in a boiling water bath for five minutes. After cooling, 100 µl of each incubation solution were spotted, 25 µl at a time under a stream of

hot air, onto a silica gel chromatography plate (LQD 5051, Quantum Industries, Fairfield, N. J.). The plates were developed with n-butanol:chloroform:methanol:ammonia (6:2:1:1, by volume) for four hours. After drying, the plates were sprayed with ninhydrin (Sigma Chemical Company, St. Louis, Mo.). The methionine was visualized by heating for approximately five minutes in an oven set to 105°C. The plates were scraped separating the reaction product methionine (R_f = 0.25) from N^5-(^{14}C-methyl)-methyltetrahydrofolate (R_f = 0.00). The scrapings were put into scintillation vials and 1.0 ml of water was added to extract the methionine. Ten milliliters of scintillation fluid were then added to each vial. The vials were capped, shaken, and assayed for ^{14}C activity using a Packard Tri-Carb 3375 Liquid Scintillation Spectrometer.

Transmethylase activity as a function of enzyme concentration was determined by varying the volume of postnuclear homogenate added to the incubation reaction and compensating with the amount of water added to maintain a constant incubation volume. Linearity of enzyme activity with time was determined by varying the incubation time from 15 minutes to two hours.

Methionine Adenosyltransferase Assay

Methionine adenosyltransferase (ATP:L-methionine S-adenosyltransferase, EC 2.4.2.13) was assayed by a modification of the double isotope method of Matthysse

et al. (1972). The assay consists of two parts. The first step involves the reaction of (^{3}H-methyl)methionine with ATP to form (^{3}H-methyl)-S-adenosylmethionine as shown in equation (6).

(^{3}H-Methyl)Methionine + ATP ⟶

(^{3}H-Methyl)-S-adenosylmethionine + PPi + Pi (6)

For the second step, a known quantity of (^{14}C-methyl)-S-adenosylmethionine is added to the incubation. The (^{3}H,^{14}C) SAMe mixture reacts with N-acetylserotonin (NAS) in the presence of hydroxyindole-O-methyltransferase (HIOMT) to produce (^{3}H,^{14}C) melatonin according to equation (7).

(^{3}H,^{14}C)Methyl-S-Adenosylmethionine + NAS ⟶

S-Adenosylhomocysteine + (^{3}H,^{14}C)Melatonin (7)

The (^{3}H,^{14}C) melatonin can be extracted into chloroform from the incubation mixture.

Solutions

A. Tris buffer, 0.3 M, pH 7.8 was prepared by dissolving 7.29 g tris base in 100 ml water, adding 69 ml 0.6 M HCl, and bringing the final volume to 200 ml with water.

B. Methionine adenosyltransferase (MAT) buffer, 0.33 M tris, pH 8.1, was prepared by combining seven parts of tris buffer (solution A) with one part each of (1) 1.2 M tris base, (2) 3.0 M $MgCl_2$, and (3) 1.0 M KCl.

C. NaOH, 1.0 N, saturated with NaCl was prepared by dissolving 40 grams of NaOH and approximately 350 grams of NaCl in 1.0 liter of water.

D. Sodium phosphate-potassium hydroxide buffer, pH 6.8, was prepared by dissolving 1.0 mole of monobasic sodium phosphate in about 200 ml water and adjusting the pH to 6.8 with KOH pellets. To this solution was added 168 ml of 0.8 M KOH and the final volume was brought to 500 ml with water.

E. Twenty percent perchloric acid (PCA):0.05 M HCl was prepared by adding 2.0 ml of 12.3 M HCl to 143 ml of 70 percent perchloric acid and making the final volume up to 500 ml with water.

Assay Procedure, Step 1

Incubations were prepared in quadruplicate in seven milliliter glass test tubes on ice. Tubes were preincubated five minutes. An incubation volume of 1.1 ml was obtained by adding to each tube (1) 200 µl MAT buffer (solution B), (2) 100 µl of 40 mM glutathione (GSH), (3) 50 µl of 200 mM ATP, (4) 100 µl of 0.022 mM SAMe, (5) 250 µl water, (6) 250 µl postnuclear homogenate from liver or brain, and (7) 50 µl (^{3}H-methyl)-methionine (14.6 Ci/mmole or 32 µCi/µmole) to start the reaction. The tubes were incubated with shaking in a 37°C water bath for 60 minutes. At the midpoint of the incubation each tube received (8) 100 µl of (^{14}C-methyl)-S-adenosylmethionine (64 µCi/µmole) containing 0.02 µCi. The incubation solution contained (1) 0.066 mM tris, (2) 0.06 M $MgCl_2$, (3) 20 mM KCl, (4) 4 mM GSH, (5) 10 mM ATP, (6) 2.2 µM SAMe,

(7) 0.28 μM (^{14}C-methyl)-S-adenosylmethionine, (9) 2.0-3.5 mg protein (liver) or 1.0-2.0 mg protein (brain), (10) 80 mM sucrose, and (11) 0.15 μM or 67 μM (^{3}H-methyl)-methionine. Backgrounds were determined by substitution of boiled (10 minutes) postnuclear homogenate for the active enzyme.

The reaction was stopped by adding 500 μl of 20 percent perchloric acid:0.05 M HCl (solution E). The tubes were chilled on ice, centrifuged, and 1.0 ml of each supernatant was transferred to a clean tube. To these supernatants, 0.5 ml of the sodium phosphate-potassium hydroxide buffer, (solution D) was added to neutralize the PCA. The tubes were then centrifuged and 1.0 ml of the supernatant was transferred to clean 15 ml test tubes for step 2 of the assay.

Assay Procedure, Step 2

Incubations were prepared in quadruplicate in 15 milliliter glass tubes on ice. A final incubation volume of 1.3 ml was obtained by adding to each tube (1) 1.0 ml of the supernatant from step 1, (2) 100 μl of 0.41 mM N-acetylserotonin, (3) 100 μl of 0.33 M L-methionine, and (4) 100 μl of the HIOMT preparation (see hydroxyindole-O-methyltransferase, partial purification). The incubation concentration of N-acetylserotonin and L-methionine was 0.032 mM and 25.3 mM, respectively. From HIOMT calibration curves, the enzyme would be expected to have 0.58 unit of activity per 100 μl of HIOMT preparation for a SAMe concentration of 13.5 μM (a unit equals 1 nmole of melatonin

produced per hour).

The tubes were incubated with shaking in a 37°C water bath for two hours. The reaction was stopped by adding 2.0 ml of 1.0 N NaOH saturated with NaCl (solution C). The reaction product (^{3}H,^{14}C-methyl)-melatonin was extracted into 7.0 ml of chloroform. After Vortexing, the aqueous layer was aspirated and washed three times with 2.0 ml of 1.0 N NaOH saturated with NaCl. A 5.0 ml aliquot of the chloroform extract was pipetted into scintillation vials, evaporated to dryness and 10 ml of scintillation fluid were added to each vial. The samples were counted for ^{3}H and ^{14}C activity in a Beckman LS-250 Liquid Scintillation System using the automatic quench correction feature.

Two backgrounds are required for the overall assay. One is necessary to correct for the ^{3}H and ^{14}C counts extracted into the chloroform but not related to the reaction product, (^{3}H,^{14}C-methyl)-melatonin. This background was determined by substituting boiled (10 minutes) post-nuclear homogenate for active enzyme in Step 1 and substituting boiled (10 minutes) HIOMT preparation in Step 2. The second background is needed to correct for the production of (^{3}H-methyl)-S-adenosylmethionine in Step 2 of the assay due to methionine adenosyltransferase activity in the HIOMT preparation. This second background was determined by substituting boiled (10 minutes) postnuclear homogenate for active enzyme in Step 1 and using active HIOMT preparation in Step 2.

Transferase activity as a function of enzyme concentration was determined by varying the volume of postnuclear homogenate added to the incubation reaction and compensating with the amount of water added to maintain a constant incubation volume. Linearity of enzyme activity with time was determined by varying the incubation time from 15 minutes to 3 hours.

For saturation studies of the enzyme with respect to L-methionine, the postnuclear tissue homogenate was prepared as described in the section on tissue preparation. This homogenate was then centrifuged at 100,000 g for 60 minutes. To remove endogenous free methionine, the supernatant was dialyzed against 200 volumes of 0.05 M Tris-HCl buffer, pH 8.1 with six changes. The resulting dialysate was used for these studies. Serial L-methionine concentrations were prepared and appropriate volumes added to the incubation solution to span the range of L-methionine concentration from 0.15 μM to 50 μM. (^{3}H-methyl)-methionine (14.6 Ci/mmole) was used for these experiments. Again, the incubation volume was maintained constant by adjusting the amount of water added. The reciprocal of the calculated reaction velocity was plotted as a function of the reciprocal L-methionine concentration to present the data in Lineweaver-Burk format.

S-Adenosylmethionine Assay

S-adenosylmethionine (SAMe) was measured by a modification of the method of Baldessarini and Kopin (1966). The assay is based on the enzymatic reaction of tritium labelled N-acetylserotonin with (^{14}C-methyl)-S-adenosyl-methionine catalyzed by HIOMT to form (1,2-^{3}H(N),^{14}C-methyl) melatonin, which is extracted into chloroform.

N-(1,2-^{3}H(N))Acetylserotonin + (^{14}C-methyl)SAMe $\xrightarrow{\text{HIOMT}}$ S-Adenosylhomocysteine + (1,2-^{3}H(N),^{14}C-Methyl)Melatonin (8)

Solutions

A. Sodium phosphate-potassium hydroxide buffer, pH 6.8, was prepared as described for the methionine adenosyl-transferase (MAT) assay.

B. NaOH, 1.0 N, saturated with NaCl was prepared as described for the MAT assay.

C. 20 percent perchloric acid:0.05 M HCl was prepared as described for the MAT assay.

Calibration Curve Determination

Serial dilutions of SAMe in liver and brain postnuclear homogenates were prepared to yield SAMe concentrations of 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 20.0 µg/ml. From each of these seven dilutions, 1.5 ml was pipetted into seven milliliter glass tubes on ice. To each tube were added (1) 150 µl of (^{14}C-methyl)SAMe (0.556 µCi/ml, 9.5 nmoles/ml) and (2) 0.5 ml of 20 percent PCA in 0.05 M HCl (solution C).

The tubes were Vortexed for 10 seconds and centrifuged at high speed in a clinical centrifuge for one minute. A volume of 1.5 ml of the supernatant in each tube was pipetted into a second set of seven milliliter glass tubes on ice. While Vortexing each tube in this second set, 500 µl of sodium phosphate-potassium hydroxide buffer, pH 6.8 (solution A) were added to neutralize the PCA. The tubes were again centrifuged. From each supernatant, three 0.5 ml aliquots were pipetted into three corresponding 15 milliliter glass tubes. In addition to the 0.5 ml aliquot, each tube received 25 µl of N-(1,2-^{3}H(N)) acetylserotonin (38.6 mCi/mmole) (see Prepared Materials) and 100 µl of HIOMT preparation (see Hydroxyindole-O-Methyltransferase, Partial Purification) to start the reaction. The incubation volume of 625 µl contained 5.68 µM N-acetylserotonin. Backgrounds were determined by substituting a boiled (10 minutes) preparation of HIOMT for the active enzyme.

The tubes were incubated for 90 minutes in a 37°C water bath with shaking. The reaction was stopped by adding 2.0 ml of 1.0 N NaOH saturated with NaCl (solution B). The reaction product (1,2-^{3}H(N),^{14}C-<u>methyl</u>)-melatonin was extracted into seven milliliters of chloroform using the same procedure outlined for the methionine adenosyltransferase assay, step 2. The ratios of ^{3}H:^{14}C were determined and plotted versus µg of SAMe per gram of liver or brain.

Assay Procedure for SAMe

Endogenous SAMe levels in liver and brain postnuclear homogenates were determined using the procedure just described. The entire procedure was performed in duplicate. After calculation of the $^{3}H:^{14}C$ ratio, the quantity of SAMe in the tissue was read directly from the calibration curve.

Methionine Assay

Radiochemical

Methionine was measured by a modification of the method of Matthysse *et al.* (1972) used for assaying the methionine adenosyltransferase. The assay is based on the $^{3}H:^{14}C$ ratio calculated for the (^{3}H-*methyl*, ^{14}C-*methyl*)-melatonin extracted into chloroform. Varying the incubation concentration of cold methionine but holding all other incubation conditions constant, the $^{3}H:^{14}C$ ratio will change as a function of the incubation methionine concentration. The dilution of tritiated methionine with cold methionine will affect the product formation of (^{3}H-*methyl*)-S-adenosyl-methionine in step 1 of the assay. This in turn will be reflected in the amount of tritium label incorporated into melatonin and thus in the calculated $^{3}H:^{14}C$ ratio. By constructing a calibration curve to define the relationship between the $^{3}H:^{14}C$ ratio and the incubation methionine concentration, an unknown methionine concentration can then be determined for conditions identical to those for which the calibration curve was derived. The calculated $^{3}H:^{14}C$

ratio is used to read the methionine concentration from the calibration curve directly.

The methionine adenosyltransferase used in the assay was prepared from rat liver. Beginning with postnuclear homogenate, prepared as detailed in the section on tissue preparation, the homogenate was then centrifuged at 100,000 g for 60 minutes. The supernatant was dialyzed against 200 volumes of 0.05 M Tris-HCl buffer, pH 8.1 with six changes. The resulting dialysate was used directly as the enzyme source in the assay.

The calibration curve was constructed by adding L-methionine to the incubation mixture described in step 1 of the methionine adenosyltransferase assay to provide a range of incubation methionine concentrations up to 50 µM. (^{3}H-methyl)-methionine (14.6 Ci/mmole) was used for these experiments. The assay procedure was identical to that detailed for the methionine adenosyltransferase with the exception of substituting 250 µl of the dialyzed liver preparation, described above, for the 250 µl of tissue postnuclear homogenate. All calibration points were determined in duplicate.

Tissues to be assayed for methionine were prepared by adding to seven milliliter glass tubes on ice (1) 500 µl of liver or brain postnuclear homogenate and (2) 250 µl of 20 percent PCA in 0.05 M HCl. The tubes were Vortexed for 10 seconds and centrifuged at high speed in a clinical centrifuge for one minute. A volume of 0.5 ml of the

supernatant from each tube was pipetted into a second set of tubes. While Vortexing, each tube in this second set then received 155 µl of 3.45 M KOH to neutralize the PCA. The tubes were again centrifuged at high speed for one minute in a clinical centrifuge. Supernatants prepared from brain of methionine injected rats were further diluted by a factor of five. A factor of ten dilution was applied to supernatants derived from liver of methionine injected rats. Liver and brain samples from saline injected rats were not diluted. These preparations were used directly for methionine assay.

Using 100 µl of this preparation and 250 µl dialyzed, soluble rat liver as methionine adenosyltransferase source, methionine was assayed by a procedure identical to the methionine adenosyltransferase assay. The incubation volume was kept constant by adjusting the volume of water added to the incubation. Sample points were determined in duplicate. The resultant $^{3}H:^{14}C$ ratio was used to determine the methionine concentration from the calibration curve. This concentration was corrected for dilutions as a result of sample preparation. From the known initial homogenization volume, the results were expressed as µmoles of L-methionine per gram of liver or brain.

Amino Acid Analysis

Methionine was measured in liver and brain postnuclear homogenates by a modification of the method of Gerritsen et al. (1965) using a Beckman automatic amino acid analyzer. The same procedure was applied to liver and brain postnuclear

homogenates and L-methionine standards (0.1 μmole/ml) run in duplicate. One hundred microliters of norleucine (1.5 μmoles/ml) were added to 2.0 ml of either the tissue homogenates or the methionine standards. These preparations were centrifuged at 100,000 g for one hour at 4°C. The supernatants were aspirated and acidified with 5.0 ml of 20 percent trichloroacetic acid. The tubes were centrifuged at 10,000 g for 10 minutes at 4°C. The clear supernatant was then washed with peroxide-free ether to remove the trichloroacetic acid. (Peroxide-free ether was prepared by dissolving 2.0 grams of $FeSO_4$ in 100 ml of 0.05 M HCl. Reagent grade ethyl ether at nine volumes ether to one volume acid was added and the supernatant was used after the mixture was vigorously shaken).

The solutions were then lyophilized overnight in a Virtis Model 10-100 V lyophilizer. The dried samples were dissolved in 250 μl of citrate buffer, pH 2.2, and loaded onto the column of the amino acid analyzer previously calibrated with a known amino acid mixture. Norleucine served as an internal standard. Methionine determinations were corrected for the percentage of norleucine recovered.

Protein Determination

Proteins were measured by the method of Lowry *et al.* (1952) which depends on color development caused by copper complexing with the amides of the peptide bonds and with

aromatic amino acids.

Solutions

A. Reagent C was prepared by combining 0.1 ml of five percent $CuSO_4$ with 0.1 ml of ten percent sodium tartrate and making the volume up to 50 ml with two percent Na_2CO_3 in 0.10 M NaOH.

B. Folin reagent was prepared by diluting one part phenol reagent (Fisher Scientific Co.) with one part water.

Procedure

All assays were done in duplicate. Calibration curves were constructed with each assay over the range 25 to 125 µg of bovine serum albumin using 25 µg increments. Protein samples were prediluted if necessary to fall on the linear region of the calibration curve. To each 0.50 ml protein sample, 2.5 ml of reagent C (solution A) were added, and mixed. The solutions were allowed to stand at room temperature for 10 minutes. A volume of 0.25 ml of Folin reagent (solution B) was then added to each tube and the contents mixed immediately. After standing one hour at room temperature, the absorbance of each solution was measured at 750 nm. The calibration curve was plotted (µg protein versus absorbance at 750 nm) and unknown proteins were read with dilution factors used to calculate original protein concentrations.

Scintillation Counting System

A Beckman LS-250 Liquid Scintillation System was used when counting ^{3}H and ^{14}C simultaneously. The single isotope, ^{14}C, was counted in a Packard Tri-Carb 3375 Liquid Scintillation Spectrometer.

Scintillation Cocktail

8.25 grams of PPO and 0.15 gram of POPOP were dissolved in one liter of toluene. 500 ml of Triton-X-100 were added and the solution was thoroughly mixed.

Procedure for Determining Efficiency Curves

A. Beckman LS-250

The automatic quench correction feature was selected and ^{3}H and ^{14}C modules were used in channels A and B, respectively. Ten milliliters of scintillation cocktail were counted in channels A and B in triplicate. Twenty-five microliters of CH_3I, a quenching agent, were added to each vial and the vials were recounted. This process was continued until a curve of background counts per minute (CPM) for channels A and B versus X-number (a measure of the degree of quenching with respect to an instrument standard) was generated.

Using the same quenching agent, efficiency (CPM/DPM) in the A channel was determined as function of X-number using (^{3}H) toluene of known disintegrations per minute (DPM). Similarly, efficiencies (CPM/DPM) in the A and B

channels were determined as a function of X-number using (^{14}C) toluene of known DPM. The following equations were used to calculate the efficiency curves shown in Figure 6.

Efficiency of ^{3}H in Channel A =

$$\frac{\text{CPMA}(^{3}H)-(\text{Background CPMA})}{\text{DPM } (^{3}H) \text{ Toluene Standard}} \times 100 \qquad (9)$$

Efficiency of ^{14}C in Channel B =

$$\frac{\text{CPMB}(^{14}C)-(\text{Background CPMB})}{\text{DPM } (^{14}C) \text{ Toluene Standard}} \times 100 \qquad (10)$$

Efficiency of ^{14}C in Channel A =

$$\frac{\text{CPMA}(^{14}C)-(\text{Background CPMA})}{\text{DPM } (^{14}C) \text{ Toluene Standard}} \times 100 \qquad (11)$$

B. Packard Tri-Carb

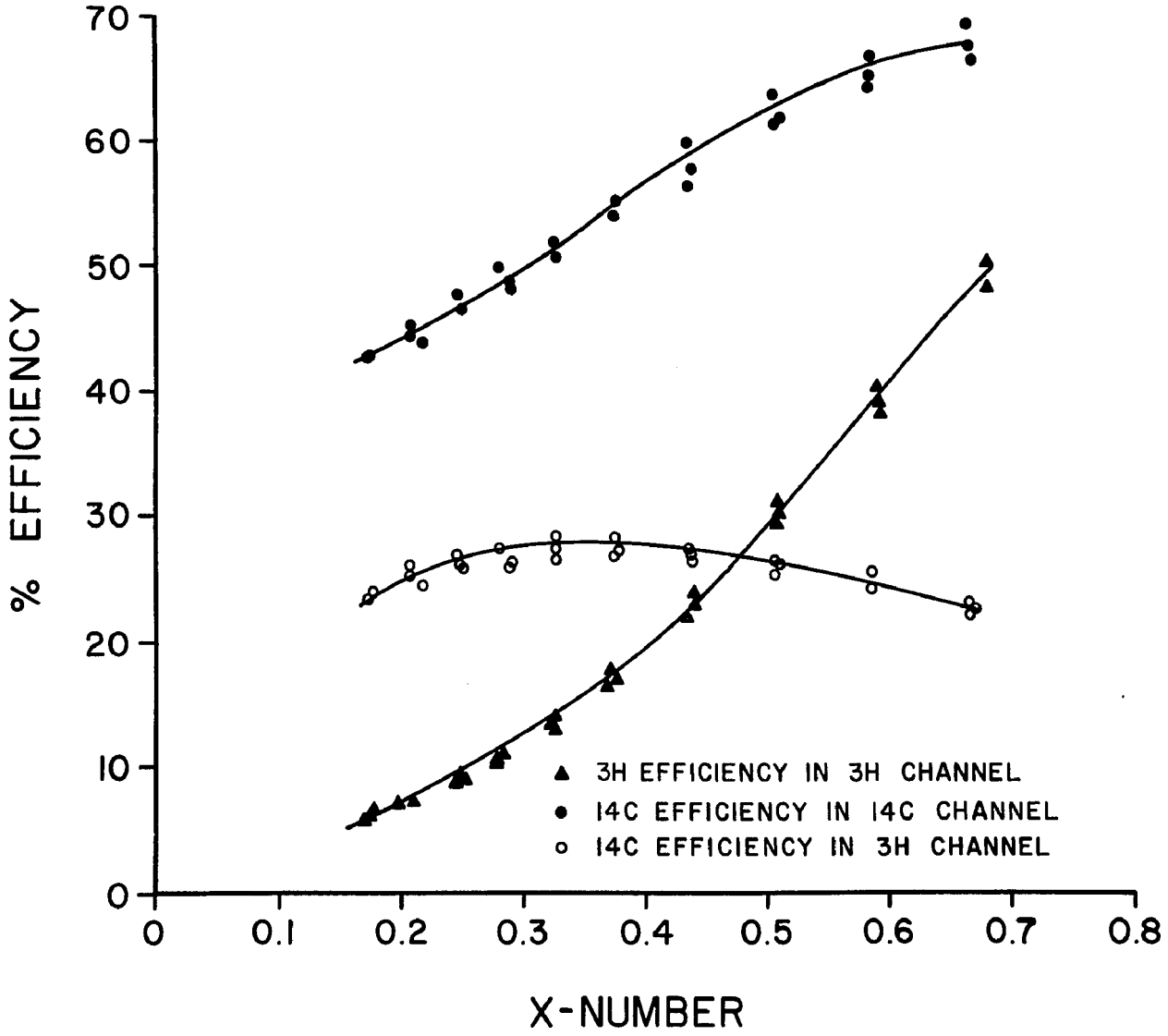
For the ^{14}C channel, a curve of background CPM versus AES (a measure of the degree of quenching with respect to an instrument standard) was generated by the procedure described for the Beckman LS-250. Similarly, efficiency (CPM/DPM) in this channel was determined as a function of AES using (^{14}C) toluene of known DPM. The following equation was used to calculate the efficiency curve shown in Figure 7:

Efficiency in ^{14}C Channel =

$$\frac{\text{CPM}(^{14}C)-(\text{Background CPM}(^{14}C))}{\text{DPM } (^{14}C) \text{ Toluene Standard})} \times 100 \qquad (12)$$

Figure 6. Efficiency Curves for Beckman LS-250 Liquid Scintillation System.

The automatic quench correction feature was used. ▲—▲ Efficiency of ^{3}H in Channel A. ●—●—● Efficiency of ^{14}C in Channel B. o—o—o Efficiency of ^{14}C in Channel A. See text for procedure of obtaining and calculating the curves.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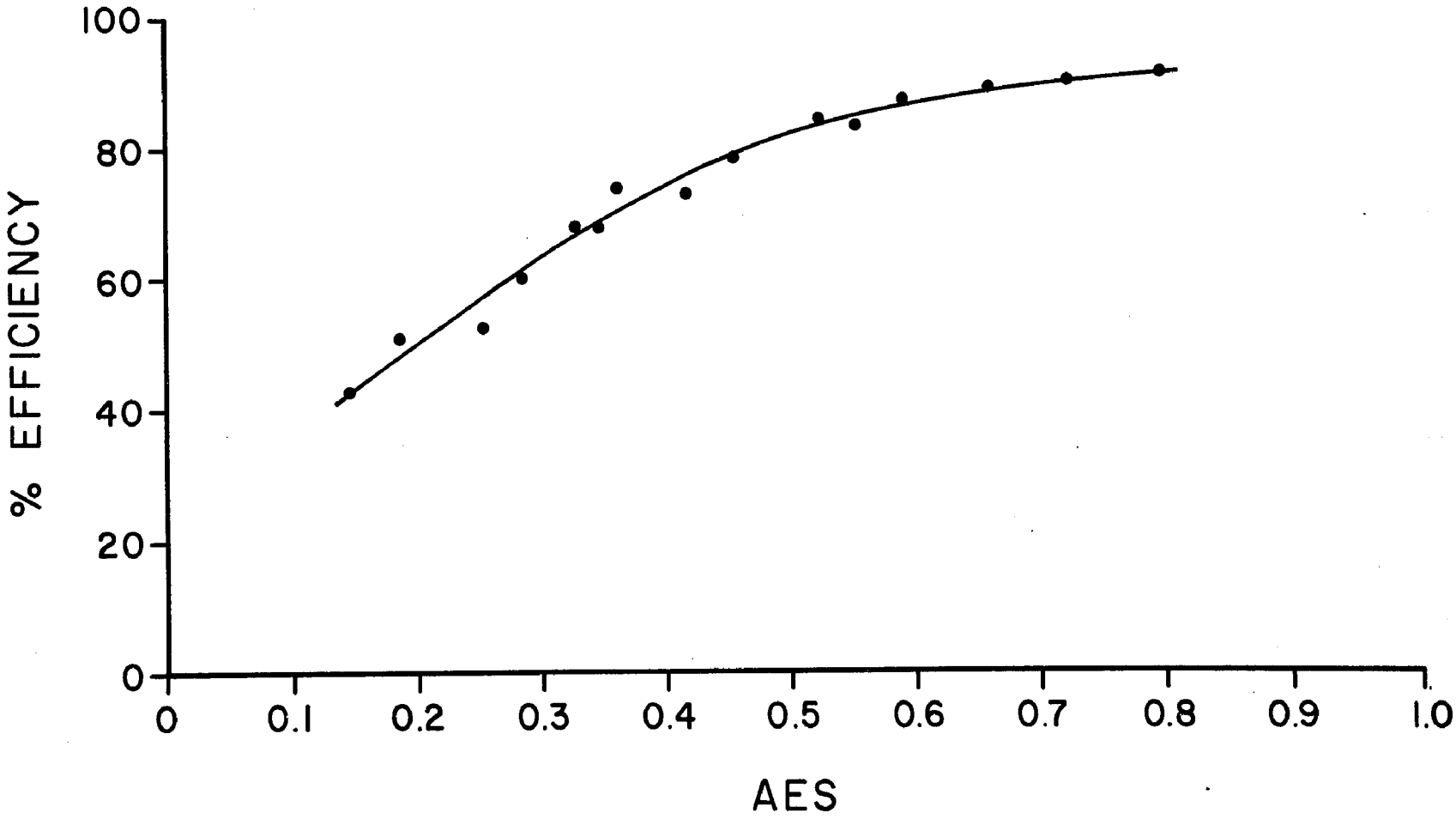
60
50
40
30
20
10
0
% EFFICIENCY
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
X-NUMBER
▲ 3H EFFICIENCY IN 3H CHANNEL
● 14C EFFICIENCY IN 14C CHANNEL
○ 14C EFFICIENCY IN 3H CHANNEL

Figure 7. ^{14}C Efficiency Curve for Packard Tri-Carb 3375 Liquid Scintillation Spectrometer.

See text for procedure of obtaining and calculating the curve.


100
80
60
40
20
0
% EFFICIENCY
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
AES

RESULTS

Standardization of Assays

Standard curves for incubation time and protein concentration for each enzyme assay were constructed by plotting liver and brain enzyme activities versus incubation time and protein concentration as described in Materials and Methods. Standard incubation times were selected from the linear region of the activity versus time curve. Standard protein concentrations were selected so that the activity under normal conditions was near the midpoint of the linear portion of the activity versus protein concentration curve in order that sensitivity to repression or induction of the enzyme be maximized.

The N^5,N^{10}-methylenetetrahydrofolic acid ($N^5N^{10}CH_2THF$) reductase derived from brain is linear in activity with respect to time to at least 30 minutes while the liver enzyme begins to show a slight departure from linearity around 20 minutes (Figure 8). Brain reductase activity under standard incubation conditions is linear with protein concentration through 3.5 milligrams (Figure 9). The activity of the liver enzyme is linear to about three milligrams of protein per incubation tube. From these data, the liver enzyme displays about two times the activity of

Figure 8. Activity versus Time Curves for N^5,N^{10}-Methyl-enetetrahydrofolic Acid Reductase in Rat Brain and Liver.

Postnuclear homogenates were prepared and assays performed as described in Materials and Methods. Incubations contained 225 µg and 960 µg of liver and brain protein, respectively. ●—●—● liver; ▲——▲ brain.

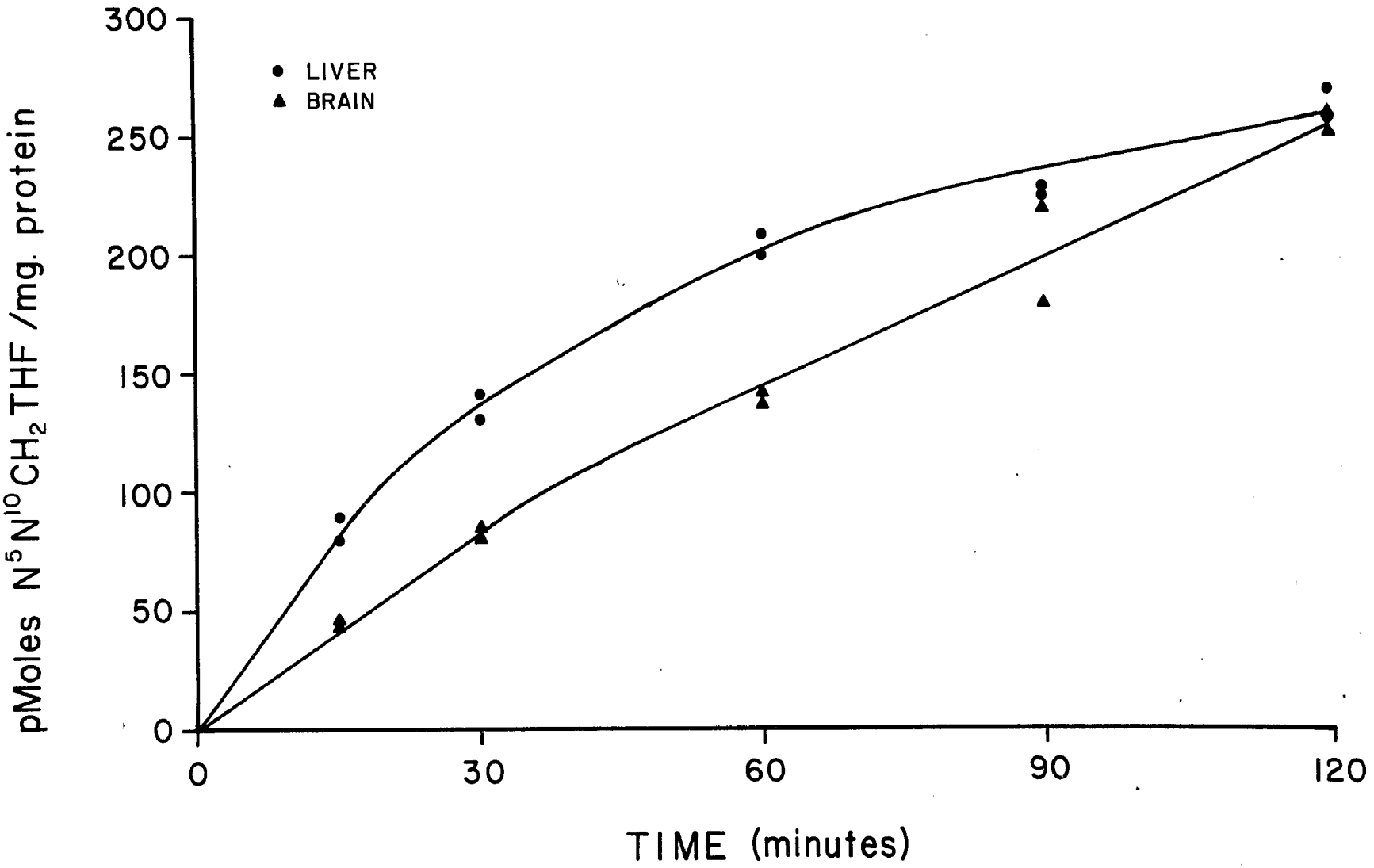
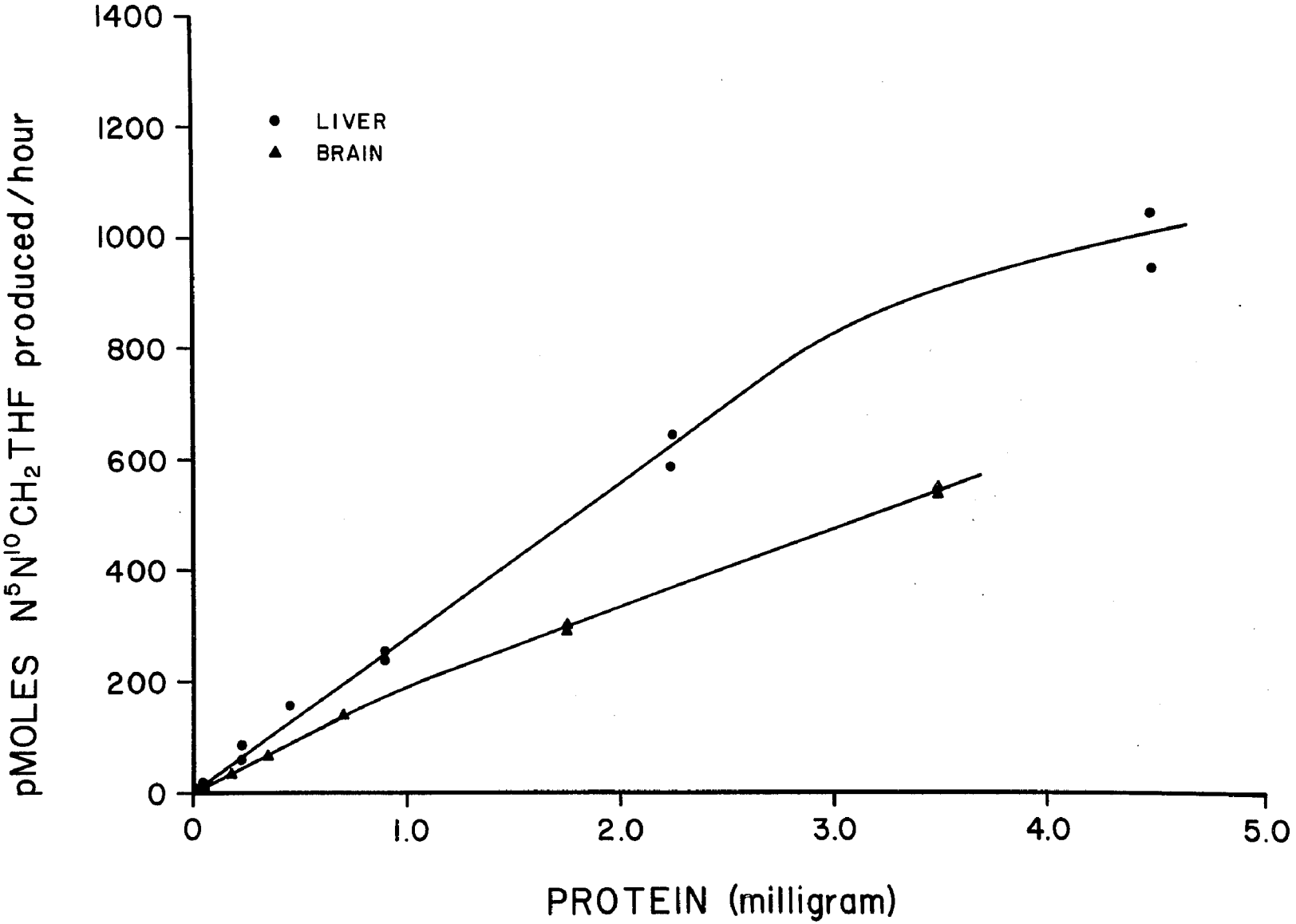

300
250
200
150
100
50
0
LIVER
BRAIN
pMoles $N^5N^{10}CH_2THF$ /mg. protein
0
30
60
90
120
TIME (minutes)

Figure 9. Activity versus Protein Concentration Curves for N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase in Rat Brain and Liver.

Postnuclear homogenates were prepared and assays performed as described in Materials and Methods. Incubations were 30 minutes for liver and brain. ●—●—● liver; ▲—▲ brain.


1400
1200
1000
800
600
400
200
0
LIVER
BRAIN
pMOLES $N^5N^{10}CH_2THF$ produced/hour
0
1.0
2.0
3.0
4.0
5.0
PROTEIN (milligram)

the enzyme derived from brain.

The activity of the N^5-methyltetrahydrofolic acid (N^5CH_3THF):homocysteine transmethylase derived from brain is linear through 165 minutes and the liver enzyme is linear through two hours (Figure 10). The liver enzyme is 2.1 times as active as the brain enzyme. The activity of the liver enzyme is linear to one milligram of protein (Figure 11). The transmethylase derived from brain shows an apparent nonlinear activity response at low protein concentrations. This may be partially due to decreased sensitivity of the assay in this region. The activity, however, is linear at higher protein concentrations going up to as high as the liver. The linear region from approximately one to two milligrams of protein was used as the standard protein concentration in the assay for this enzyme from brain.

Methionine adenosyltransferase (MAT) from liver is linear in activity with respect to time to one hour (Figure 12). The transferase from brain shows a linear response in activity with time to 100 minutes. The activity of the liver enzyme is approximately three times as active as the enzyme derived from brain. The liver enzyme is nonlinear in activity at low protein concentrations (Figure 13). This may again be partially due to an attenuation of assay sensitivity at low concentrations of enzyme. Sample readings under these conditions approach background. The linear region from approximately 0.8 to

Figure 10. Activity versus Time Curves for N^5-Methyltetrahydrofolic Acid:Homocysteine Transmethylase in Rat Brain and Liver.

Postnuclear homogenates were prepared and assays performed as described in Materials and Methods. Incubations contained 0.8 mg and 1.32 mg of liver and brain protein, respectively. ●—●—● liver; ▲—▲—▲ brain.

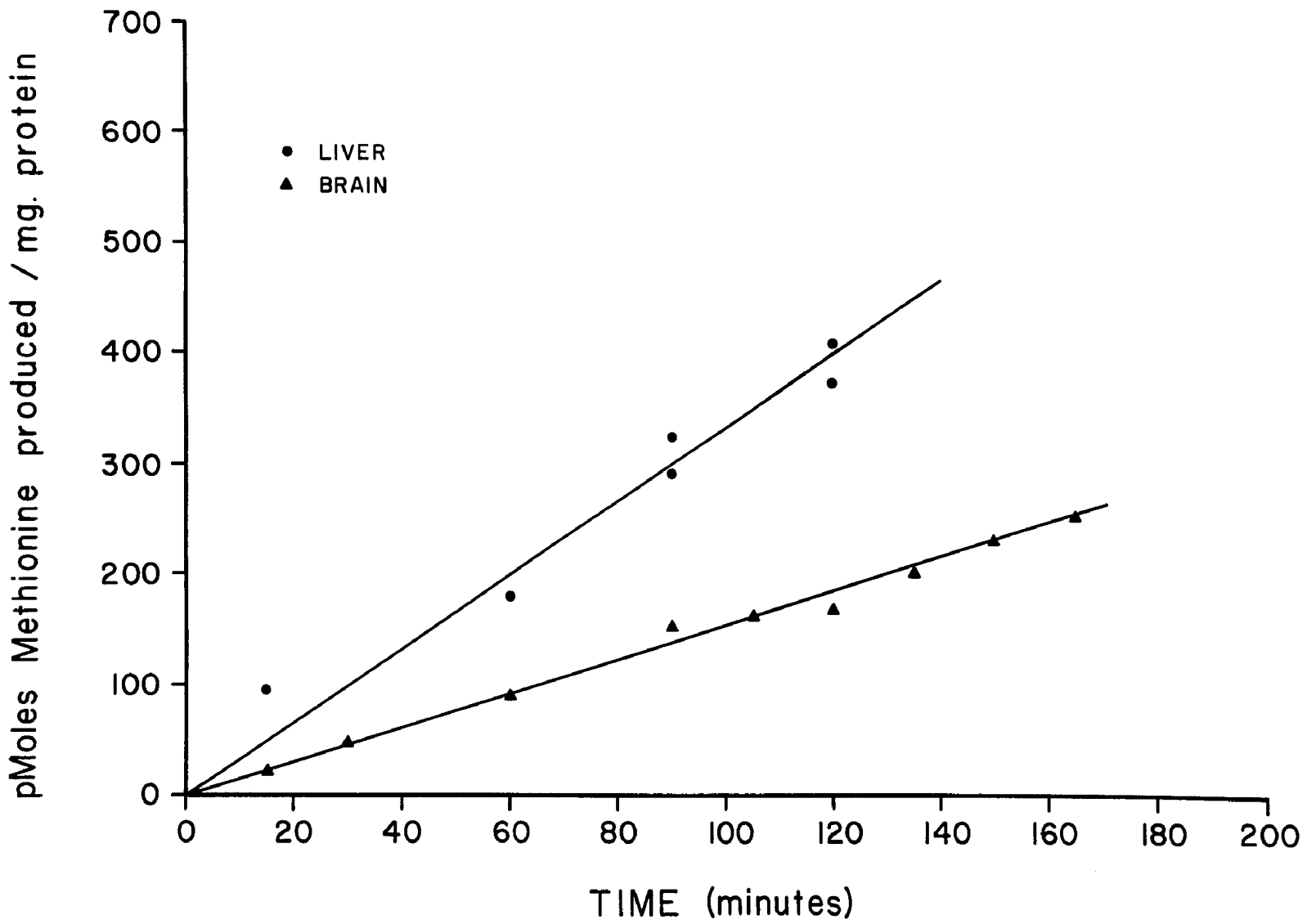

700
600
500
400
300
200
100
0
LIVER
BRAIN
pMoles Methionine produced / mg. protein
0
20
40
60
80
100
120
140
160
180
200
TIME (minutes)

Figure 11. Activity versus Protein Concentration Curves for N^5-Methyltetrahydrofolic Acid:Homocysteine Transmethylase in Rat Brain and Liver.

Postnuclear homogenates were prepared and assays performed as described in Materials and Methods. Incubations were 90 minutes for liver and brain. ●—●—● liver; ▲—▲—▲ brain.

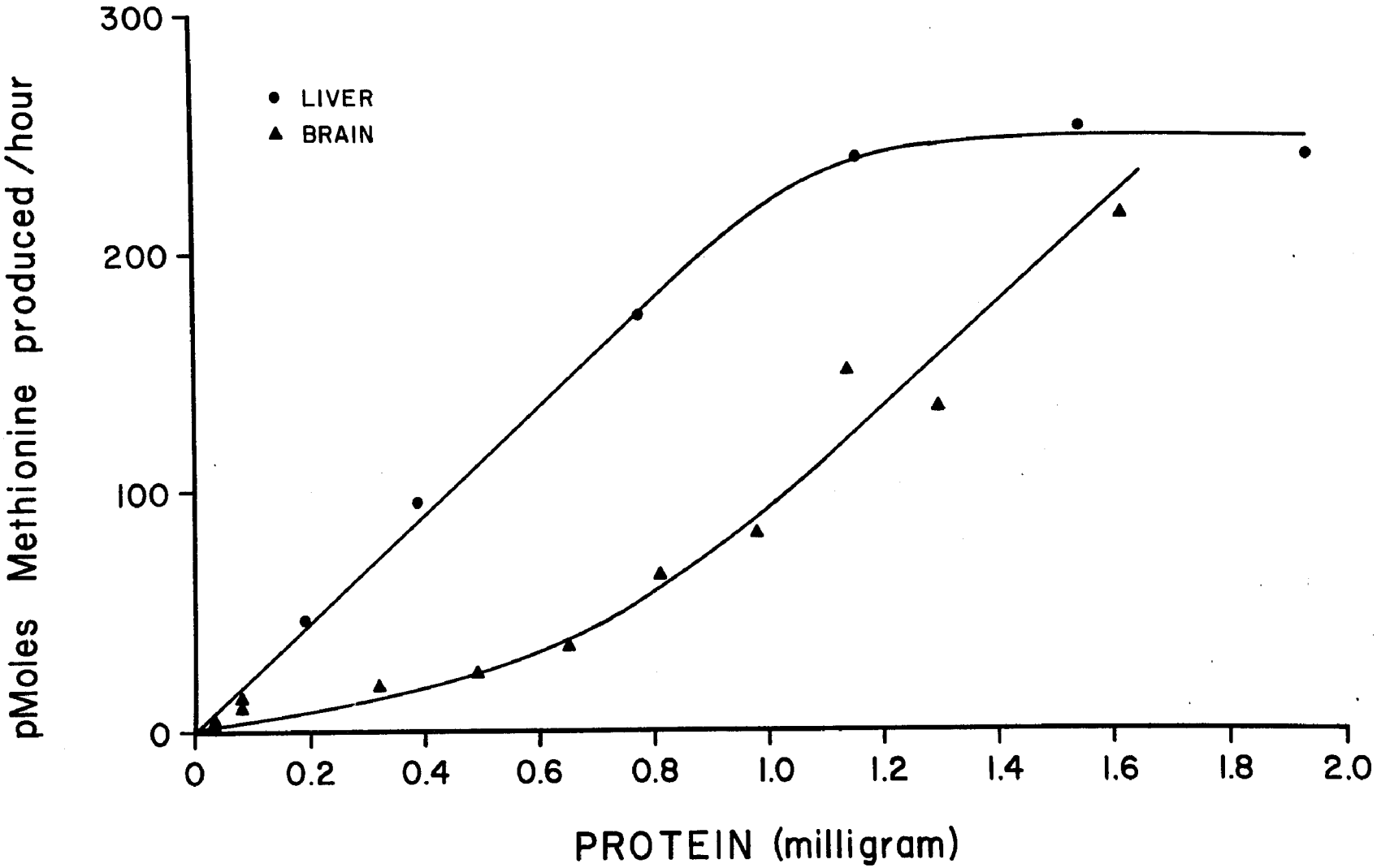

300
200
100
0
pMoles Methionine produced /hour
LIVER
BRAIN
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
PROTEIN (milligram)

Figure 12. Activity versus Time Curves for Methionine Adenosyltransferase in Rat Brain and Liver.

Postnuclear homogenates were prepared and assays performed as described in Materials and Methods. Incubations contained 1.5 mg protein for liver and brain. ●—●—● liver; ▲—▲—▲ brain.

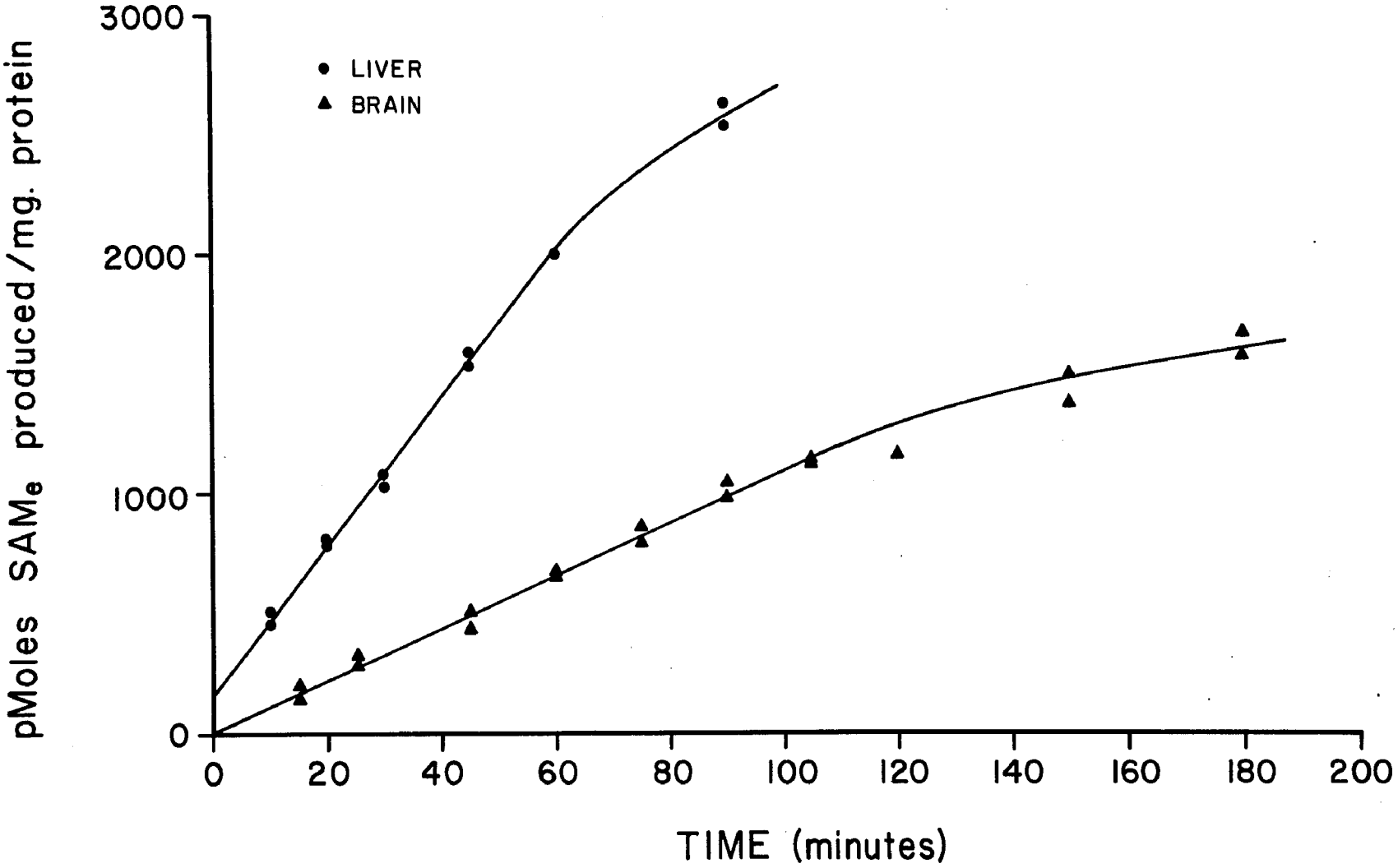
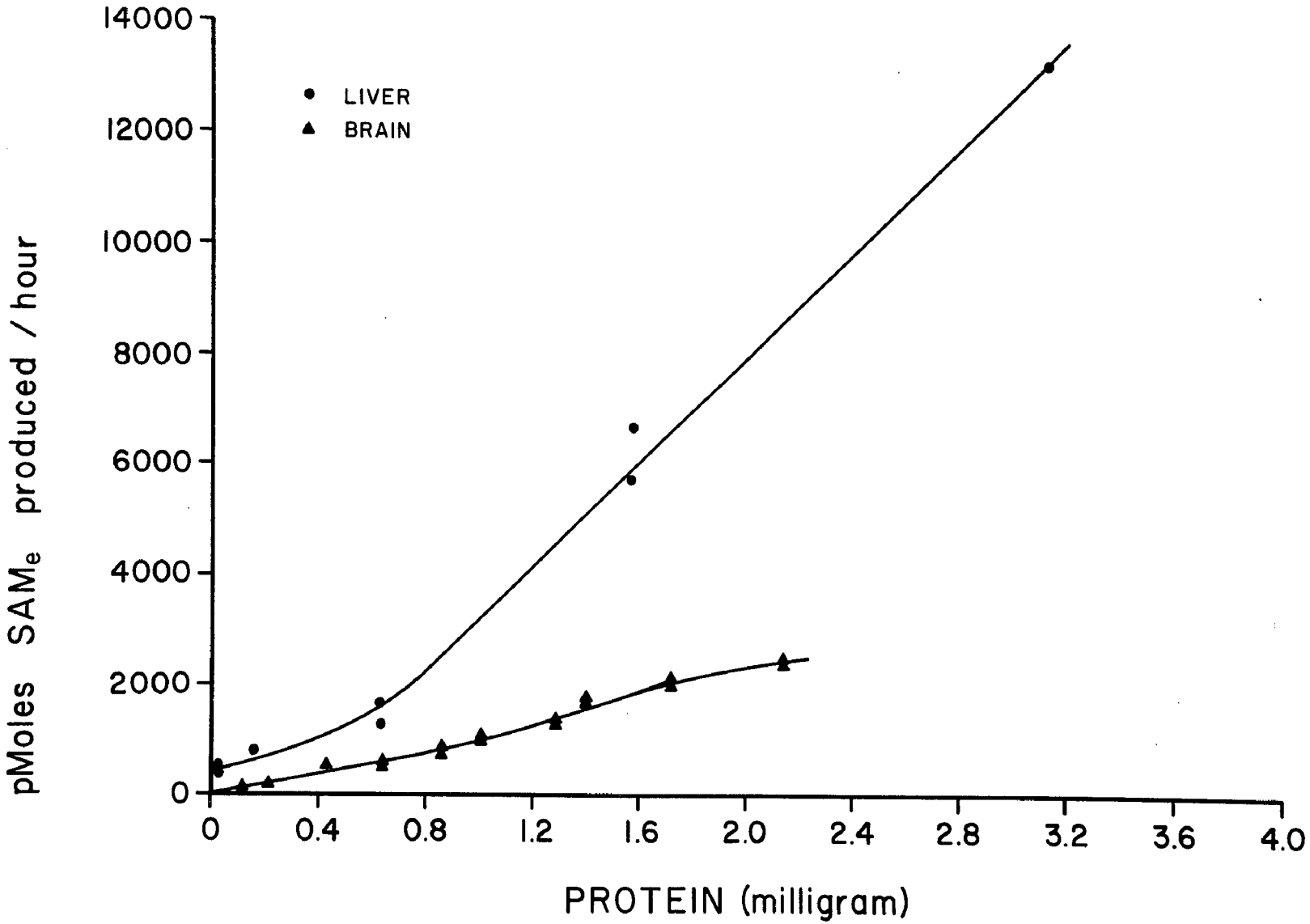

3000
2000
1000
0
LIVER
BRAIN
pMoles SAMe produced/mg. protein
0
20
40
60
80
100
120
140
160
180
200
TIME (minutes)

Figure 13. Activity versus Protein Concentration Curves for Methionine Adenosyltransferase from Rat Brain and Liver.

Postnuclear homogenates were prepared and assays performed as described in Materials and Methods. Incubation times were 90 minutes for brain and 60 minutes for liver. ●—●—● liver; ▲—▲—▲ brain.


14000
12000
10000
8000
6000
4000
2000
0
LIVER
BRAIN
pMoles SAMe produced / hour
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
4.0
PROTEIN (milligram)

3.2 milligrams of protein was used as the standard protein concentration in the assay for this enzyme from liver. The brain activity is essentially linear to about 1.6 milligrams protein (Figure 13).

The calibration curve used for assaying tissue levels of S-adenosylmethionine (SAMe) is shown in Figure 14. The curve was obtained by the procedure described in Materials and Methods. The method is based on the isotope dilution of (^{14}C-methyl)SAMe which reacts with N-(1,2-^{3}H(N)) acetylserotonin to form (1,2-^{3}H(N), ^{14}C-methyl)-melatonin. The reaction is catalyzed by hydroxyindole-O-methyltransferase (HIOMT). The calibration curve is corrected for endogenous levels of SAMe present in the tissues used for generation of the curve. The ^{3}H:^{14}C ratio is a linear function of liver or brain SAMe levels through 250 µg of SAMe per gram of liver or brain.

The calibration curves for the assay of tissue levels of methionine were obtained by the procedure outlined in Materials and Methods. The curve which defines the relationship between the calculated ^{3}H:^{14}C ratio and the incubation concentration of methionine is shown in Figure 15. Plotting the reciprocal of the ^{3}H:^{14}C ratio versus the incubation methionine concentration yields a linear relationship between ten and forty micromolar methionine (Figure 16). Tissues assayed for methionine were pre-diluted as necessary to enable methionine concentrations to be determined within the range defined by this curve.

Figure 14. Standard Curve for S-Adenosylmethionine Assay.

The curve was constructed by the procedure detailed in Materials and Methods and used for assaying S-adenosyl-methionine levels in brain and liver from methionine loaded rats and saline injected controls.

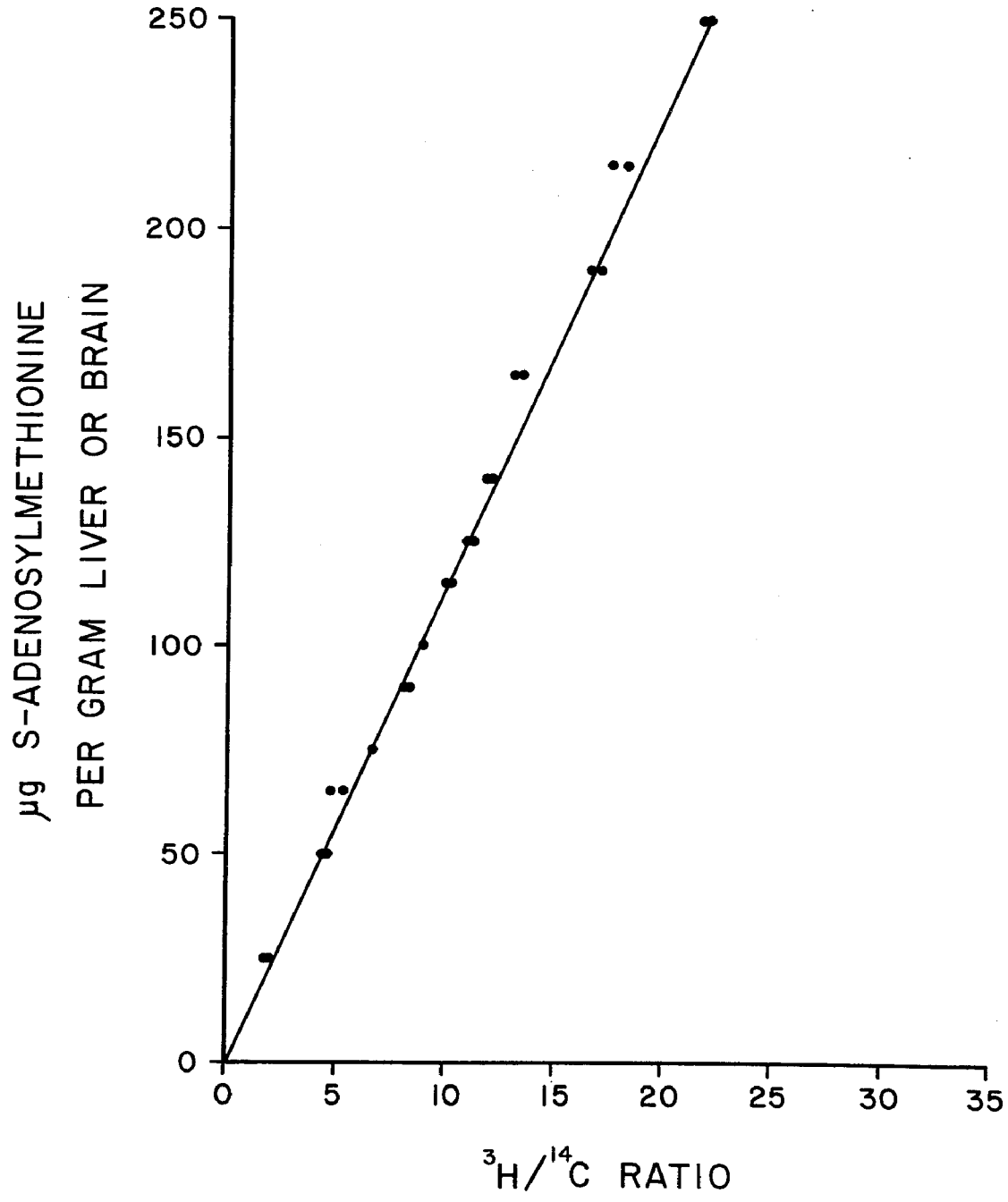

250
200
150
100
50
0
0
5
10
15
20
25
30
35
μg S-ADENOSYLMETHIONINE
PER GRAM LIVER OR BRAIN
$^{3}H/^{14}C$ RATIO

Figure 15. Methionine Concentration as a Function of $^{3}H:^{14}C$ Ratio in Methionine Adenosyltransferase Dependent Methionine Assay.

Assay conditions were defined in Materials and Methods. $^{3}H:^{14}C$ ratio is plotted versus incubation methionine concentration.

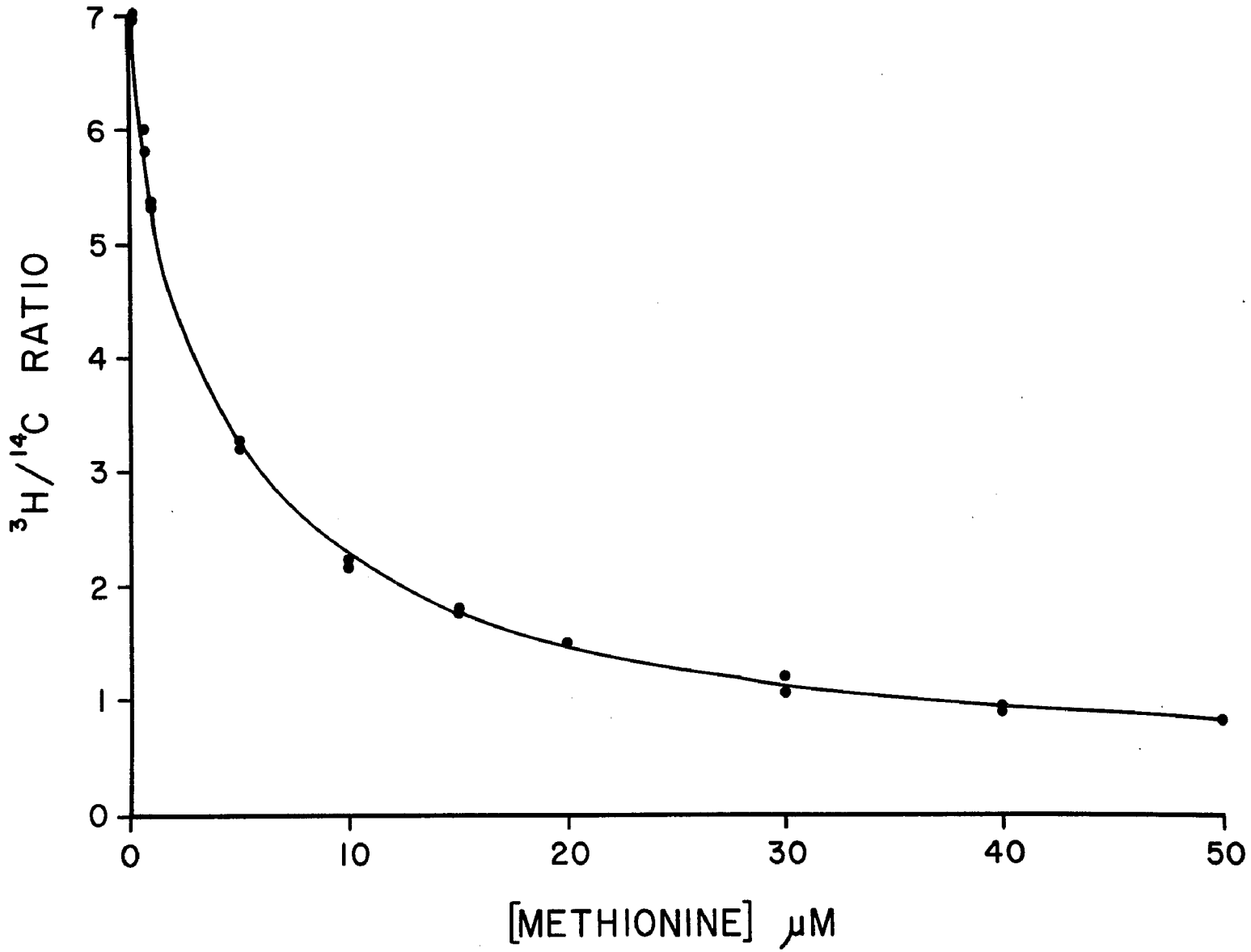
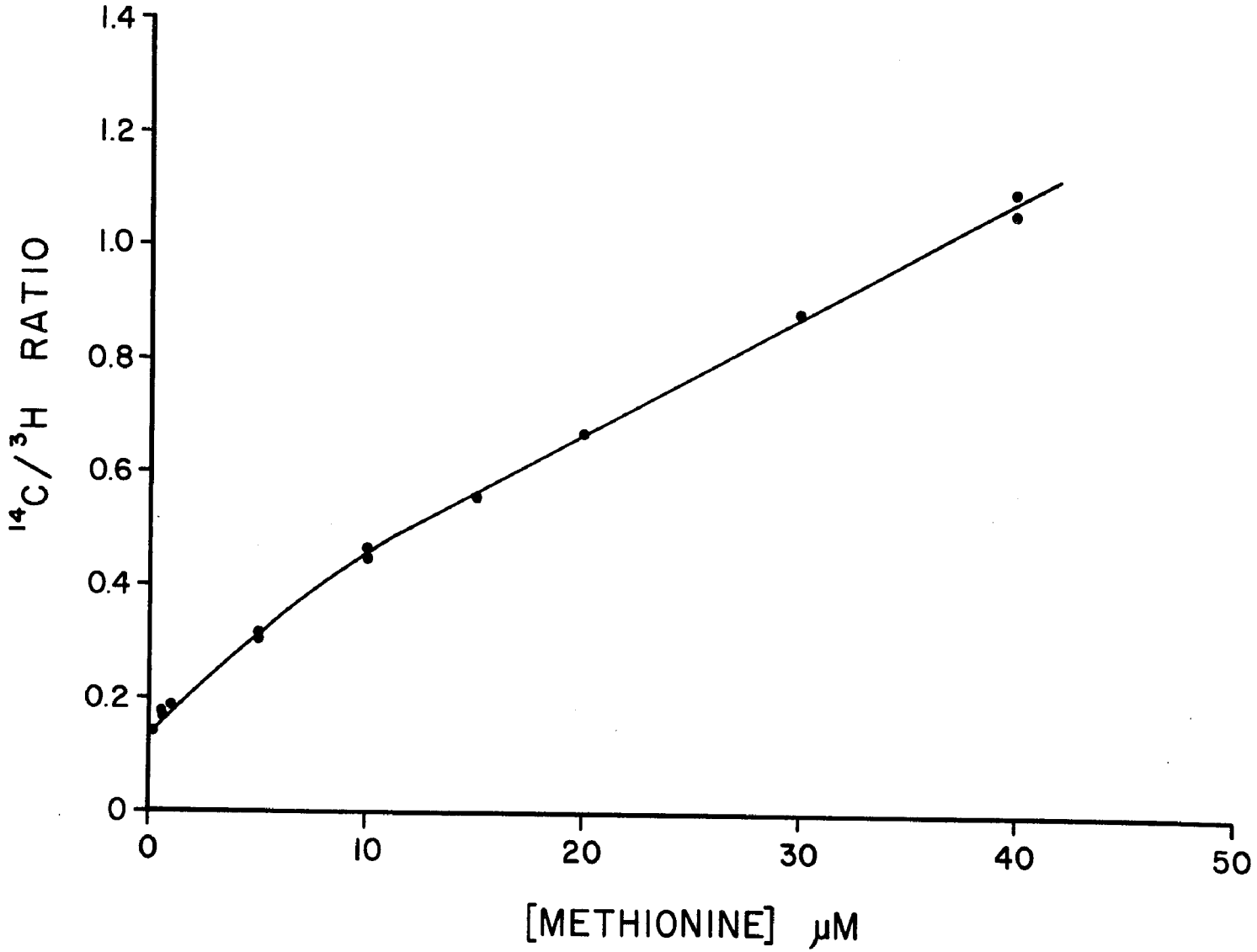

7
6
5
4
3
2
1
0
$^{3}H/^{14}C$ RATIO
0
10
20
30
40
50
[METHIONINE] μM

Figure 16. Typical Calibration Curve for Methionine Assay.

This curve served as the calibration for assaying methionine levels in brain and liver from methionine loaded rats and saline injected controls. The reciprocal of the ordinate shown in Figure 15 is plotted versus incubation methionine concentration.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
$^{14}C/^{3}H$ RATIO
0
10
20
30
40
50
[METHIONINE] μM

Methionine Loading

Effect on Enzyme Activities

Rat brain activities of N^5,N^{10}-methylenetetrahydrofolic acid reductase, N^5-methyltetrahydrofolic acid:homocysteine transmethylase, and methionine adenosyltransferase in response to L-methionine loading and saline injection are shown in Table I. The corresponding activities in rat liver are given in Table II. The $N^5N^{10}CH_2THF$ reductase activity in brain is not altered. The activity of this enzyme in liver of methionine loaded rats is 26 percent of the activity in liver from saline injected controls. Dialysis abolishes the inhibition in liver and results in a 33 percent increase in the activity of $N^5N^{10}CH_2THF$ reductase in this tissue from saline injected rats (Table II). This suggests the presence of an endogenous inhibitor. The N^5CH_3THF:homocysteine transmethylase was unaffected by methionine loading in either brain or liver.

Experiments were designed to measure changes in methionine adenosyltransferase activity due either to changes in enzyme concentration or changes in substrate concentration. Under relatively high concentrations of added methionine, 67 μM, the contribution of methionine to the incubation from the tissue to be assayed is minimized. Assays performed under these conditions should detect differences in enzyme concentration (methionine

Table I. Rats were treated with methionine or saline as described in Materials and Methods and sacrificed one hour after final injection. Enzyme assays are described in Materials and Methods. Values are expressed as means ± standard deviation. Numbers in parentheses designate number of rats from which data were derived. Probabilities were calculated using Student's t-test. For methionine adenosyltransferase (a), incubation concentrations of L-methionine, corrected for methionine present in enzyme samples, are 96 μM and 70 μM for methionine and saline injected rats, respectively. For methionine adenosyltransferase (b), incubation concentrations of L-methionine, corrected for methionine present in enzyme samples, are 29 μM and 2.8 μM for methionine and saline injected rats, respectively. Dialysis of methionine adenosyltransferase was against 60 volumes of 0.32 M sucrose, overnight, in cold with six changes. Subsequent incubation was in presence of 67 μM L-methionine.

Table I. Response of One-Carbon Pathway Enzymes to Methionine Loading in Rat Brain

nmoles/mg protein/hour

ENZYME	METHIONINE	SALINE	P
$N^5N^{10}CH_2THF$ Reductase	0.177 ± 0.049 (20)	0.174 ± 0.053 (20)	>0.1
N^5CH_3THF:Homocysteine Transmethylase	0.070 ± 0.022 (12)	0.063 ± 0.019 (12)	>0.1
Methionine Adenosyltransferase			
(a)	2.34 ± 1.81 (8)	1.28 ± 0.62 (8)	>0.1
(b)	0.80 ± 0.16 (4)	0.22 ± 0.14 (4)	<0.01
Dialyzed	0.45 ± 0.05 (8)	0.40 ± 0.04 (8)	>0.05

Table II. Rats were treated with methionine or saline as described in Materials and Methods and sacrificed one hour after final injection. Enzyme assays are described in Materials and Methods. Values are expressed as means ± standard deviation. Numbers in parentheses designate number of rats from which data were derived. Probabilities were calculated using Student's t-test. Dialysis of $N^5N^{10}CH_2THF$ reductase was against 60 volumes of 0.32 M sucrose, overnight, in cold with six changes. For methionine adenosyltransferase (a), incubation concentrations of L-methionine, corrected for methionine present in enzyme samples are 104 μM and 92 μM, respectively. For methionine adenosyltransferase (b), incubation concentrations of L-methionine, corrected for methionine present in enzyme samples, are 37 μM and 15 μM for methionine and saline injected rats, respectively. Dialysis of methionine adenosyltransferase was the same as $N^5N^{10}CH_2THF$ reductase dialysis. Subsequent incubation was in presence of 67 μM methionine.

Table II. Response of One-Carbon Pathway Enzymes to Methionine Loading in Rat Liver.

nmoles/mg protein/hour

ENZYME	METHIONINE	SALINE	P
$N^5N^{10}CH_2THF$ Reductase	0.072 ± 0.027 (12)	0.283 ± 0.064 (12)	<0.001
Dialyzed	0.307 ± 0.057 (4)	0.377 ± 0.074 (4)	>0.1
N^5CH_3THF:Homocysteine Transmethylase	0.086 ± 0.007 (4)	0.100 ± 0.023 (4)	>0.1
Methionine Adenosyltransferase			
(a)	4.63 ± 1.89 (8)	4.01 ± 0.58 (8)	>0.1
(b)	3.03 ± 1.82 (4)	0.46 ± 0.13 (4)	<0.05
Dialyzed	0.10 ± 0.03 (8)	0.11 ± 0.03 (8)	>0.1

adenosyltransferase (a) in Tables I and II). This assay condition results in no significant difference in enzyme activity due to methionine loading for either the brain or liver enzyme. Assays performed under conditions of low concentration of exogenous methionine, 0.15 μM, should detect alterations in enzyme activity due to changes in endogenous methionine concentration (methionine adenosyltransferase (b) in Tables I and II). Table I shows a 2.6-fold increase in brain enzyme activity under these conditions. The corresponding increase in activity of the liver enzyme (Table II) is 5.5-fold. All methionine adenosyltransferase activities were calculated after correction for the contribution of endogenous methionine to the incubation concentration. Tables I and II also show that dialysis of the postnuclear homogenates from liver and brain results in a considerable decrease in methionine adenosyltransferase activity when assayed at 67 μM methionine.

Effect on Levels of Methionine and S-Adenosylmethionine

The restoration of liver $N^5N^{10}CH_2THF$ reductase activity by dialysis and the report by Kutzbach and Stokstad (1967) of *in vitro* inhibition of this enzyme in rat liver by SAMe led to measurement of tissue SAMe levels. Tissue levels of methionine were also assayed since this quantitation was required for calculating the actual methionine concentration in the incubation for the methionine adenosyltransferase assay. This concentration was then used to determine the specific activity of methionine in the

incubation which in turn is related to the enzyme activity.

The concentration of methionine in brain of methionine loaded rats increases by 964 percent as determined by the radiochemical assay (Table III). The concentration of S-adenosylmethionine displays only a ten percent increase in this tissue which is not statistically significant. A reciprocal effect occurs in the liver where methionine concentration increases by 139 percent (Table III, radiochemical assay) while the concentration of S-adenosylmethionine increases by 609 percent. Samples were subjected to amino acid analysis to double check the values of methionine obtained by the radiochemical technique. By this method, methionine levels increased by 545 percent in brain in response to methionine loading. The corresponding increase in liver was 95 percent. The lower net increases in tissue methionine obtained by this method may be due to differences in preparatory procedures applied to the tissue.

Saturation of Methionine Adenosyltransferase by L-Methionine

Calculation of the contribution of tissue methionine (Table III, radiochemical assay) to the methionine concentration in the methionine adenosyltransferase assay yields an additional 29 μM and 2.8 μM methionine from brains of methionine and saline injected rats, respectively. For livers of methionine and saline injected rats, the tissue

Table III. Rats were injected with methionine or saline as described in Materials and Methods and sacrificed one hour after final injection. Methionine and S-adenosyl-methionine (SAMe) were assayed as described in Materials and Methods. Preparation of samples for amino acid analysis is given in Materials and Methods. Values are expressed as means $\pm$ standard deviation. Numbers in parentheses indicate number of rats from which data were derived. Probabilities were calculated using Student's t-test.

Table III. Brain and Liver Methionine and S-Adenosylmethionine After Methionine Loading

μmoles/gram tissue

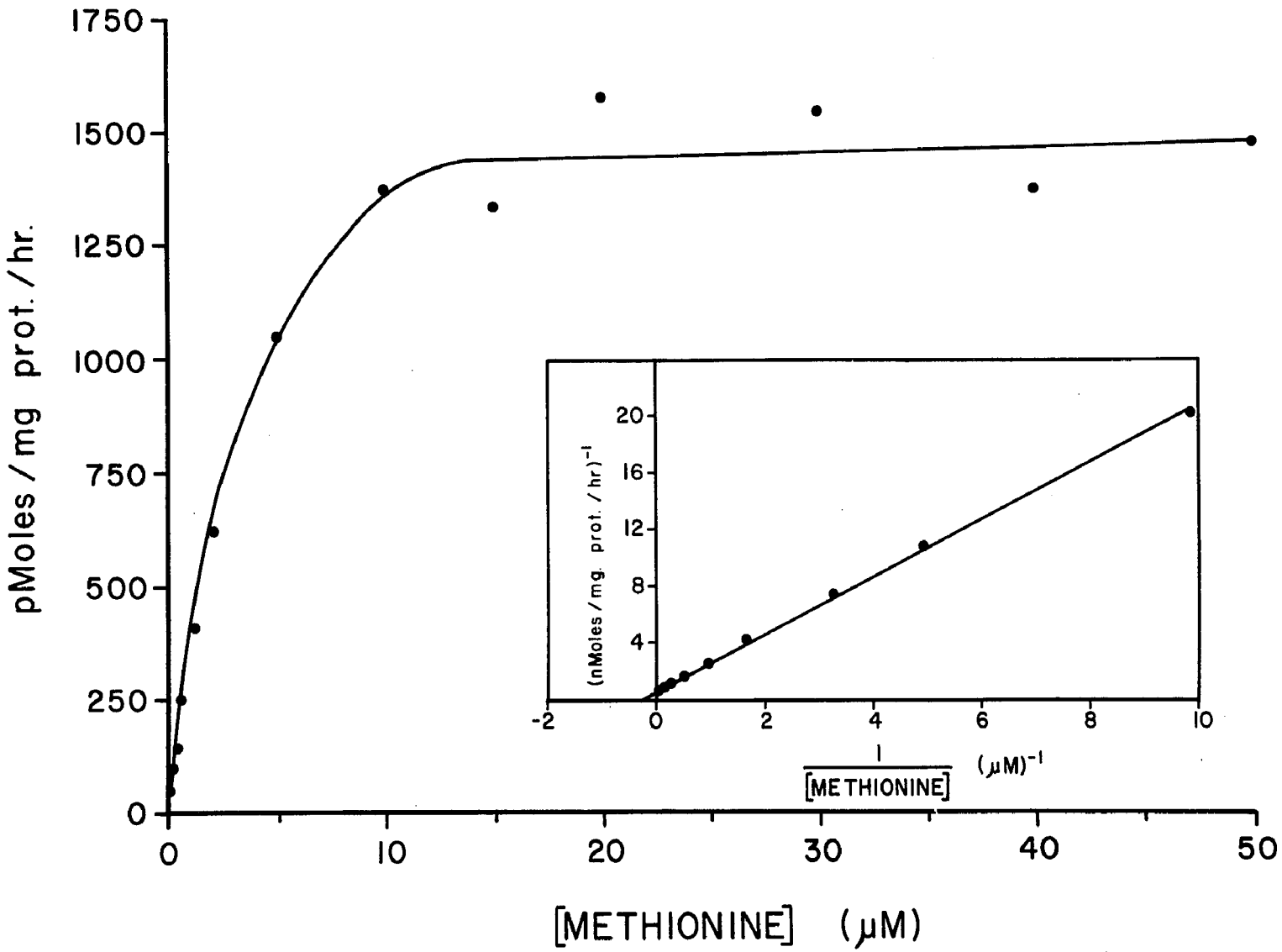
	BRAIN			LIVER		
	METHIONINE	SALINE	P	METHIONINE	SALINE	P
Methionine						
Radiochemical	1.17±0.23 (7)	0.11±0.06 (8)	<0.001	1.46±0.55 (8)	0.61±0.11 (8)	<0.001
Amino Acid Analysis	0.71	0.11	-	1.85	0.95	-
SAMe	0.053±0.005(8)	0.048±0.07 (8)	>0.1	0.78 ±0.08 (8)	0.11±0.02 (8)	<0.001

contributions are 37 μM and 15 μM, respectively. When using an additional 0.15 μM (^{3}H-methyl)-methionine to assay methionine adenosyltransferase activity, it was noted that the 1.4-fold increase in methionine concentration for the liver assay stimulated a 5.5-fold increase in methionine adenosyltransferase activity (Table II, methionine adenosyltransferase (b)). In brain, the 9.6-fold increase in methionine concentration stimulated only a 2.6-fold increase in the activity of this enzyme (Table I, methionine adenosyltransferase (b)). Because of the difference in response of the enzyme to elevation of substrate concentration in brain and liver, analyses of the methionine concentration dependence of the enzyme from both sources were undertaken.

A saturation curve for brain methionine adenosyltransferase is shown in Figure 17. The curve is hyperbolic in shape. The inset of Figure 17 is a Lineweaver-Burk plot of the same data. Previous results indicate a 2.6-fold increase in enzyme activity between 2.9 μM and 29 μM methionine (Table I, methionine adenosyltransferase (b)). This is clearly not in accord with Figure 17. Over the same concentration range in Figure 17, an approximate increase of only 0.7-fold is expected with the enzyme being completely saturated at 29 μM. It should be noted that the enzyme source used for generation of Figure 17 was dialyzed. Brain samples from methionine and saline

Figure 17. Substrate Dependence of Methionine Adenosyltransferase Activity in Rat Brain.

The enzyme was assayed as described in Materials and Methods using dialyzed (4X vs. 500 volumes 0.10 M sucrose) 100,000 g supernatant from normal, adult, male, rat brain as enzyme source. Inset is a Lineweaver-Burk plot over the range of methionine concentrations used.

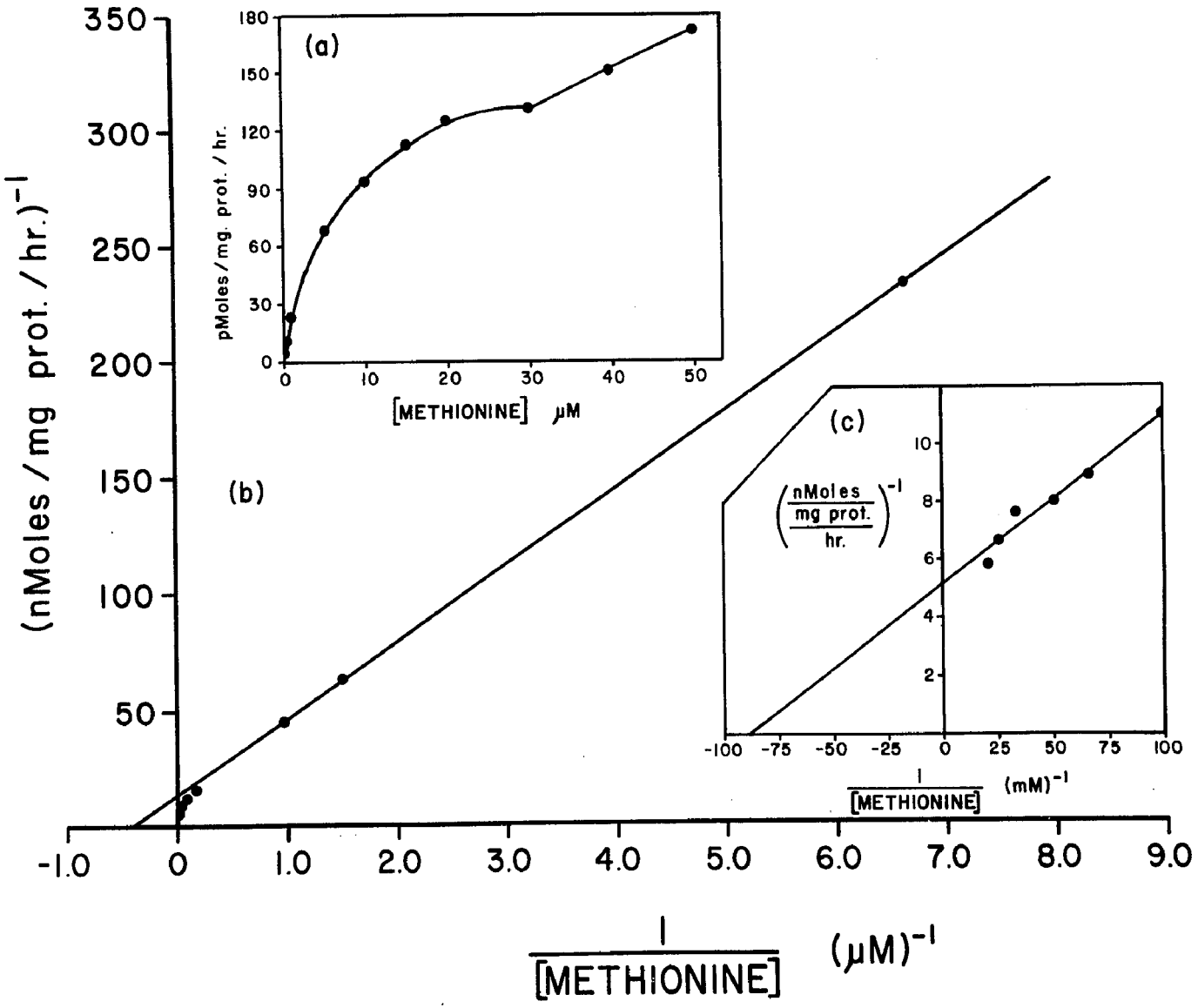

1750
1500
1250
1000
750
500
250
0
pMoles / mg prot. / hr.
0
10
20
30
40
50
[METHIONINE] (μM)
20
16
12
8
4
(nMoles / mg. prot. / hr)⁻¹
-2
0
2
4
6
8
10
1
[METHIONINE]
(μM)⁻¹

injected rats were not subjected to this procedure. Therefore, Figure 17 may not be representative of the undialyzed enzyme. It has already been stated that dialysis of the postnuclear homogenate results in a considerable decrease in methionine adenosyltransferase activity. An alternate explanation is that the determinations of free methionine by the techniques used for its assay may be spuriously high. Specifically, the effects of tissue preparation on the values of tissue methionine reported by the two methods employed have not been evaluated. A 2.6-fold increase in enzyme activity can result from a 9.6-fold increase in methionine concentration if the latter increase were to occur over a lower concentration range, as can be seen from Figure 17.

A saturation curve for liver methionine adenosyltransferase is shown in Figur 18(a). Figure 18(b) is a Lineweaver-Burk plot of the saturation curve in the methionine concentration range of 0.15-50 μM. The Lineweaver-Burk plot in the concentration region of 10-50 μM is shown in Figure 18(c). Methionine adenosyltransferase, assayed under low incubation concentrations of methionine, indicated a 5.5-fold increase in liver activity between 15 μM and 37 μM methionine. A sigmoidal saturation curve which is suggested by these data is in agreement with the experimentally derived saturation curve (Figure 18(a)). While an upward inflection in this curve does occur around 30 μM methionine, the curve does not

Figure 18. Saturation and Lineweaver-Burk Plots for Liver Methionine Adenosyltransferase.

The enzyme was assayed as described in Materials and Methods using dialyzed (4X vs. 500 volumes 0.10 M sucrose) 100,000 g supernatant from normal, adult, male rat liver as enzyme source. (a) Saturation curve of liver methionine adenosyltransferase in concentration range of 0.15-50 μM methionine. (b) Lineweaver-Burk plot for the range of methionine concentration, 0.15-50 μM. (c) Lineweaver-Burk plot for the range of methionine concentration, 10-50 μM.


(a)
pMoles / mg. prot. / hr.
[METHIONINE] μM
(b)
(c)
(nMoles / mg prot. / hr.)⁻¹
1 / [METHIONINE] (μM)⁻¹
1 / [METHIONINE] (mM)⁻¹

predict a 5.5-fold increase in enzyme activity in the range from 15 μM to 37 μM methionine. The same comments made for the brain enzyme may be relevant here. Figure 18(a) was obtained by dialysis of the liver enzyme whereas tissues assayed for methionine adenosyltransferase were not treated in this manner. Additionally, the methionine assays employed may be accurate relative measures of methionine, but experimental error may have been introduced into the absolute determination by virtue of tissue preparation required for the assay. Figure 18(a) covers the range of methionine concentration up to 50 μM. Due to the nature of the assay used, concentrations above 50 μM substantially decrease the specific activity of (^{3}H-methyl)-methionine so as to provide unreliable results at higher concentrations. A downward deflection of the Lineweaver-Burk plot (Figure 18(b)) is seen at higher concentration indicative of negative cooperativity of the enzyme for methionine. Expanding the plot for high methionine concentrations (10-50 μM) permits the calculation of an apparent Km for the enzyme with respect to methionine over this concentration range (Figure 18(c)) which differs from the Km calculated for a much lower concentration range (Figure 18(b)). This behavior is in contrast to the brain enzyme for which the double reciprocal plot is linear over the entire range of methionine concentrations (Figure 17). The Km's and Vmax's derived from the Lineweaver-Burk plots of Figures 17 and 18 are given in

Table IV. The methionine adenosyltransferase was assayed as described in Materials and Methods. Apparent constants were calculated from the Lineweaver-Burk plots using the following equations:

$$Km = -\frac{1}{\text{Abscissal Intercept}}$$

$$Vmax = \frac{Km}{\text{Slope}}$$

Table IV. Kinetic Parameters for Methionine Adenosyltransferase in Rat Brain and Liver

METHIONINE CONCENTRATION (μM)	BRAIN Km (M)	BRAIN Vmax $\frac{\text{nmoles}}{\text{mg protein}}{\text{hour}}$	LIVER Km (M)	LIVER Vmax $\frac{\text{nmoles}}{\text{mg protein}}{\text{hour}}$
0.15-1.5	4.0×10^{-6}	2.0	2.6×10^{-6}	0.08
10-50	-	-	1.2×10^{-5}	0.20

Table IV. An increase in the apparent Km's and Vmax's for the liver enzyme is indicated while the brain enzyme has constant kinetic parameters throughout the methionine concentration range of 0.15-50 μM.

Inhibition of N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase by S-Adenosylmethionine

The saturation curve of liver $N^5N^{10}CH_2THF$ reductase is hyperbolic with respect to N^5CH_3THF concentration (Figure 19). The inset of Figure 19 is a Lineweaver-Burk plot of the same data. The inhibition of $N^5N^{10}CH_2THF$ reductase activity in liver of methionine injected rats appears to be due to the six-fold increase in SAMe levels in this tissue (Table III). To investigate the nature of this inhibition, Lineweaver-Burk plots of $N^5N^{10}CH_2THF$ reductase activity versus N^5CH_3THF concentration for different incubation concentrations of SAMe were generated. These studies indicate that SAMe inhibition of this enzyme is of the classical noncompetitive type in both brain (Figure 20) and liver (Figure 21). Calculation of the apparent Km's, Vmax's, and K_I (SAMe)'s from these plots (Table V) indicates that the reductase concentration is higher in liver than brain by 1.3-fold [Vmax(liver) = 2.3 Vmax(brain)]. This is in agreement with the approximate one-fold higher liver activity over brain determined from the standardization curves for this enzyme. Table V also shows that the Km for N^5CH_3THF and the K_I for SAMe are

Figure 19. Dependence of N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase Activity on Substrate (N^5CH_3THF) Concentration in Rat Liver.

Tissue preparation and assay procedure are detailed in Materials and Methods. Inset is a Lineweaver-Burk plot of the same data.

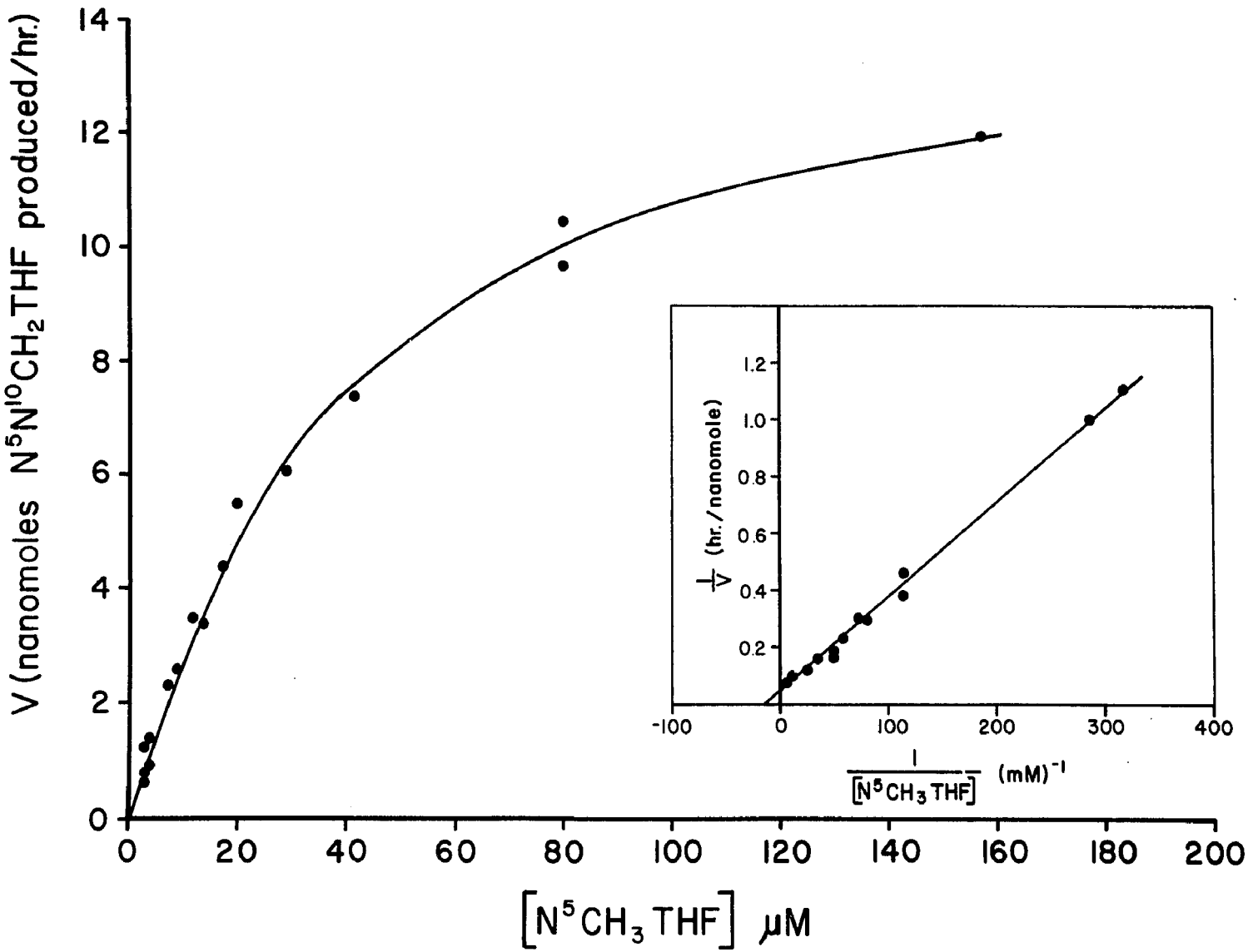

V (nanomoles $N^5N^{10}CH_2THF$ produced/hr.)
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
160
180
200
$[N^5CH_3THF]$ μM
$\frac{1}{V}$ (hr./nanomole)
1.2
1.0
0.8
0.6
0.4
0.2
-100
0
100
200
300
400
$\frac{1}{[N^5CH_3THF]}$ (mM)$^{-1}$

Figure 20. Lineweaver-Burk Plots of Rat Brain N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase Activity versus N^5CH_3THF Concentration in Presence of SAMe.

The enzyme was assayed as described in Materials and Methods. Postnuclear homogenate of normal, adult, male rat brain was used as the enzyme source.

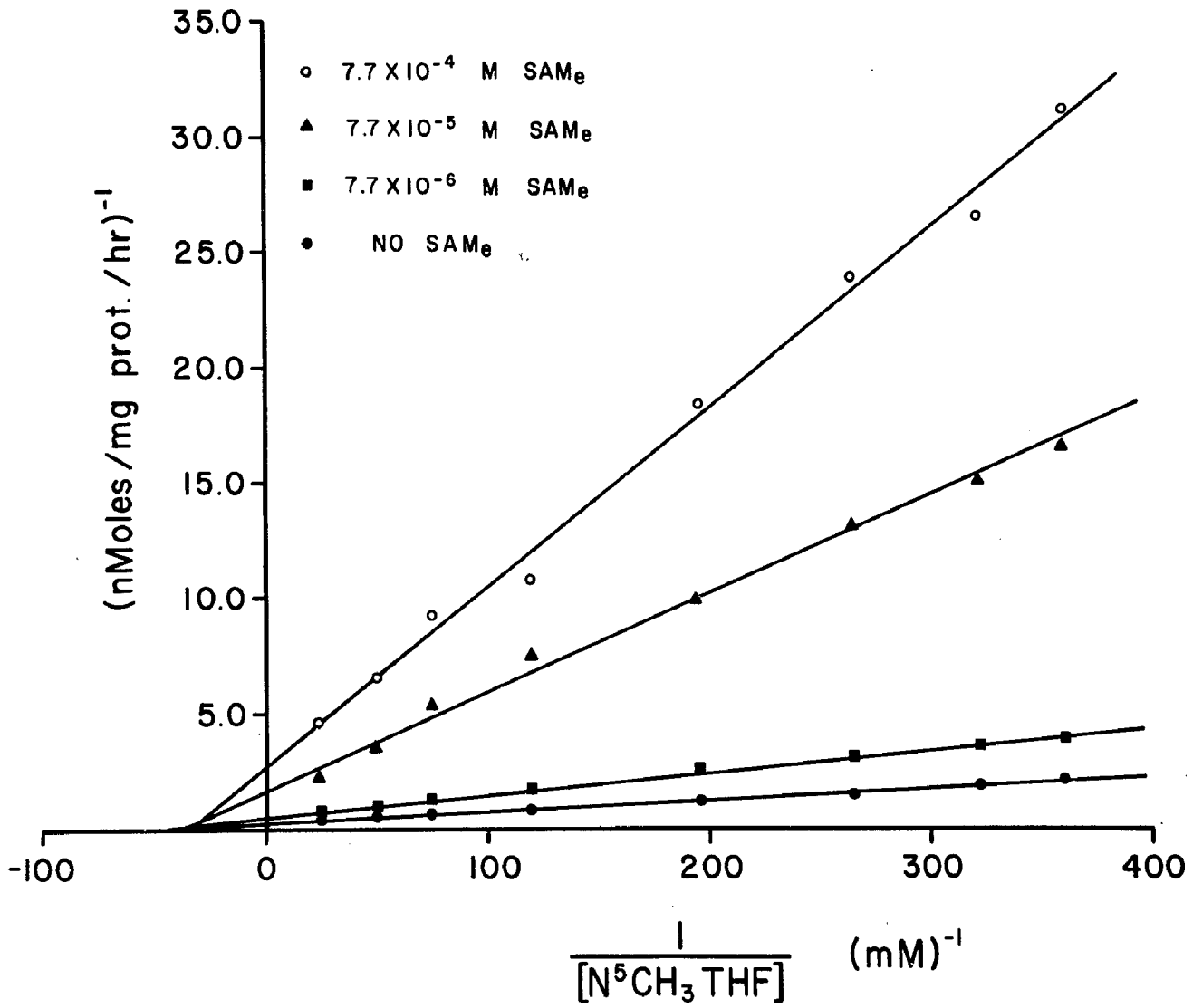

35.0
30.0
25.0
20.0
15.0
10.0
5.0
-100
0
100
200
300
400
(nMoles/mg prot./hr)$^{-1}$
7.7×10^{-4} M SAMe
7.7×10^{-5} M SAMe
7.7×10^{-6} M SAMe
NO SAMe
$\frac{1}{[N^5CH_3THF]}$ $(mM)^{-1}$

Figure 21. Lineweaver-Burk Plots of Rat Liver N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase Activity versus N^5CH_3THF Concentration in Presence of SAMe.

The enzyme was assayed as described in Materials and Methods. Postnuclear homogenate of normal, adult, male rat liver was used as the enzyme source.

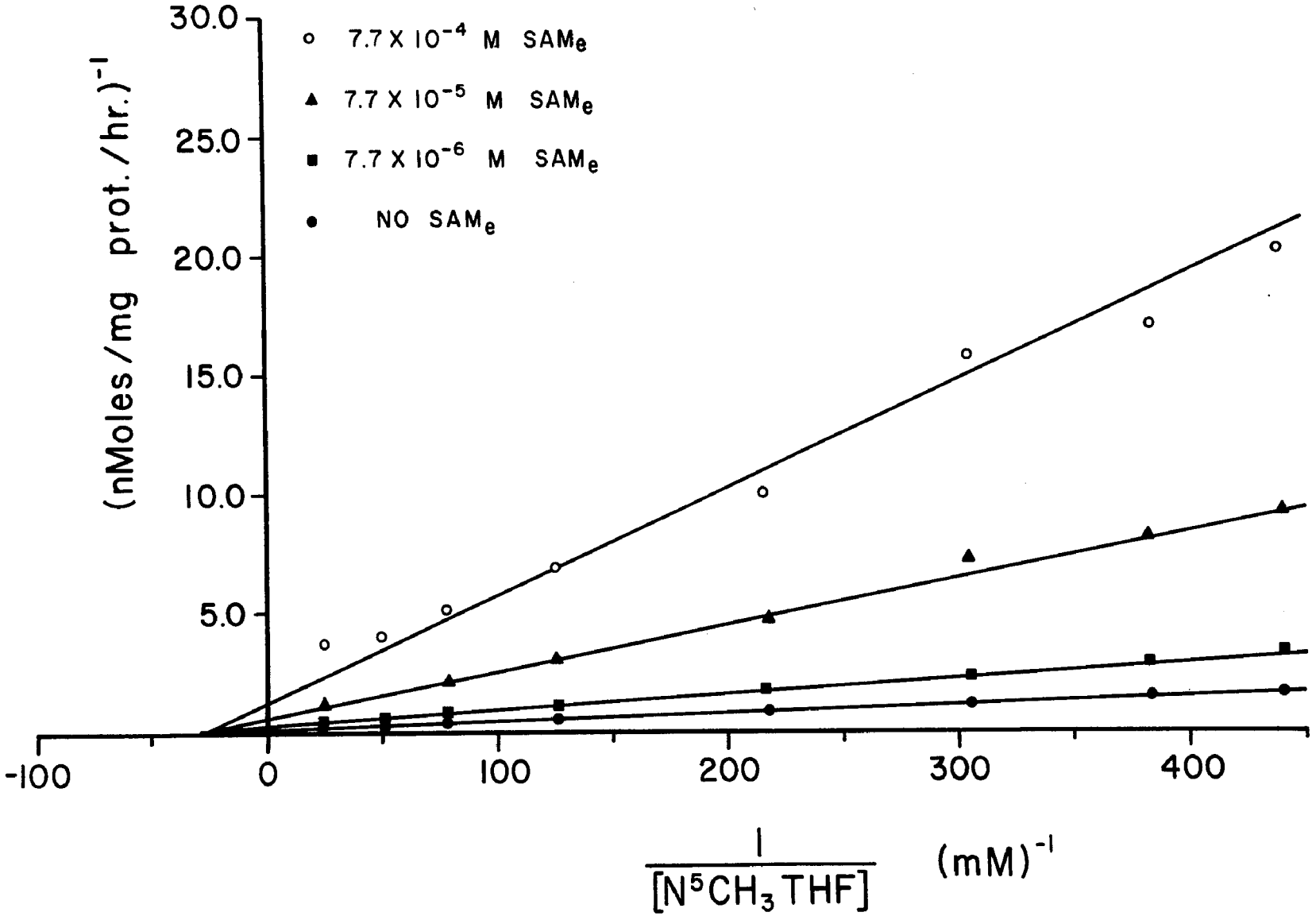

30.0
25.0
20.0
15.0
10.0
5.0
(nMoles/mg prot./hr.)$^{-1}$
$\circ$ 7.7×10^{-4} M SAM_e
$\blacktriangle$ 7.7×10^{-5} M SAM_e
$\blacksquare$ 7.7×10^{-6} M SAM_e
$\bullet$ NO SAM_e
-100
0
100
200
300
400
$\frac{1}{[N^5CH_3THF]}$ $(mM)^{-1}$

Table V. The $N^5N^{10}CH_2THF$ reductase was assayed as described in Materials and Methods. Apparent constants were calculated from the Lineweaver-Burk plots using the following equations:

$$Km = - \frac{1}{\text{Abscissal Intercept}}$$

$$Vmax = \frac{Km}{\text{Slope}}$$

$$K_I = \frac{VmaxI}{Vmax-VmaxI}[I]$$

where [I] is the inhibitor concentration. The substrate (N^5CH_3THF) concentration range was 2.3-40 μM.

Table V. Kinetic Parameters for N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase in Rat Brain and Liver.

	BRAIN	LIVER
Km (m)	2.9×10^{-5}	3.7×10^{-5}
Vmax $\frac{\text{nmoles}}{\text{mg protein} \cdot \text{hour}}$	5.3	12.1
K_I (SAMe) (M)	3.0×10^{-5}	3.9×10^{-5}

similar for liver and brain. Translation of the concentration of SAMe in livers from methionine loaded rats (Table III) to the assay system gives a concentration of 1.15×10^{-5} M SAMe in these assays. A 74 percent inhibition of enzyme activity at this concentration *in vitro* compares with inhibitions of 83 and 54 percent at SAMe concentrations of 7.7×10^{-5} M and 7.7×10^{-6} M, respectively (Figure 21).

Effect of Metabolites on N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase

S-adenosylhomocysteine (SAH) is an inhibitor of many methylating enzymes. Due to the inhibition of $N^5N^{10}CH_2THF$ reductase by SAMe, experiments were devised to examine the effect of SAH on this enzyme. Lineweaver-Burk plots show an activation of the $N^5N^{10}CH_2THF$ reductase in brain (Figure 22) and liver (Figure 23) by 1.0 mM SAH. The activation is 0.3-fold and 0.4-fold in brain and liver, respectively. There is a slight activation by both homocysteine and cysteine in brain (Figure 22) and liver (Figure 23) which can be replicated by 10 mM mercaptoethanol (Figure 24), and the effects are not additive. Thus, activation by cysteine and homocysteine appears to be due to the thiol group possessed by both metabolites. The activation by 10 mM mercaptoethanol is further enhanced by 1.0 mM SAH (Figure 24). This latter metabolite appears to be a specific activator of the $N^5N^{10}CH_2THF$ reductase.

Figure 22. Effect of Metabolites on Brain N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase Activity.

The enzyme was assayed as described in Materials and Methods. The reciprocal of the reaction velocity is plotted versus the reciprocal of N^5CH_3THF concentrations. The effects of 1.0 mM concentrations of glycine, cysteine, homocysteine and S-adenosylhomocysteine on enzyme activity are compared to the activity in the absence of added metabolites.

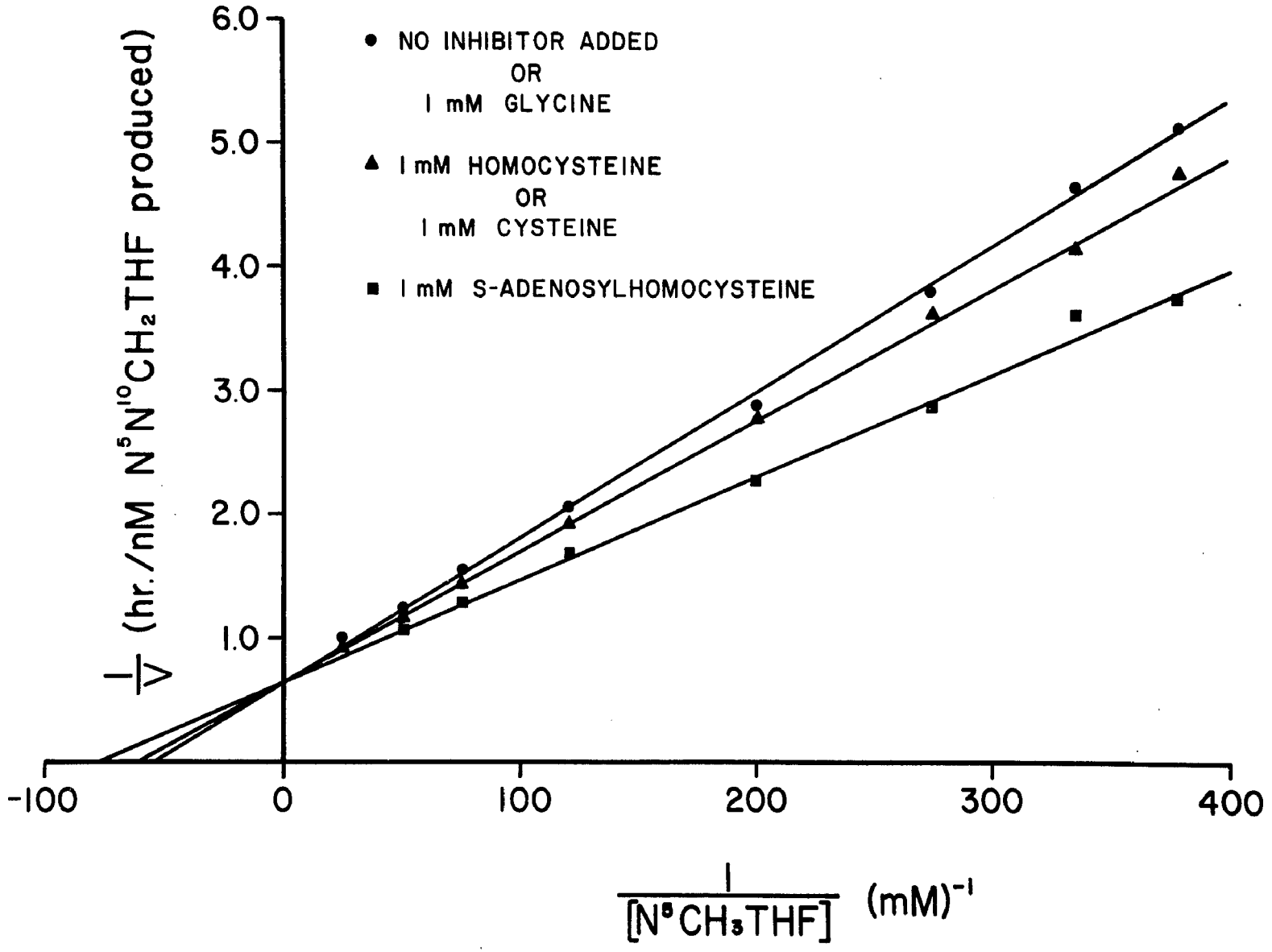

6.0
5.0
4.0
3.0
2.0
1.0
-100
0
100
200
300
400
1/V (hr./nM N5N10CH2THF produced)
NO INHIBITOR ADDED
OR
1 mM GLYCINE
1 mM HOMOCYSTEINE
OR
1 mM CYSTEINE
1 mM S-ADENOSYLHOMOCYSTEINE
1/[N5CH3THF] (mM)-1

Figure 23. Effect of Metabolites on Liver N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase Activity.

The enzyme was assayed as described in Materials and Methods. The reciprocal of the reaction velocity is plotted versus the reciprocal of N^5CH_3THF concentration. The effects of 1.0 mM concentrations of glycine, cysteine, homocysteine, and S-adenosylhomocysteine on enzyme activity are compared to the activity in the absence of added metabolites.

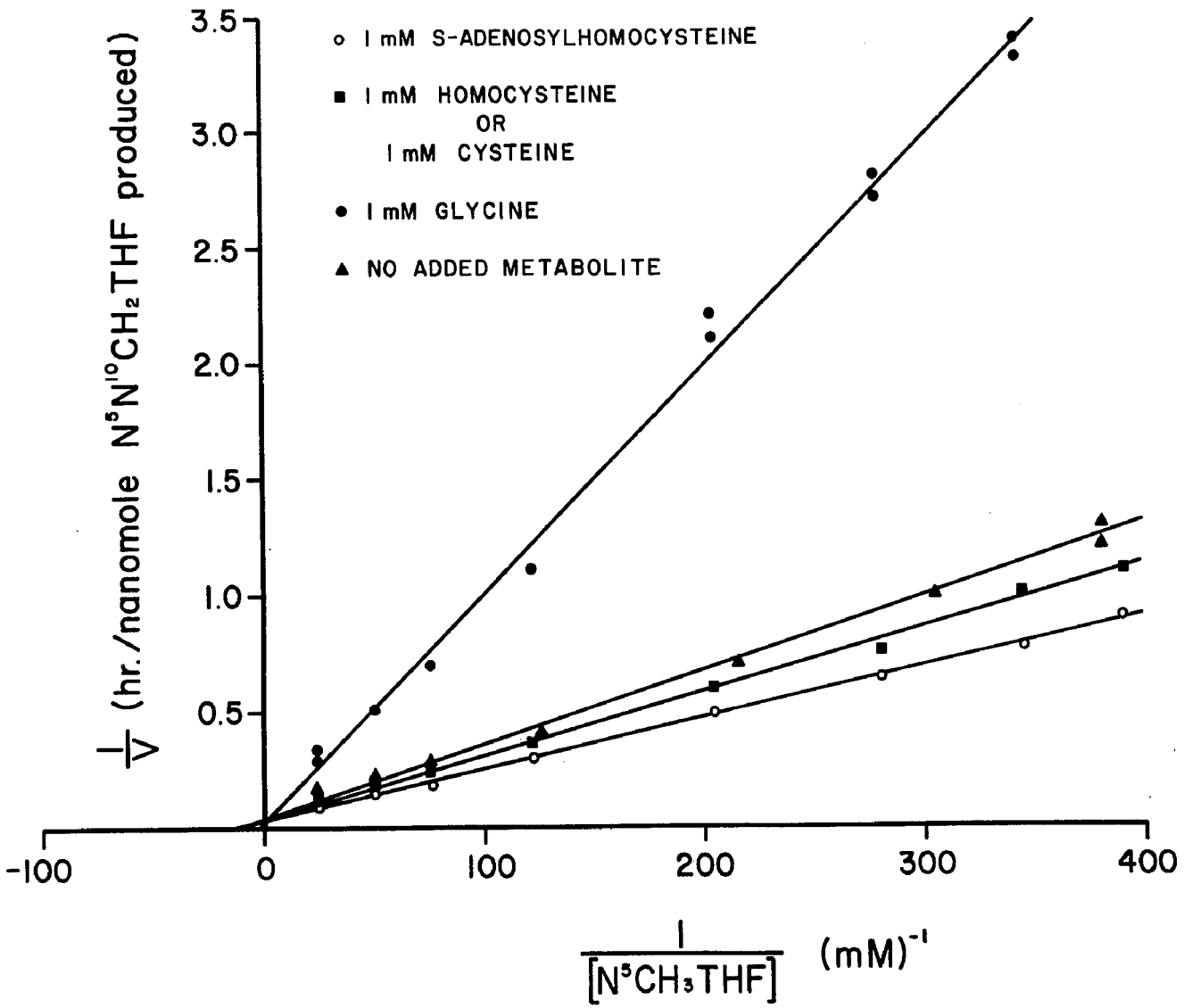
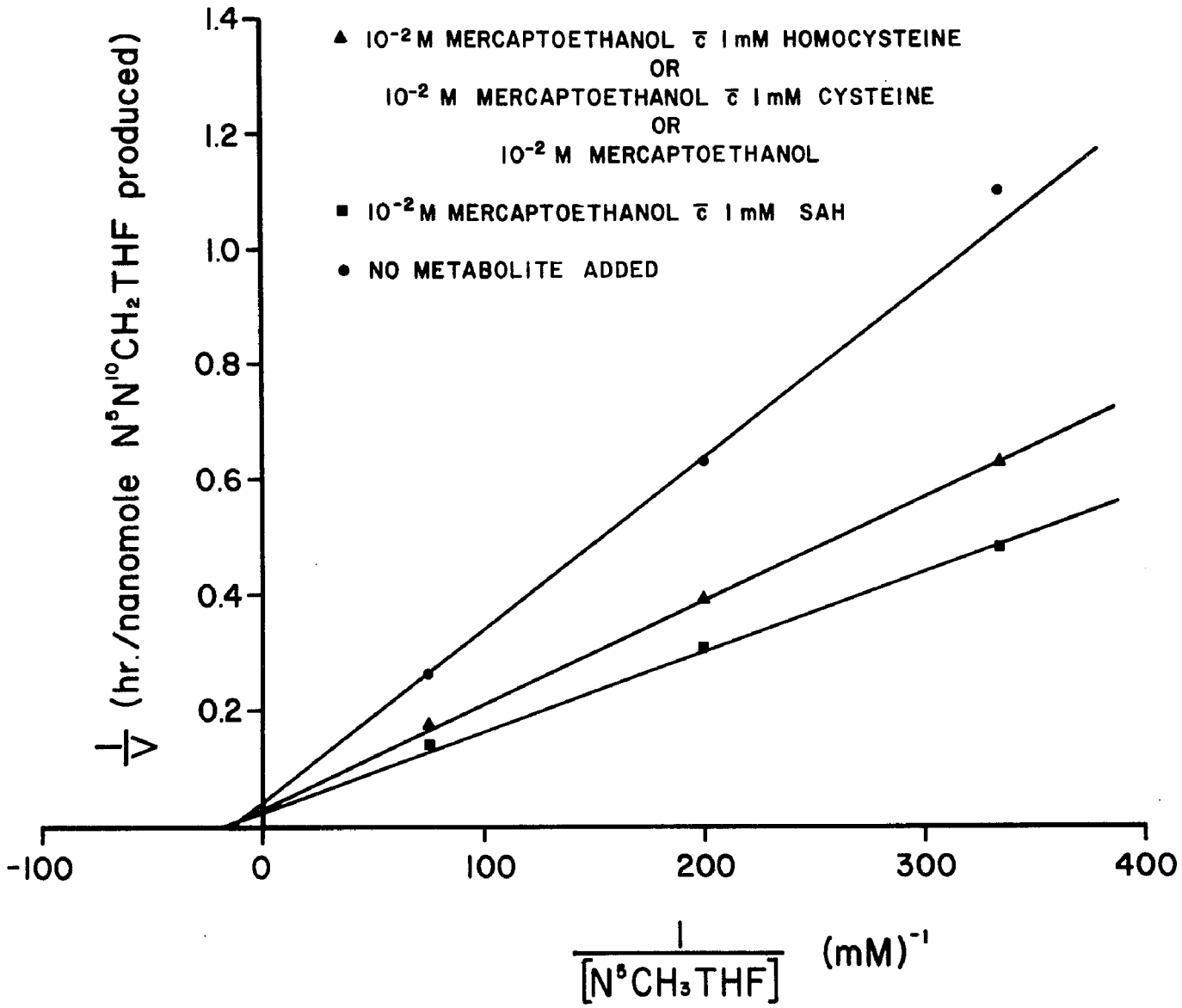

o 1 mM S-ADENOSYLHOMOCYSTEINE
■ 1 mM HOMOCYSTEINE
OR
1 mM CYSTEINE
● 1 mM GLYCINE
▲ NO ADDED METABOLITE
3.5
3.0
2.5
2.0
1.5
1.0
0.5
1/V (hr./nanomole N5N10CH2THF produced)
-100
0
100
200
300
400
1/[N5CH3THF] (mM)-1

Figure 24. Effect of Thiol Groups on Liver N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase Activity.

The enzyme was assayed as described in Materials and Methods. The reciprocal of the reaction velocity is plotted versus the reciprocal of N^5CH_3THF concentration. The effects on enzyme activity of 10 mM mercaptoethanol above and in the presence of 1.0 mM homocysteine, cysteine, and S-adenosylhomocysteine (SAH) are compared to the activity in the absence of added metabolites.


▲ 10^{-2} M MERCAPTOETHANOL c̄ 1 mM HOMOCYSTEINE
OR
10^{-2} M MERCAPTOETHANOL c̄ 1 mM CYSTEINE
OR
10^{-2} M MERCAPTOETHANOL
■ 10^{-2} M MERCAPTOETHANOL c̄ 1 mM SAH
● NO METABOLITE ADDED
$\frac{1}{V}$ (hr./nanomole $N^5N^{10}CH_2THF$ produced)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
-100
0
100
200
300
400
$\frac{1}{[N^5CH_3THF]}$ $(mM)^{-1}$

Among other amino acids, methionine, serine, and cystathionine were without effect on this enzyme. Glycine at 1.0 mM exerted a differential effect in the assay system on the brain and liver enzymes. Brain $N^5N^{10}CH_2THF$ reductase activity was unaffected (Figure 22). However, a 66 percent inhibition occurred for the liver enzyme in the presence of this concentration of glycine. The inhibition by glycine appears to be competitive in nature (Figure 23).

DISCUSSION

Tissue Concentrations of Methionine and S-Adenosylmethionine

Chronic methionine loading (250 mg/kg/day) causes differential elevations of this amino acid in rat brain and liver, 9.6-fold and 1.4-fold, respectively (Table III, radiochemical assay). The smaller increase in liver methionine after seven weeks of loading may be due to a catabolic rate for methionine in liver which can better follow its uptake. Dietary methionine supplementation for shorter periods and acute methionine loads at doses higher than used in this study have been reported to increase methionine levels in liver to a greater extent than in brain (Daniel and Waisman, 1969). These authors found brain and liver methionine levels to be elevated by eight-fold and thirteen-fold, respectively, in response to feeding a three percent methionine supplemented diet for two weeks to weanling rats. One hour after a single intraperitoneal injection of 1.64 g/kg of L-methionine, the same investigators reported this difference to be magnified. Levels of methionine increased 25-fold and 69-fold in brain and liver, respectively.

Table III also shows that conversion of methionine to S-adenosylmethionine (SAMe) follows a pattern inverse to the

methionine increases in brain and liver. Brain SAMe levels do not increase by a statistically significant amount whereas liver levels increase by six-fold. A similar relationship between rat brain methionine and SAMe concentrations was observed by Rubin et al. (1974) who found that one hour after a single intraperitoneal injection of 100 mg/kg of L-methionine, a 9-fold increase in brain methionine levels was accompanied by a much smaller (35 percent) increase in SAMe. Using Sprague-Dawley rats, Baldessarini (1966) compared the effects of a single intraperitoneal injection of L-methionine (100 mg/kg) on SAMe levels in brain and liver. After 30 minutes SAMe levels increased 3.4-fold in liver but only 30 percent in brain. In contrast to the moderate increases in brain SAMe reported by other investigators and verified in this study, Schatz and Sellinger (1975) found a one-fold increase in rat brain SAMe levels three hours after administration of a much higher dose of L-methionine (700 mg/kg). This was attributed to increased precursor availability. Another factor may be the time of sacrifice after injection. A 9-fold increase in rat brain methionine levels was found one hour after a dose of 100 mg/kg (Rubin et al., 1974). Daniel and Waisman (1969) showed that methionine concentrations in rat brain reach a maximum about four hours after injection. Rabbit brain methionine levels increased 10-fold four hours after an intraperitoneal injection of 500 mg/kg of L-methionine (Tudball and Griffiths, 1976) but the rates of

methionine incorporation into brains of rabbits and rats may differ. The evidence then indicates that methionine loading, acute or chronic, causes huge increases in brain methionine with only marginal increases in SAMe. The extent of brain SAMe elevation is dependent on the dose of methionine and the time after its administration. While it is difficult to elevate SAMe, but not methionine, in brain, the opposite pertains to liver. The relationship in brain between methionine and SAMe is further elucidated by the observations that repeated doses of 3,4-dihydroxy-phenylalanine (L-DOPA) (100 mg/kg) failed to affect brain methionine concentrations while a single such dose depressed brain SAMe levels by 60 percent (Ordonez and Wurtman, 1973). Baldessarini (1975b) reported similar results for SAMe depletion by 100 mg/kg L-DOPA in rat brain, but liver SAMe levels were unaffected by this treatment.

The concentration of methionine found in brains of saline injected rats (0.11 μmole/gram tissue) is in excellent agreement with the value (0.10 μmole/gram tissue) reported by Daniel and Waisman (1969). These authors report a comparable value (0.12 μmole/gram tissue) for liver which is substantially below the concentrations determined by radiochemical assay and amino acid analysis (0.61 and 0.95 μmole/gram tissue, Table III). Daniel and Waisman (1969) reported liver methionine concentrations for rats 23 days and 35 days of age. During this time a 64 percent increase in liver methionine concentration

occurred. If liver methionine concentration does increase with age, the higher values reported in Table III may be accounted for since adult rats were used for these studies.

The SAMe concentrations found in brain and liver from saline injected rats were 0.048 and 0.11 μmole/gram tissue, respectively (Table III). Similar determinations were obtained from rats by Taylor and Randall (1975) for brain (0.049 μmole/gram tissue) and liver (0.134 μmole/gram tissue) and by Lombardini and Talalay (1973) for brain (0.043 μmole/gram tissue) and liver (0.123 μmole/gram tissue).

Enzyme Responses to Methionine Loading

For the enzymes assayed, neither induction nor repression of enzyme synthesis occurred in either tissue in response to methionine loading. This is at variance with the report by Kutzbach *et al.* (1967) who reported a repression of N^5CH_3THF:homocysteine transmethylase in rat liver in response to dietary supplementation of 1.5-4 percent methionine for 11-15 weeks. Enzyme activity was reduced to 48 percent of normal. These authors also reported no change in $N^5N^{10}CH_2THF$ reductase activity by this treatment. However, their tissue homogenates were frozen at -15°C for an unspecified time until assayed. The *in vivo* inhibition of this enzyme by SAMe, as

demonstrated by the present data, would not have been detected since extracts of tissues kept frozen more than 24 hours lose significant amounts of SAMe (Baldessarini and Kopin, 1966). A repression of rat liver N^5CH_3THF: homocysteine transmethylase to 68 percent of normal levels was also reported by Finklestein et al. (1971) in response to intraperitoneal injection of one millimole of L-methionine administered in two spaced injections.

N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase

Metabolic control of the de novo synthesis of methyl groups in liver is accomplished by $N^5N^{10}CH_2THF$ reductase. A 74 percent inhibition of the activity of this enzyme in liver occurred in response to methionine loading. The inhibition is due to the elevated concentration of SAMe in livers of methionine treated animals. This conclusion is based on the restoration of normal activity with dialysis of the enzyme and the demonstration that exogenous SAMe added to the incubation in a concentration comparable to that of endogenous SAMe will inhibit the activity to the same extent. De novo methyl group synthesis in brain can also be regulated by the inhibitory effects of SAMe on the $N^5N^{10}CH_2THF$ reductase as demonstrated by the in vitro inhibition of the brain enzyme when SAMe is added to the assay (Figure 20). However, SAMe does not accumulate in brain in response to methionine loading. Consequently, an alteration in the

activity of brain $N^5N^{10}CH_2THF$ reductase was not detected. The extent of the regulation of this enzyme in brain by endogenous levels of SAMe and the importance of such regulation remain to be determined. The concentration of SAMe in brain for saline injected rats (0.048 μmole/gram tissue, Table III) is 80 μM assuming that SAMe distributes in tissue water (60 percent by net weight). An 88 percent inhibition of the brain enzyme was obtained in response to 77 μM SAMe in vitro (Figure 20). Thus, an endogenous SAMe concentration of this magnitude seems to be an inhibitory regulator of the enzyme in vivo. It should also be noted that SAMe inhibition of this enzyme was measured in the non-physiological, oxidative direction of the enzyme and the assumption made that the inhibition also applies to the reductive direction. A direct demonstration of SAMe inhibition in this direction has yet to be presented. A physiological role for the oxidative direction of this enzyme during early development, as implied by Ordonez and Villarroel (1976), and the function of SAMe under these conditions merit further investigation.

Burton and Sallach (1975) reported liver $N^5N^{10}CH_2THF$ reductase to be five times as active as the brain enzyme in rats. An approximate 0.7-fold increase was found in the present study. Rats of different strains and age were used in each study which may partially account for the difference in the reported factors. Direct comparison

of enzyme activities obtained with those reported by other investigators is not meaningful due to variations in assay procedures and incubation conditions. Kutzbach and Stokstad (1967) reported the Km with respect to N^5CH_3THF for $N^5N^{10}CH_2THF$ reductase from rat liver to be about 100 μM. This value is high relative to those given in Table V for liver (37 μM) and brain (29 μM) using the mixed monoglutamate diastereoisomers of N^5CH_3THF. With respect to the same isomer mixture, Cheng et al. (1975) reported Km values of 32 μM and 45 μM for crude and purified rat liver enzyme, respectively, in excellent agreement with the values reported here. The Vmax for the crude liver enzyme prepared by dialysis of the postnuclear homogenate was given by the same authors as 6.5 nmoles/mg protein/hour which is comparable to the values of 5.3 and 12.1 nmoles/mg protein /hour for brain and liver, respectively, in Table V. The inhibition of the enzyme by SAMe is noncompetitive with respect to N^5CH_3THF in both tissues (Figures 20 and 21). Cheng et al. (1975) also found liver $N^5N^{10}CH_2THF$ reductase to be inhibited by SAMe in a noncompetitive fashion when either the mono- or pentaglutamate form of N^5CH_3THF was used as substrate. A half maximal inhibition was reported at a SAMe concentration of 51.2 μM in reasonable agreement with the present determinations of 30 μM and 39 μM for brain and liver, respectively (Table V).

Kutzbach and Stokstad (1971a) observed that inhibition of the $N^5N^{10}CH_2THF$ reductase activity by S-adenosylmethionine *in vitro* was partially overcome by S-adenosylhomocysteine. They further noted that S-adenosylhomocysteine added alone to a concentration of two micromolar did not stimulate the enzyme. The present data indicate that a higher concentration of S-adenosylhomocysteine (1.0 mM) does activate the brain and liver enzymes (Figures 22 and 23). The $N^5N^{10}CH_2THF$ reductase is also activated by metabolites possessing a free thiol group such as cysteine, homocysteine, and mercaptoethanol. The liver enzyme was inhibited to 34 percent of normal activity by 1.0 mM glycine whereas the brain enzyme was unaffected. This effect may be due to a greater serine hydroxymethyltransferase activity in liver than brain. Since this enzyme catalyzes the reaction of $N^5N^{10}CH_2THF$ with glycine to form serine, the product of the $N^5N^{10}CH_2THF$ reductase, $N^5N^{10}CH_2THF$, would be alternately used in the presence of high glycine concentrations simulating an inhibitory effect on the enzyme. Another explanation which may partially account for the effect of glycine on liver $N^5N^{10}CH_2THF$ reductase involves the much greater activity of the glycine cleavage system in liver than brain (Daly *et al.*, 1976). This system donates the alpha carbon of glycine to tetrahydrofolic acid to form $N^5N^{10}CH_2THF$. Since the $N^5N^{10}CH_2THF$ reductase was assayed in the oxidative direction with

$N^5N^{10}CH_2THF$ formed as product, a high endogenous concentration of this coenzyme in liver due to glycine cleavage system activity may result in feedback inhibition of the reductase.

N^5-Methyltetrahydrofolic Acid:Homocysteine Transmethylase

Despite the fact that the activity of N^5CH_3THF: homocysteine transmethylase is lower than the $N^5N^{10}CH_2THF$ reductase and could therefore be rate-limiting in the de novo synthesis of methyl groups in brain and liver, the activity of this enzyme is not affected by methionine loading in the assay employed. It seems that the activity of this enzyme is not susceptible to product inhibition by methionine at concentrations below 100 μM. The elevated methionine concentrations in liver and brain from methionine loaded rats would result in incubation concentrations of 66 μM and 82 μM methionine for brain and liver, respectively. The activity of N^5CH_3THF: homocysteine transmethylase in liver was 0.6-fold more active than in brain of control animals (Tables I and II), a factor which is identical to that reported by Burton and Sallach (1975). Rassin and Gaull (1975) found the liver activity of this enzyme in Nelson-Wistar male, adult rats to be 4.7-fold greater than the brain activity while Finklestein et al. (1971) reported brain activity in Sprague-Dawley rats to be 0.2-fold higher than liver activity.

Methionine Adenosyltransferase

No significant difference was observed in methionine adenosyltransferase activity in either tissue between treated and control animals when the enzyme was assayed using relatively high concentrations of methionine (methionine adenosyltransferase (a), Tables I and II) indicating that methionine loading has no significant effect on the enzyme concentration in either tissue.

To determine whether an increase in methionine adenosyltransferase activity occurred in response to elevated tissue concentrations of methionine, the enzyme assay was performed under conditions of low incubation methionine concentration (methionine adenosyltransferase (b), Tables I and II). Significant increases in activity in brain (2.6-fold) and liver (5.5-fold) were measured as a result of methionine loading. To evaluate whether these increases also occurred *in vivo*, saturation curves with respect to methionine for brain and liver methionine adenosyltransferase were obtained. The curve for brain (Figure 17) is hyperbolic yielding a linear double reciprocal plot indicative of a lack of cooperative effects. Saturation in brain occurs at approximately 20 μM methionine. From Table III (radiochemical assay), methionine concentration in brain of saline injected rats is 0.11 μmole/gram tissue. Assuming that methionine distributes in tissue water (60 percent by net weight), this value converts to a brain concentration of

183 μM. Baldessarini (1975b) gives the brain methionine concentration as 80-150 μM. Even at the lower limit of this range, methionine concentration is four times greater than the saturating value of 20 μM determined from Figure 20. Thus, it appears that rat brain methionine adenosyltransferase is saturated at normal tissue levels of methionine. In this context, it is not surprising that methionine loading did not elevate brain SAMe levels even though methionine concentrations in brain were elevated 9.6-fold above normal.

The saturation curve for rat liver methionine adenosyltransferase (Figure 18(a)) differs from the curve for rat brain. This curve has a sigmoidal appearance suggestive of cooperativity with respect to methionine. This behavior provides an explanation for the 5.5-fold increase in activity observed in response to the 1.4-fold increase in methionine concentration in the assay of liver methionine adenosyltransferase activity (methionine adenosyltransferase (b), Table II). In Table II, the actual methionine concentrations in the assay of saline liver methionine adenosyltransferase ((a) and (b)) are 82 μM and 15 μM, respectively. A 4.5-fold increase in methionine concentration yields an 7.7-fold increase in enzyme activity. These results suggest a sigmoidal saturation curve in liver which has been verified experimentally (Figure 18(a)).

Lombardini et al. (1973) showed that the activity versus methionine concentration curve for rat liver methionine adenosyltransferase is sigmoidal in the substrate range of 7-175 μM and positive cooperativity was claimed from the upward-sloping double reciprocal plots. Tallan and Cohen (1976), investigating the requirements of this enzyme for K^+ and Mg^{++}, obtained data supporting the existence of positive cooperativity. In contrast, Finklestein et al. (1975a) using liver enzyme from rats and humans, reported negative cooperativity based on a downward-sloping double reciprocal plot which permitted the calculation of two Km's for methionine, one for the concentration range of 6-42 μM methionine and another for the range of 0.1-1.0 mM methionine. Negative cooperativity of the rat liver enzyme is supported by the present studies as evidenced in the downward-sloping double reciprocal plot (Figure 19(b)). For a negatively cooperative enzyme, the apparent Km increases with substrate concentration. Thus, a unique Km at high substrate concentration can not be determined from a Lineweaver-Burk plot due to its nonlinearity. By straight line approximation through data points in two ranges of methionine concentration, Finklestein et al. (1975a) obtained apparent Km's of 23.6 μM and 507 μM for the methionine ranges of 6-42 μM and 0.1-1.0 mM, respectively. A similar approach provided two apparent Km's in the present

study with the higher methionine concentration range yielding the larger Km (Table IV).

Negative cooperativity with respect to methionine for rat liver methionine adenosyltransferase has also been reported by Liau et al. (1977). These investigators found that liver contains two isofunctional forms of the enzyme, a minor form with a Km (methionine) of 21 μM as well as a major form with a Km of one millimolar. The high Km value is in good agreement with the 0.91 mM value reported by Pan and Tarver (1967) and 0.94 mM reported by Matthysse et al. (1972). The assay for methionine adenosyltransferase used in the current studies is not suitable for determining the high Km value. However, the apparent Km's reported in Table IV for the liver enzyme may represent additional isofunctional forms of the enzyme since at 2.6 μM and 12 μM they are considerably lower than the 21 μM reported by Liau et al. (1977) and the 23.6 μM reported by Finklestein et al. (1975).

From the standardization curves and data derived from saline injected rats (Tables I and II, methionine adenosyltransferase (a)), liver methionine adenosyltransferase is 2.1-fold more active than brain. Due to the presence of cooperativity for the liver enzyme, this factor will vary depending on the methionine concentration. Thus, under high incubation concentrations of methionine, the liver enzyme was found to be more active by factors of 11.4

(Rassin and Gaull, 1975), 10.3 (Mudd et al., 1965), and 9.1 (Lombardini et al., 1973). Comparable activites for the hepatic and brain enzymes have been claimed, however, by Altares and Sellinger (1976) on the basis of a greater solubilization of the brain enzyme.

In summary, the activation of the methyl group in rat brain and liver is under different regulatory control. In normal rat brain, the methionine adenosyltransferase operates near its maximal velocity. An influx of methionine will result in its accumulation due to a lower capacity to metabolize it. Methionine loading will result in a decreased ratio of SAMe to methionine in brain. In liver, an influx of methionine can be metabolized to SAMe due to the existence of negative cooperativity and a major Km with respect to methionine which approaches one millimolar. The ratio of SAMe to methionine in liver is thus found to increase.

The transmethylation hypothesis of schizophrenia postulates the production of a psychotomimetic compound due to abnormal methylation. This theory implies an excess methylating capacity. The report by Freeman et al. (1975) of a folate responsive schizophrenia due to deficient $N^5N^{10}CH_2THF$ reductase favors the hypothesis that a decreased methylating capacity may precipitate schizophrenia. This led Levi and Waxman (1975) to propose a deficiency in methionine adenosyltransferase in schizophrenia. Mudd and Freeman (1974) postulated that the exacerbation of

schizophrenic psychoses by methionine may be due to a redistribution of folate coenzyme forms in brain. The present study indicates that such a redistribution in normal rat brain is unlikely to occur since it is effected by the regulatory control of SAMe on the $N^5N^{10}CH_2THF$ reductase and the concentration of this metabolite does not significantly increase in brain with methionine loading. The evidence gathered in this study also favors an aberrant methionine adenosyltransferase activity as a possible factor in schizophrenia. Instead of a deficiency of the enzyme, however, an enzyme mutation would be more in line with the evidence. A difference in the regulation of this enzyme in rat brain and liver has been demonstrated. If the brain enzyme in schizophrenics were to behave similarly to the liver enzyme as a result of a gametic or somatic mutation, brain SAMe levels could become greatly elevated in response to methionine loading. The exacerbation of psychosis could then conceivably occur through excessive transmethylation, redistribution of folate coenzyme forms in brain or these two effects acting in concert. The common ground between the transmethylation hypothesis and the folate effect implied by Levi and Waxman (1975) may be an aberrant brain methionine adenosyltransferase as described. The behavioral disruption of rats in response to methionine loading does not seem to be mediated through the enzymes involved in production and activation of methyl groups

in rat brain. This effect may be peripheral to methylation. However, a detailed kinetic analysis of the methionine adenosyltransferase in normal and schizophrenic brain or possibly other tissues may prove to be fruitful.

CONCLUSIONS

The major conclusions of this study are:

1. Chronic methionine loading produces (a) a 74 percent inhibition of liver $N^5N^{10}CH_2THF$ reductase activity in the assay while the activity of the brain enzyme is unaffected, (b) a six-fold increase of SAMe levels in liver but an insignificant increase in brain, and (c) a 9.6-fold increase of methionine levels in brain and a 1.4-fold increase in liver.

2. Chronic methionine loading does not affect the *de novo* synthesis of methyl groups and their activation in rat brain.

3. The methionine adenosyltransferase in rat brain is near saturation with respect to methionine. A single apparent Km value exists for this enzyme.

4. Methionine adenosyltransferase in rat liver is negatively cooperative with respect to methionine at low substrate concentrations.

5. SAMe inhibition of the $N^5N^{10}CH_2THF$ reductase *in vivo* is responsible for the decreased activity of this enzyme in liver due to methionine loading. The inhibition is noncompetitive in nature.

6. S-adenosylhomocysteine activates the $N^5N^{10}CH_2THF$ reductase in liver and brain. The liver enzyme is

competitively inhibited by high concentrations of glycine (1.0 mM).

APPENDIX

Sample Calculations

N^5,N^{10}-Methylenetetrahydrofolic Acid Reductase

The following data (CPM) were obtained for recovery, background, and sample and converted to DPM by subtracting the instrumental background (20 CPM for the ^{14}C channel) and dividing by the efficiency of counting ^{14}C (90 percent for this example):

	^{14}C (CPM)	^{14}C (DPM)
Recovery	588	631
Background	1806	1984
Sample	6114	6771

For 2000 DPM of ^{14}C-formaldehyde added to the recovery tube and since 1.0 ml of the two milliliter toluene extract was counted,

$$\text{Percent Recovery} = \frac{(631) \times 2}{2000} \times 100 = 63\%.$$

Background was then subtracted from sample and corrected for formaldehyde recovery to yield sample product formed in DPM as in the following:

$$\text{Corrected Sample DPM} = \frac{(6771 - 1984) \times 2}{0.63}$$

$$= 15{,}197$$

For a specific activity of (^{14}C-methyl)N^5CH_3THF of 100 DPM per picomole, the enzyme activity (v) can be calculated for an incubation time of one hour with one milligram of protein by:

$$v = \frac{15{,}197 \text{ DPM}}{(100 \text{ DPM/pmole})(1 \text{ hour})(1 \text{ mg protein})}$$

$$= 152 \text{ pmoles/mg protein/hour}$$

N^5-Methyltetrahydrofolic Acid:Homocysteine Transmethylase

The following data (CPM) were obtained for recovery, background, and sample for two bands A and B isolated by thin layer chromatography with methionine migrating into the B band:

	(^{14}C) CPMA	(^{14}C) CPMB
Recovery	827	2620
Background	35689	386
Sample	26323	9044

Since 100 µl of the 470 µl incubation mixture was spotted on thin layer plates, a volume correction facto of 4.7 was used to calculate total DPM in the incubation. Spotting a cold methionine standard produced a typical background CPMA and CPMB of 30. For a counting efficiency of 80 percent,

$$\text{Total DPM(A+B)}_{\text{methionine}} = \frac{(827 + 2620 - (2 \times 30)\ 4.7}{0.8}$$

$$= 19899 \text{ DPM}$$

To account for volume spotting errors, the following is calculated for 20,000 DPM of ^{14}C-methionine added to the

recovery tube.

$$\text{Recovery (A+B)}_{\text{methionine}} = \frac{19899}{20{,}000} \times 100$$

$$= 99\%$$

Similar recoveries were calculated for background and sample. For 220,000 DPM of (^{14}C-methyl)N^5CH_3THF added to background and samples tubes,

$$\text{Recovery (A+B)}_{\text{background}} = \frac{(35{,}689 + 386 - 2 \times 30)\ 4.7}{0.8\ (220{,}000)} \times 100$$

$$= 96\%$$

$$\text{Recovery (A+B)}_{\text{sample}} = \frac{(26323 + 9044 - 2 \times 30)\ 4.7}{0.8\ (220{,}000)} \times 100$$

$$= 94\%$$

Spotting a (^{14}C) methionine standard, 1977 DPM were recovered in band A and 10,574 DPM in band B. The percentage of methionine recovered in band B is

$$\frac{10574}{1977 + 10574} \times 100 = 84 \text{ percent}$$

The recovery of methionine in band B was then calculated as

$$\text{Recovery (B)}_{\text{methionine}} = \frac{\frac{(2620 - 30)}{0.8} \times 4.7}{(0.99)(0.84)(20{,}000)} \times 100$$

$$= 91 \text{ percent}$$

Sample DPM in the B band, corrected for background, was calculated from the following formula.

$$\text{Corrected DPM}_{\text{sample}} = \frac{4.7\ (\text{Sample DPM in B band})}{\text{Recovery (A+B)}_{\text{sample}}} - \frac{4.7\ (\text{Background DPM in B band})}{\text{Recovery (A+B)}_{\text{background}}}$$

$$= \frac{\dfrac{4.7\ (11{,}268)}{0.94} - \dfrac{4.7\ (445)}{0.96}}{\text{Recovery B}_{\text{methionine}}} = \frac{\dfrac{4.7\ (11{,}268)}{0.94} - \dfrac{4.7\ (445)}{0.96}}{0.91} = 59518 \text{ DPM}$$

For a specific activity of (^{14}C-methyl)N^5CH_3THF of 100 DPM per picomole, the enzyme activity (v) can be calculated for an incubation time of one hour with one milligram of protein by:

$$v = \frac{59518 \text{ DPM}}{(100 \text{ DPM/pmole})(1 \text{ hour})(1 \text{ mg protein})}$$

$$= 595 \text{ pmoles/mg protein/hour}$$

Methionine Adenosyltransferase

The following data were obtained for sample and backgrounds:

	^{3}H (CPM)	^{14}C (CPM)
Background I (Boiled MAT, Boiled HIOMT)	88	19
Background II (Boiled MAT, Active HIOMT)	1218	4481
Sample (Active MAT, Active HIOMT)	12285	4254

background II counts.

For background II,

$$^{14}C_{(DPM)} = \frac{4481 - 19}{0.71} = 6284 \text{ DPM}$$

assuming an efficiency of 71 percent in the ^{14}C channel.

$$^{3}H \text{ (DPM)} = \frac{1218 - 88 - 6256\ (0.13)}{0.35} = 905 \text{ DPM}$$

assuming a ^{3}H efficiency of 35 percent and a ^{14}C efficiency in the ^{3}H channel of 13 percent.

Thus, the $^{3}H/^{14}C$ ratio for background II is 0.14.

For the sample,

$$^{14}C \text{ (DPM)} = \frac{4254 - 19}{0.71} = 5965 \text{ DPM}$$

$$^{3}H \text{ (DPM)} = \frac{12285 - 88 - 5965\ (0.13)}{0.35} = 32643 \text{ DPM.}$$

The $^{3}H/^{14}C$ ratio for the sample is 5.5.

For a specific activity of (^{3}H) methionine of 2000 μCi/μmole and 0.02 μCi of ^{14}C added to the incubation, enzyme activity (v) can be calculated for an incubation time of one hour with one milligram of protein by:

$$v = \frac{(5.5 - 0.14)\ (0.02\ \mu Ci)\ (10^{6}\ \text{pmoles})}{(2000\ \mu Ci/\mu mole)\ (1\ \text{hour})\ (1\ \text{mg protein})\ (\mu mole)}$$

$$= 53.6 \text{ picomoles/mg protein/hour}$$

Nomenclature of Radioactive Compounds

The isotope employed is designated by its chemical symbol. The superscript which precedes this symbol is the mass number. The position of the label in the compound is indicated in parentheses. Thus, N^5-(^{14}C-methyl)-methyltetrahydrofolate and (^{3}H-methyl)-methionine indicate that the methyl groups on the respective compounds are labeled. Methyl is underlined to emphasize that the methyl group on the parent compound is labeled.

The specificity of the radioactive label is adjacent to the isotope symbol according to the terminology used by New England Nuclear Corporation. Nominally labeled (N) is used when the label is at a specific site or sites but no further information is provided as to the extent of labeling at other positions. N-(1,2-^{3}H(N))-acetyl-serotonin indicates that the compound is nominally labeled at positions one and two of the ethyl side chain. The (N) designation is not used when all labeled positions are included in the name of the compound and the radioactivity at these positions is greater than 95 percent of the total incorporated into the compound.

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