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THE EFFECT OF DIETARY SULFUR ON OPIATE
ANALGESIA, CONJUGATIONS AND BINDING

A Dissertation
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Degree
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ABSTRACT

Female Sprague Dawley rats weighing between 80 and 120 grams were individually housed and fed diets with a basal formulation of 15 percent casein with inorganic sulfur supplementation at 0.0002 percent, 0.02 percent, and 0.42 percent. Three variations in organic sulfur were achieved by no supplement, supplemental methionine (0.625 percent), and supplemental cysteine (0.505 percent).

Five animals on each of the nine diets (45 tests) were investigated for each phase of the study. The tests were made following a period of ad libitum feeding lasting 17 days.

The animals received a subcutaneous injection of ^3H -etorphine (2.6×10^6 CPM) in saline solution. Counts per minute in blood and brain homogenates were determined. The animals were sacrificed and cerebroside sulfates were isolated from the brain homogenates by florisil column chromatography.

Ratios of sulfated:glucuronidated ^3H -etorphine in 24-hour urine collections from injected animals were calculated from differences in radiation levels following hydrolysis with type IX glucuronidase and type V sulfatase. The freed etorphine was extracted by dichloromethane.

Incubation of brain homogenate in varying concentrations of ^3H -etorphine and ^3H -naloxone followed by

displacement of the unbound drug by centrifugation determined the levels of drug specifically bound to receptors. Number of binding sites was determined by Scatchard analysis.

No differences were detected in analgesic response with the opiate tested due to the fractional occupancy of etorphine receptors necessary for eliciting the effect. Differences in conjugation ratios were significant for level of dietary inorganic sulfate and form of organic sulfur supplementation. Increased levels of inorganic sulfate increased sulfated:glucuronidated ratios (S:G) and supplementation with cysteine resulted in higher S:G than methionine supplementation.

Dietary sulfur raised the number of binding sites for the agonist and antagonist tested. There was an increase in effect on apparent number of binding sites for the agonist etorphine with methionine supplementation. Conversely, inorganic sulfate at 0.42 percent supplementation increased the apparent number of binding sites for the antagonist naloxone.

This study provides evidence of a requirement for sulfate for the development of the opiate receptor. Sulfur status of the rat was shown to affect the number of receptors for opiates in brain homogenates.

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CHAPTER I

INTRODUCTION

Most opiates have the same ring skeleton and bind to a specific receptor site. This stereoselective match of drug with receptor elicits the pharmacological effects. Knowledge of the distribution and biochemical properties of the drug receptors has been accumulated and provides insight into their mechanism of action.

Previous work by Loh using commercial cerebroside sulfate led to the proposal of cerebroside sulfate functioning as binding sites for opiates in vivo. Demonstration of decreased opiate binding by several methods of sulfatide hydrolysis has been reported by Loh. Increased amounts of morphine are required to elicit analgesia in the Jimpys, a strain of mice with decreased cerebroside formation in comparison to normals. Work in this laboratory has demonstrated an effect of methionine supplementation on the sleep time of pentobarbital-treated animals indicating a physiological significance of organic sulfur supplementation on pharmacological response.

The importance of inorganic sulfate to drug toxicity has been well-established by previous research in this laboratory. Conjugation with sulfate is one mechanism by which the body eliminates drugs. Salicylamide and acetaminophen have been shown to be increasingly conjugated with

sulfate as the level of inorganic sulfate increased. An effect on the conjugating enzyme UDP-glucuronyltransferase has been demonstrated. The effect of inorganic sulfate on sulfotransferase has not been established.

Since sulfate is a requirement for the formation of cerebroside sulfates and the use of binding sites for opiates requires conjugation of the drug with sulfate for elimination, the competition for sulfate between these two pathways during use of opiates could explain some of the biological alterations leading to tolerance in chronic users.

The purpose of this investigation was to determine the effects of dietary sulfur on the binding sites, metabolism and pharmacological response of opiates. With the possibility of sulfate serving as the recognition site for opiates, it was of interest to determine whether sulfur status produced alterations in the actions of opiates.

CHAPTER II

REVIEW OF LITERATURE

Opiates and Their Receptors

Man's struggle to alleviate pain began very early. Mythological writings recorded accounts of culture and religious rituals of ancient Hebrews, Egyptians, Greeks, and Romans in their search for pain-relieving preparations (1). Analgesia-producing narcotics were discovered in plants. (2). Opium, for example, is found in the dried sap of the unripened seed capsule of the poppy plant, Papaver somniferum (3).

The isolation of pure alkaloids from the poppy plant constituted the second phase of narcotic development (1). In 1805 Frederick Serturmer, a young German apothecary's assistant, was responsible for the isolation from the poppy plant of a substance which he named morphine after the Greek god of dreams, Morpheus. He also determined that it had alkaline or basic properties, while the plants themselves were usually either neutral or acidic (2). In later years other alkaloid components were isolated from the plant. The third phase in the development of narcotics began with man's ability to synthesize alkaloids. Today's pharmacological methods are being utilized to synthesize even more potent forms of the product.

Most narcotics of either natural or synthetic origin exhibit a T-shaped configuration, and the active form is usually levorotatory (-). Very small changes in structure can transform an agonist into its antagonist, which blocks the effect of a narcotic. By comparing the structure of morphine, codine, heroin, and etorphine as depicted in Figure 1, it becomes evident that they have the same ring skeleton. They are also often classified as phenanthrene derivatives. Figure 2 depicts two very common antagonists, naloxone and naltreone, which are produced by substitution of an allyl group on the nitrogen. As stated earlier, their action is by counteracting the effects of agonists such as morphine. Figure 3 illustrates two synthesized narcotics, methadone and a meperidine (Demerol). It is not easy to detect similarities in their two-dimensional structures; however, three-dimensional models of these two synthesized drugs have conformations that correspond to the d(-) isomer of morphine (3). Another generalization regarding narcotics is that extremely potent drugs bind to a specific receptor site even at very low concentrations.

All of the preceding observations led to the development of a drug-receptor site theory. Beckett and Casy (4) recognized the importance of the general features of a molecule, such as shape and size, in the stereoselective match of drug with receptor. They investigated the activity versus configuration of several optical isomers.

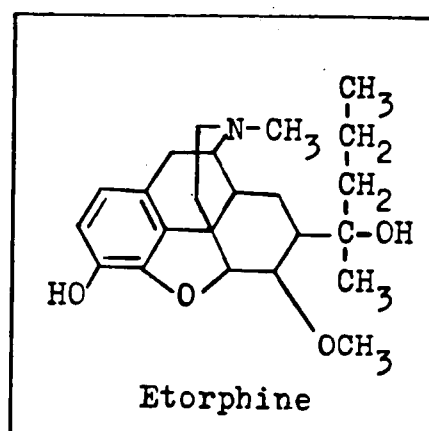
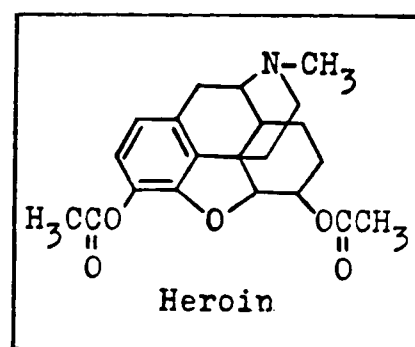
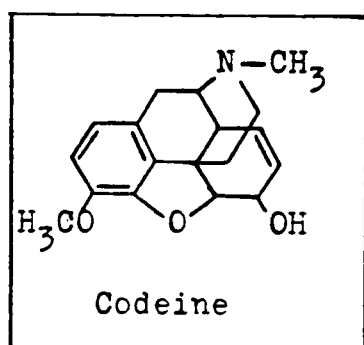
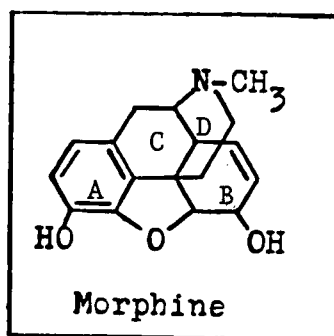


Figure 1. Structures of Various Constituents of Opium: Morphine and two of its Derivatives, Codeine and Heroin (2). Etorphine is the Opiate used in this Study.

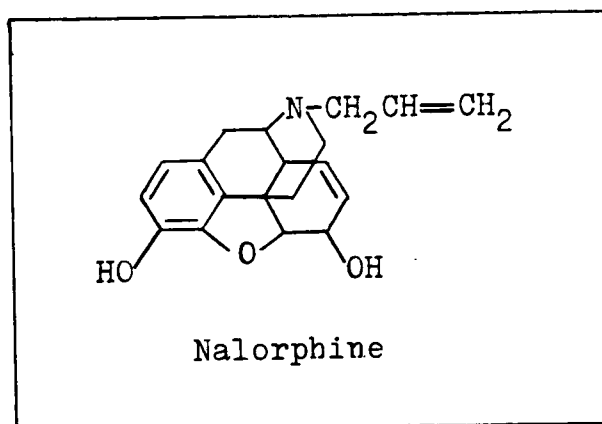
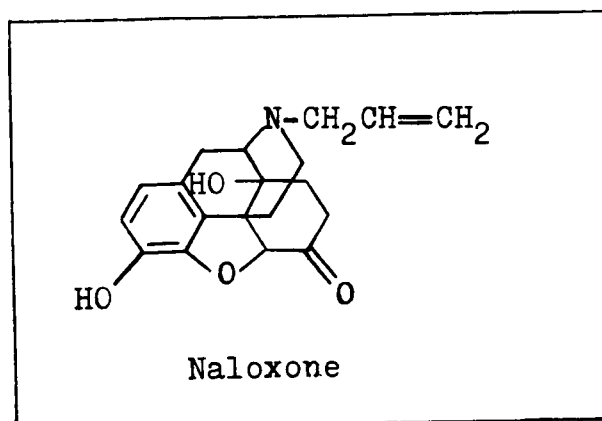


Figure 2. Two Antagonists of Opiates (3).

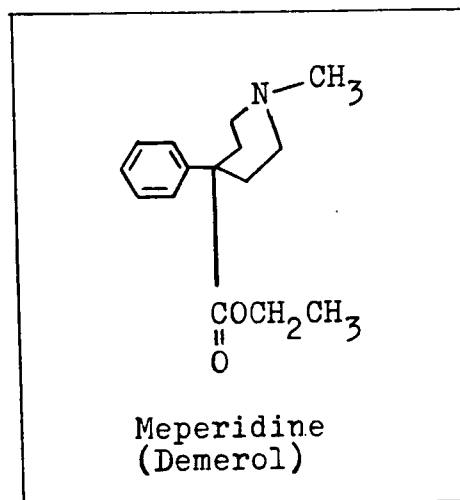
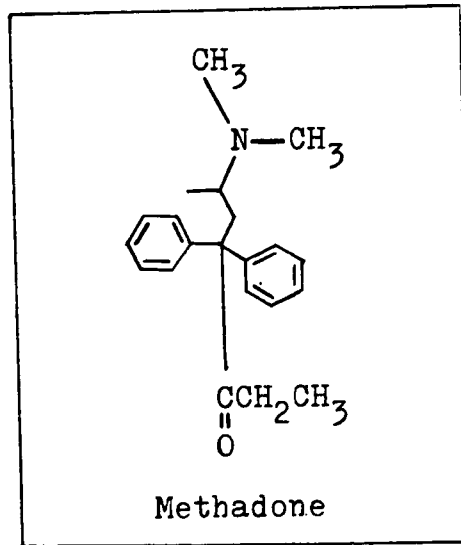


Figure 3. Two Synthesized Narcotics (2).

Since many of the isomers had similar distribution and metabolism traits, they concluded that the differences noted in activity were due to a difference in the nature of the drug-receptor interaction.

Both analgesic compounds and their antagonists fit the receptor surface, the former initiating a reaction sequence; and the latter, due to the slight difference in its structure, failing to evoke the reaction sequence. The following are three features Beckett and Casy proposed as essential for proper fit.

1. A flat region for the binding of a benzene ring through weak intermolecular attractions.
2. An anionic site for association with the protonated nitrogen present at physiological pH.
3. A cavity properly situated with regard to sites 1 and 2 which would be capable of accomodating the projecting CH₂ of the D ring (2).

The benzene ring and nitrogen are common features of active opiates, agonist as well as antagonist. There are correlations in the effective width of the basic group of the opiates with analgesic activity. Whereas the receptor proposed by Beckett and Casy was essentially rigid, Portoghese (5) suggested a conformationally flexible receptor. Instead of the highly distance-specific Van der Waal forces, he proposed that hydrophobic-attractive forces were operative in binding of the aromatic ring. He noted

that differences in the distance between ring and basic nitrogen of conformationally restricted phenolic compounds are in obvious disagreement with Beckett and Casy's finding. He envisioned the anionic site as a pivotal site around which various modes of binding could occur. This is analogous to the conformational mobility of an active site of an enzyme that enables the substrate to be correctly aligned with the catalytic groups necessary for action.

Goldstein et al. (6) developed a procedure for determining stereospecific binding to opiate receptors. Figure 4 illustrates their use of levorphenol and its three specific types of interactions. The trapped and dissolved interactions are known as nonsaturable interactions and are mere associations of the drug with some subcellular fraction. The second category, nonspecific saturable interactions, arises through ionic bonds, hydrogen bonds, and hydrophobic forces. Cationic drugs like the opiate narcotics would be expected to interact nonspecifically with anionic groups such as are present in proteins, nucleic acids, mucopolysaccharides, etc. The stereospecific binding is the relatively small amount of specific saturable interaction at the receptor site where drug binding triggers the chain of events that leads to the characteristic effect. In Figure 4, Section A depicts the participation of the labeled drug, levorphenol, in all three kinds of interactions. Section B demonstrates that the incubation with

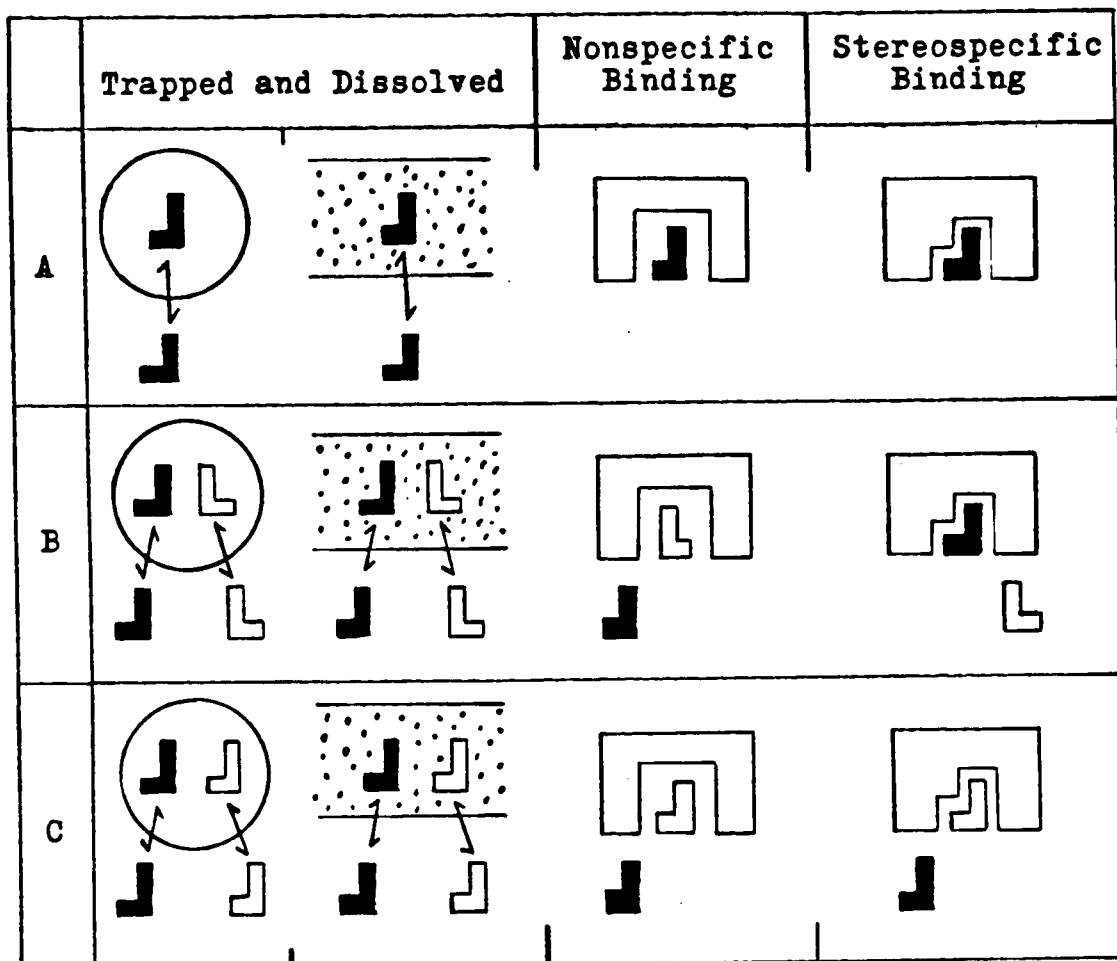


Figure 4. Goldstein's Illustration of Procedure for Determining Stereospecific Binding (6).

100-fold excess of the unlabeled L(+) isomer, dextrorphan, blocks the label from entering the nonspecific saturable sites. The nonsaturable sites are not hindered. The difference between Sections A and B would determine the nonspecific saturable amounts. Finally, 100-fold excess of the nonradioactive levorphenol would produce the results as shown in Section C. The label is blocked from saturable sites, but it still participates in nonsaturable associations. The difference between Sections B and C represents the stereospecific binding. This development paved the way for recent research that has focused on identification and isolation of opiate receptors. Other necessary criteria for identifying the opiate receptor are: saturability, target cell specificity, correlation between the affinity of various opiates and analgesia, and correlation between concentration and pharmacologic action.

The distribution and biochemical properties of these receptors have been thoroughly investigated. Using tritiated (^3H) naloxone, Pert and Snyder (7) independently demonstrated opiate receptors present in the guinea pig ileum and central nervous system tissue. They found the greatest enrichment of opiate receptors in the microsomal fraction of the rat brain. The chemical makeup of the receptor was investigated by Pasternak and Snyder (8) by using enzymatic treatments to modify membrane constituents. Both lipase and protease destroyed opiate binding.

RNase and DNase were both shown to be ineffective. A protein-lipid complex was postulated as the opiate receptor. Lin and Simon (9) gave support to the role of phospholipids in the binding process by demonstrating the sensitivity of the opiate receptor to ionic and nonionic detergents. A fluid-mosaic model for the structure of a biological membrane was proposed by Singer and Nicholson (10). Membrane perturbation upon binding of a drug to components of the receptor may result in redistribution of membrane components by diffusion through the viscous two-dimensional solution, thereby allowing new interactions to occur. The role of lipid in the receptor mechanism is summarized as follows:

1. The lipid, alone or in combination with a membrane protein, serves as the recognition site for the ligand.
2. The lipid surrounding the receptor molecule and that of the receptor modulates the three-dimensional structure and, therefore, regulates affinity of the ligand for the receptor.
3. The lateral mobility of the receptor is regulated by the physical state of the lipid in which the receptor is situated and, in turn, the interaction of the ligand-receptor complex with the effector molecule such as adenylate cyclase or the ion channel (11).

If a specific lipid has a role in the recognition of the ligand, the purified and synthetic lipid has a high affinity for this ligand and is able to compete for this same ligand with the biological membrane containing the receptor.

Since most neural transmitters and drug molecules are cationic at physiological pH, acidic lipids were considered by Loh (12) as the most likely candidates for binding sites of opiates. Carboxylate, phosphate and sulfate anionic derivatives are all present in nerve membranes. Loh stated compelling reasons that favored sulfate as having similarities to the opiate receptor postulated by Beckett and Casy:

If we fixed the ceramide moiety of the cerebroside sulfate in the antiplanar structure (which is believed to be the most stable), the freedom of motion of the cerebroside sulfate is much more restricted and the galactose moiety can only move around the axis bonding it to the cerebroside moiety. If an opiate with a rigid ring structure such as morphine is allowed to approach cerebroside sulfate, the opiate molecule may induce the flexible cerebroside sulfate molecule to fit its rigid structure. Thus, the highly polarizable phenyl group of morphine could interact with the complimentary part of the ceramide moiety by Van der Waals bonding which is proportional to the polarizability due to its bonds. In addition, the morphine group could bind the hydroxyl group in the ceramide moiety through hydrogen bonding. The piperidine ring of morphine would then fit the space between the ceramide moiety and galactose group of CS by hydrophobic bonding and allow electrostatic interaction between the protonated nitrogen and anionic sulfate group of cerebroside sulfate (12).

Figure 5 shows a comparison between the molecular model of cerebroside sulfate (lower right) and that of the receptor

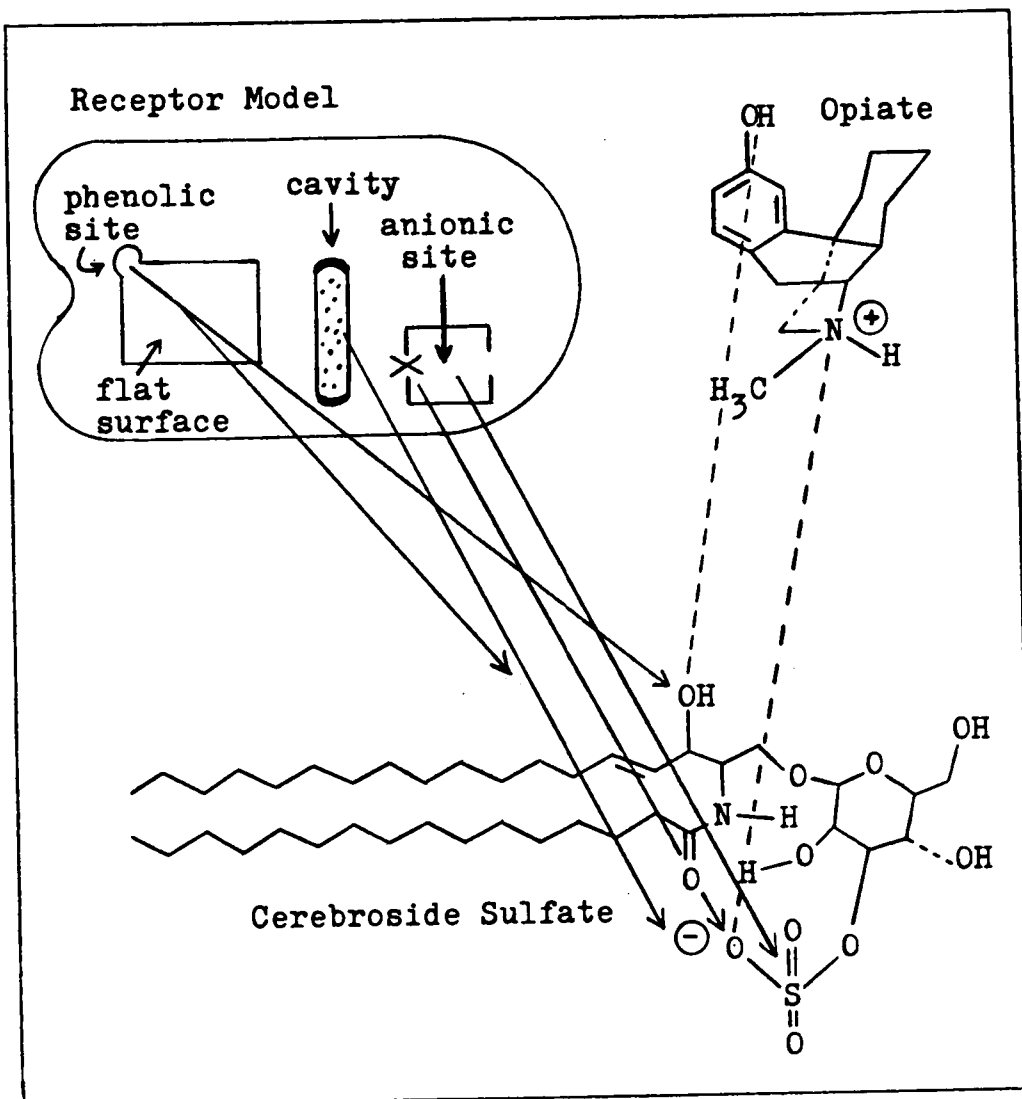


Figure 5. Loh's Postulated Opiate Receptor (Cerebroside Sulfate) Compared to Beckett and Casy's Model Receptor as Adapted from Loh (12).

as proposed by Beckett and Casy (upper left). The possible sites of interaction with a narcotic such as morphine (upper right) are shown. Using an organic-aqueous solvent partitioning system, Loh demonstrated that opiates bind stereospecifically to cerebroside sulfate (CS) with high affinity and that these binding sites could be saturated. There was a high correlation between in vivo potencies of various opiates and their affinities for cerebroside sulfate. The CS-agonist was demonstrated to be more hydrophobic than the CS-antagonist.

To investigate the possibility of cerebroside sulfate involvement as the cellular recognition site for opiate, an elution profile was obtained by chromatographing the acidic lipid on a Sephadex LH-20 column. When this profile was compared to one obtained by another researcher in the purification of an opiate receptor, the two were virtually identical. Loh has further demonstrated the involvement of cerebroside sulfate and opiate receptor binding by the use of Azure A, a cationic dye, which caused inhibition of opiate binding (11). Treating the membrane with a sulfatide degradative enzyme, cerebroside sulfatase, also resulted in inhibition. Fifty percent of the specific binding of the antagonist ^3H -naloxone was inhibited, while only two percent of the cerebroside sulfate present was hydrolyzed. Scatchard analysis indicated a significant decrease in the number of binding sites with no

statistically significant alteration in affinity of the ligand for the receptor (13).

To further accumulate evidence that lipids play an integral role in the opiate receptor, Cranes et al. (14) developed antibodies to cerebroside sulfate by repeated injections of a hapten. In rats that were anesthetized with pentobarbital, injections of morphine or β -endorphin inhibited the wet-shake response to ice water dipping. Treatment with the antibody to cerebroside sulfate prevented this inhibition by the opiates. Antiserum to triphosphoinositol, another acidic lipid, failed to antagonize morphine.

Using a mouse brain homogenate, Ahmed and Byrne (15) demonstrated inhibition of opiate binding with the use of 4,4'-dithiopyridine, a sulfhydryl modifying agent. The reversibility of this inhibition was demonstrated by re-suspension in dithiothreitol or β -mercaptoethanol.

Evidence that could dispute Loh's CS receptor role was established when Marzullo and Friedhoff (16) found no correlation between opiate binding capacity and cerebroside sulfate levels in neural tissues of the developing chick. They reported that frontal lobes of the brain, which have the highest opiate binding activity, contained only as much sulfatide as cerebellum, which has very low binding activity. Myelin, on the other hand, contained very high concentrations of cerebroside sulfate and demonstrated no

binding affinity. If the A-1 basic protein is removed from myelin cerebroside sulfate, opiates are then able to bind.

Formation of Cerebroside Sulfates

Cerebrosides are carbohydrates containing both glycolipid and nitrogen (but not phosphate or glycerol as the phospholipid lecithin and cephalin do). Cerebroside sulfates (sulfolipids or sulfatides) have a sulfate group attached in an ester linkage to the galactosyl residue (17) (Figure 6). Their formation begins with the synthesis of sphingosine (Figure 7). The pathway of these sphingosines to ceramides, cerebroside, and sulfatides is also illustrated in Figure 8, denoting the nucleotide carrier uridine diphosphate (UDP) as essential to the formation of cerebroside and phosphoadenosine phosphosulfate (PAPS) to sulfatide formation (18).

It has been demonstrated that once the period of myelination is complete, the sulfolipid content in brain tends to remain stationary (19). The incorporation of ^{35}S -sulfate into brain sulfolipid of rats was investigated by Davison and Gregson (20). The maximum rate was apparent in 20-day-old animals, a development which parallels the period of myelination.

A strain of mice known as Jimpy (B6CBA) have low levels of brain cerebroside but normal levels of phospholipids and gangliosides. Loh (12) showed the effects of this reduced availability of brain sulfatide on morphine

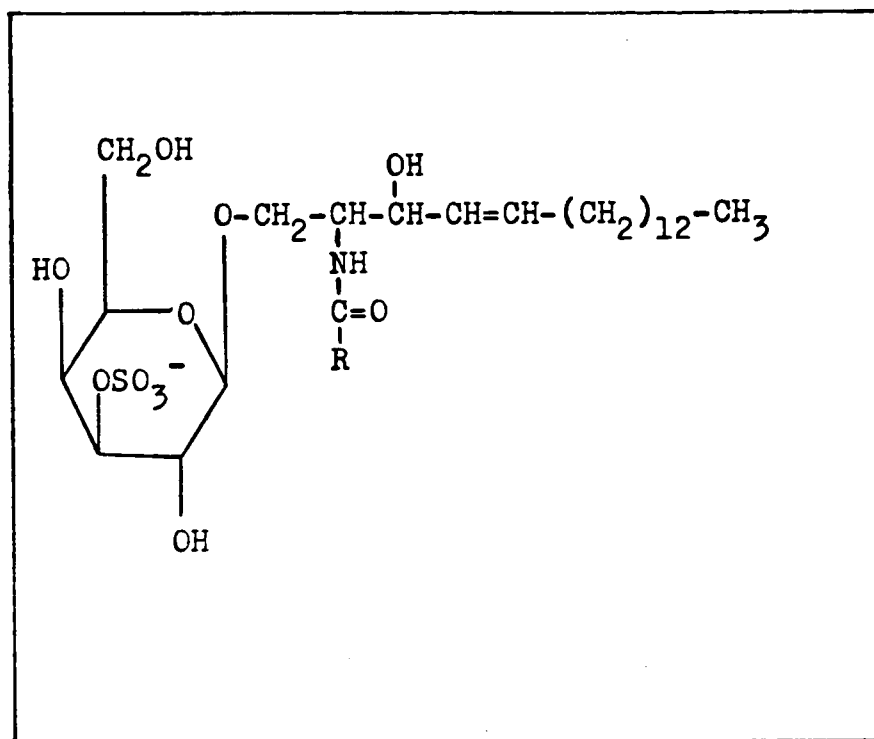


Figure 6. Cerebroside Sulfate (17).

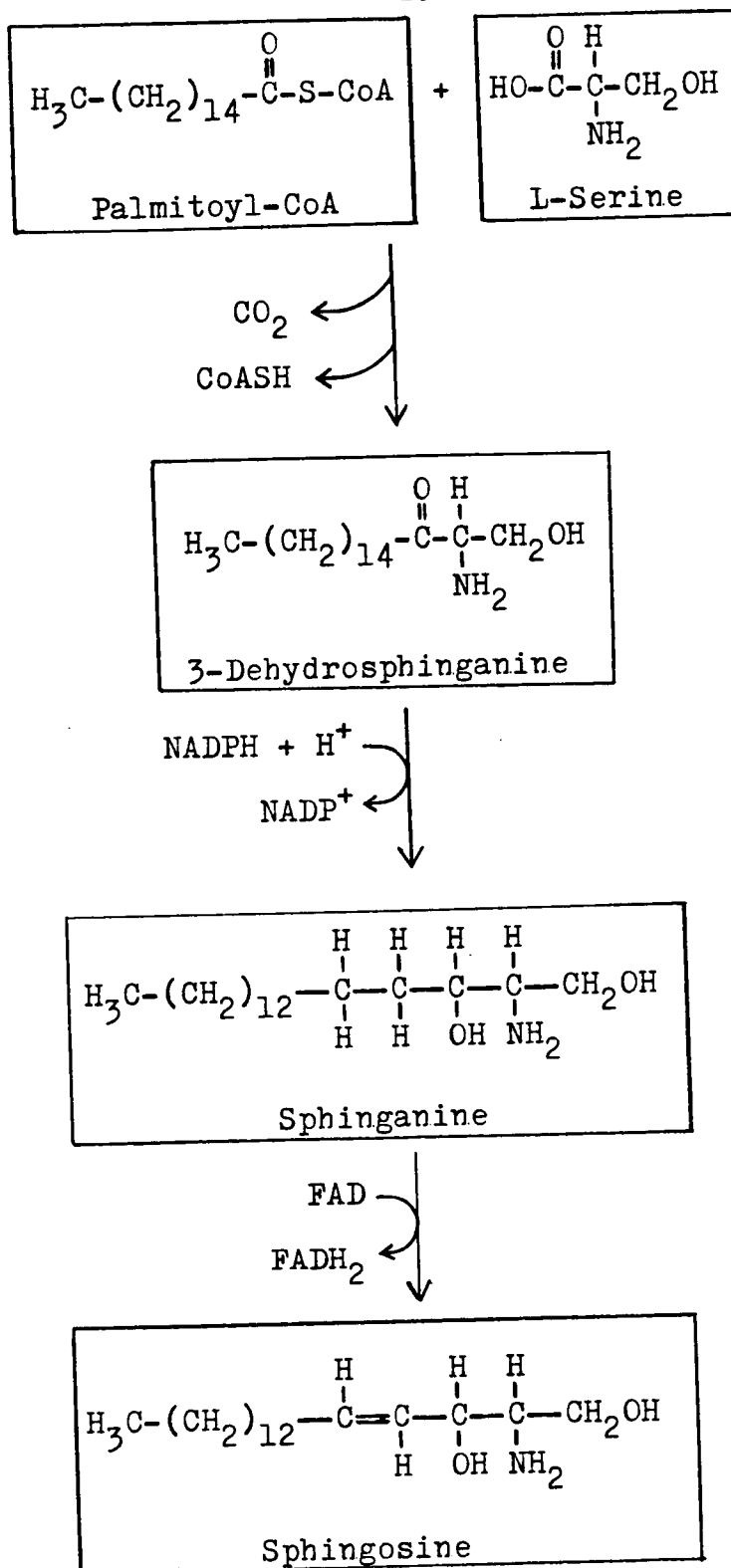


Figure 7. Sphingosine Synthesis as Adapted from Bhagavon (18).

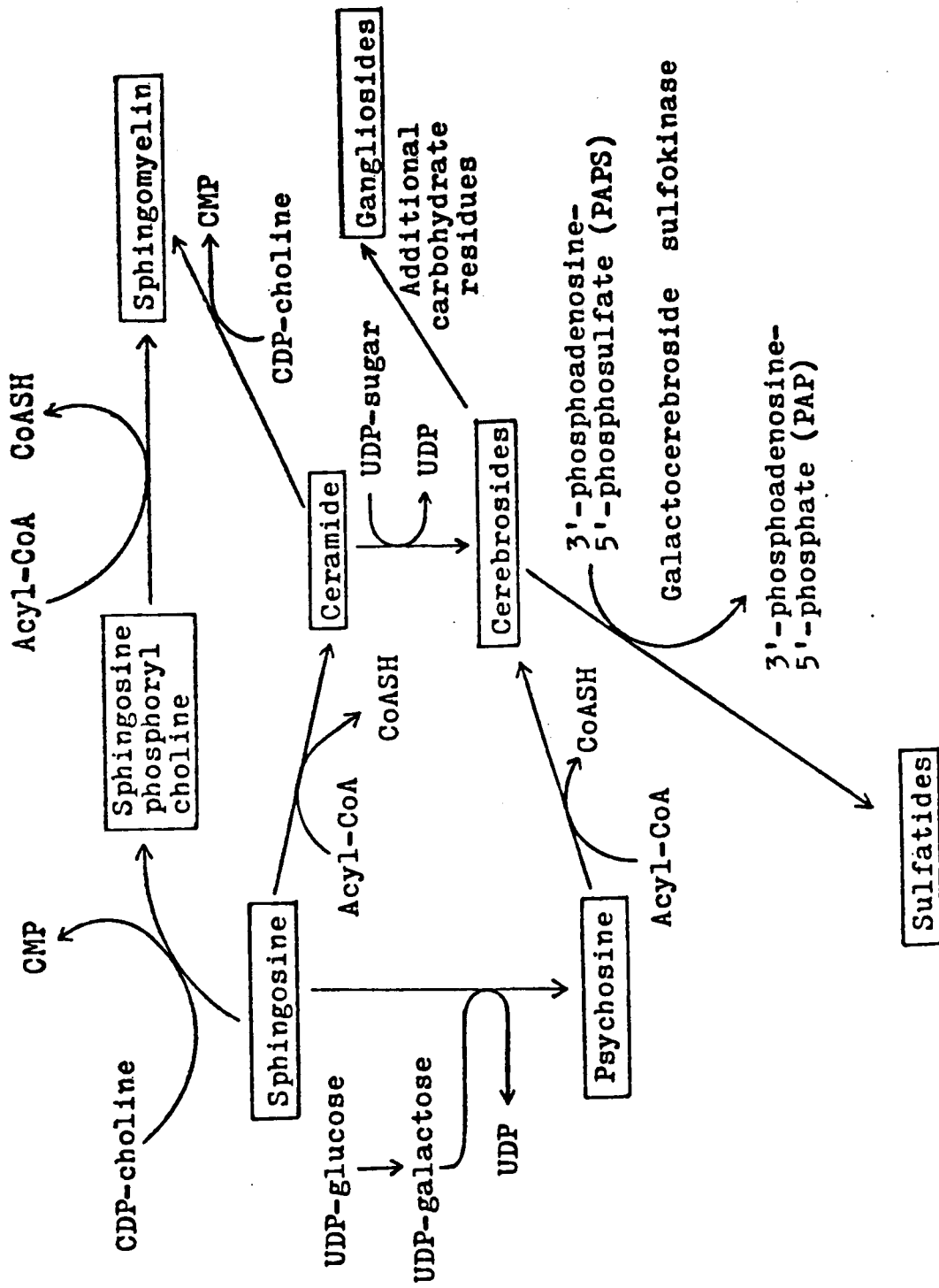


Figure 8. Pathway for Formation of Sulfatides from Sphingosine as Adapted from Bhagavon (18).

analgesia. The median analgesic dose required for the Jimpys was 11-fold higher than that required for normal animals.

Dietary Sulfur

Due to the requirement for sulfur in the formation of cerebroside sulfate, previous work in this laboratory is of special pertinence. Sulfur-containing hormones, coenzymes, as well as amino acids, play significant roles in cellular regulation. The need for organic sulfur is met primarily by methionine, cysteine, and cystine. Methionine is the source of methyl groups that are donated in the formation of a number of compounds (21). Cysteine can serve to spare methionine and thus allow it to remain in the remethylation pathway (22). In the diet of rats, cysteine has been shown to be able to supply over 50 percent of the total sulfur amino acid requirement (23), and 80 percent replacement has been demonstrated in man (24).

Metabolic alterations that parallel the levels of inorganic sulfate in the diet of experimental rats have been demonstrated by several researchers under the direction of Smith (25, 26, 27). Inorganic sulfate as a dietary component also plays a role in sparing methionine by influencing the utilization of methionine. The omission of inorganic sulfur from the diet of a rat forces it to satisfy sulfate requirements by oxidizing sulfur to sulfate, which is a process wasteful of the sulfur-containing amino

acids. The feed efficiency of rats was improved with the addition of inorganic sulfate to their diet (25). This dietary inorganic sulfate can be incorporated into sulfolipids (18) as well as hepatic fibrinogen, taurine, and cysteine. Acuff obtained decreased sulfate recovery from brain lipid fraction in rats fed 15 percent casein diet supplemented with 0.625 percent methionine and treated with pentobarbital. The drug-metabolizing enzyme, cytochrome P₄₅₀ was elevated in this same group of animals [an expected result that had been demonstrated earlier (26)], but the sleep time of the animals was longer. It has been postulated that this effect is due to a lower level of drug eliciting sleep by occupying fewer receptors, yet enough to produce the effect. Less drug should be required, and the effect would, thereby, be longer lasting (28).

Low inorganic sulfur levels increased the mobilization of tissue sulfur from malathion-intoxicated rats (27). Brown (29) found that even with adequate methionine and cysteine in the diets, a vitamin E deficiency produced a requirement for inorganic sulfur for normal collagen formation.

Both carbohydrate and fat metabolism have been affected by manipulation of dietary sulfur. The incorporation of cysteine (U-¹⁴C-cysteine) into glycogen was increased in rats fed the optimal level of inorganic sulfate. Alterations in rat liver phosphoenol pyruvate carboxykinase and

pyruvate carboxylase activity were reported (30). Lipogenesis has also been demonstrated to be sensitive to the level of dietary inorganic sulfate. The enzymes acetyl-CoA carboxylase, citrate cleavage, and malic all showed altered activity levels by dietary sulfur manipulation (31, 32).

Smith (33) established that the optimum level of inorganic sulfate for a rat is 0.02 percent. This was determined by measuring expired $^{14}\text{CO}_2$ labeled by 1- ^{14}C -methionine catabolism. The diets tested had levels of inorganic sulfate ranging from 0.01 to 0.05 percent. A reduction in expired $^{14}\text{CO}_2$ was obtained with the 0.02 percent level, thus the optimal sparing of methionine had been achieved.

Metabolism of Opiates

The duration and intensity of action of opiates in animal and man is limited by their metabolism, represented by a complex interplay of several factors. Route of administration, absorption, degree of binding of the drug to protein, excretion, as well as the actual metabolism of the drug are all factors that determine the efficacy.

Dobbs (34) studied the effective dose (ED_{50}) for etorphine analgesia. He found the dose for subcutaneous administration to be $1.7 \mu\text{g/kg}$. By contrast, the ED_{50} for dihydromorphine is 1.8 mg/kg subcutaneous. Dobbs et al. (35) found the potencies of etorphine to be $2.0 \mu\text{g/kg}$ with intravenous administration, $2.5 \mu\text{g/kg}$ in intramuscular administration, and $5\text{--}10 \mu\text{g/kg}$ for sublingual administration.

Intracisternal and intraventricular routes of administration would largely, but not entirely, obviate distribution differences between the groups of opiates.

The rate of absorption of opiates is principally determined by the extent of ionization (pK_a), lipid solubility, and molecular size. Diffusion through lipoidal biological membranes as lipid soluble, nonionized molecules is at a rate of transfer related to their lipid-water partition coefficient. The hypothesis that enhanced lipophilic character favors selected uptake in nervous tissue, and as a consequence may contribute in part to the very high potency of the drug, was suggested by Boura and Fitzgerald (36) in their work with another endoethenooripavane derivative (M 320). Dobbs (34) investigated the ratio of brain concentration at the onset of peak activity compared to administered dose as a measure of overall relative selectivity of the uptake of etorphine in the brain. He found an approximate 10-fold difference relative to morphine, which he stated accounted for only a portion of the approximately 1,000-fold overall increase in potency. Misra (37) reported that the ionized species of these drugs are relatively impermeable to biological membranes. Absorption, therefore, must occur in the nonionized form by passive diffusion.

Albumin is the major plasma protein that binds opiates; however, these drugs may also bind to gamma globulin

fraction antibodies. Extensive binding results in reduction of rates of metabolism and excretion and increases the half-life of the drug.

Primary pathways for excretion of several opiates are renal and biliary. Conjugation of the drug with D-glucuronic acid or sulfuric acid increases the polarity of the molecule, which leads to its solubility in bile or urine and its consequent excretion from the body. Glucuronidation and sulfation require the formation of a purine nucleotide derivative, which donates the conjugating group. Conjugation occurs primarily in the liver by the Phase 2 enzymes of xenobiotic metabolism. Figure 9 shows the reaction of glucuronidation of etorphine catalyzed by the microsomal enzyme UDP-glucuronyltransferase. The sulfation of etorphine catalyzed by sulfotransferase, a cytoplasmic enzyme, is shown in Figure 10. UDP-glucuronic acid (UDP-GA) and PAPS are required for effective conjugation (38).

Figure 11 illustrates the sulfoxidation of cysteine to inorganic sulfate. Since methionine sulfur can also be converted into cysteine sulfur by transsulfuration, either amino acid can supply sulfate. In the rat, deprivation of sulfur-containing amino acids produced large decreases in urinary excretion of sulfate. Investigation by Sabry (39) indicated a linear relationship between intake of cysteine and methionine and excretion of inorganic sulfate.

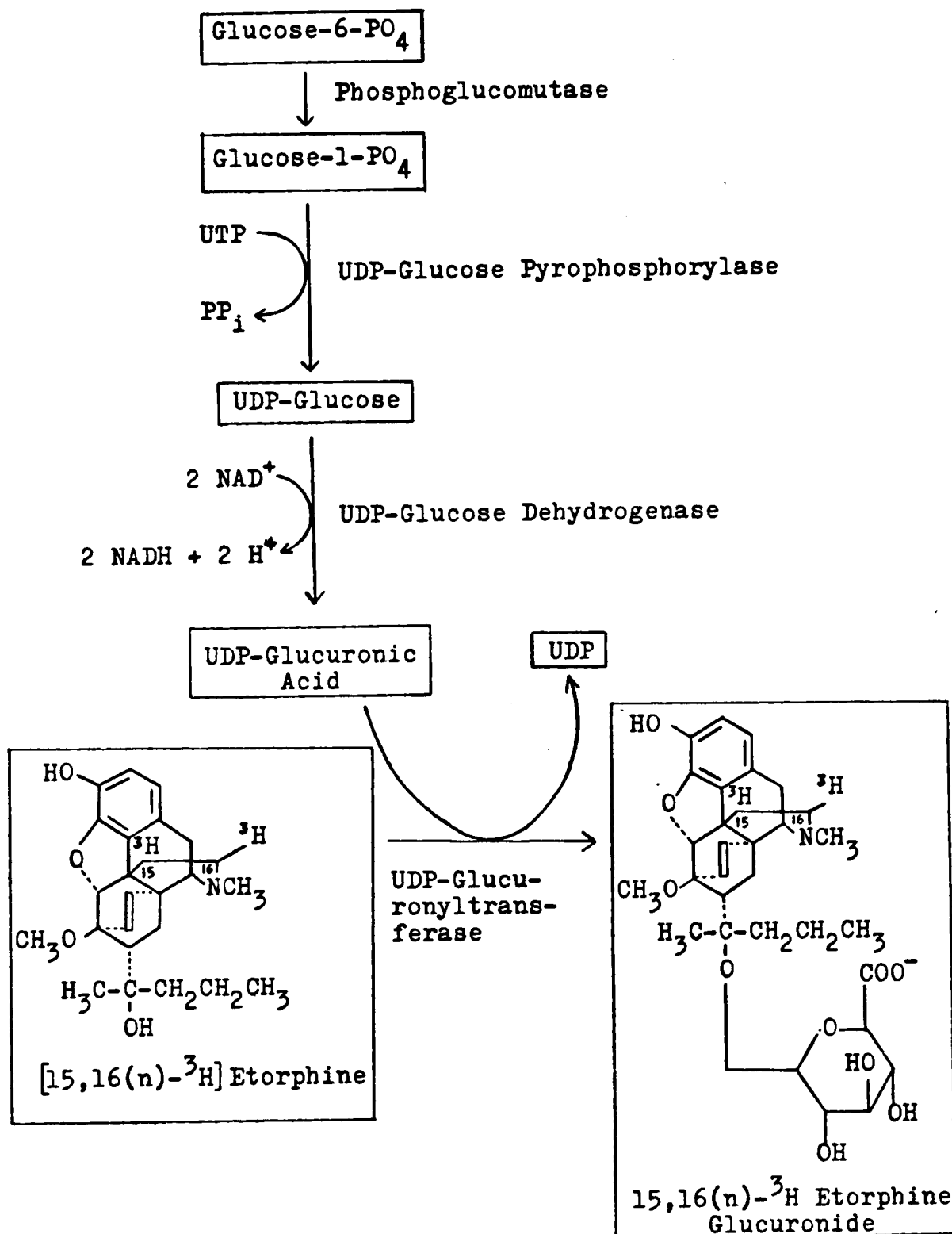


Figure 9. Conjugation of Etorphine with Glucuronide as Adapted from Dutton (38).

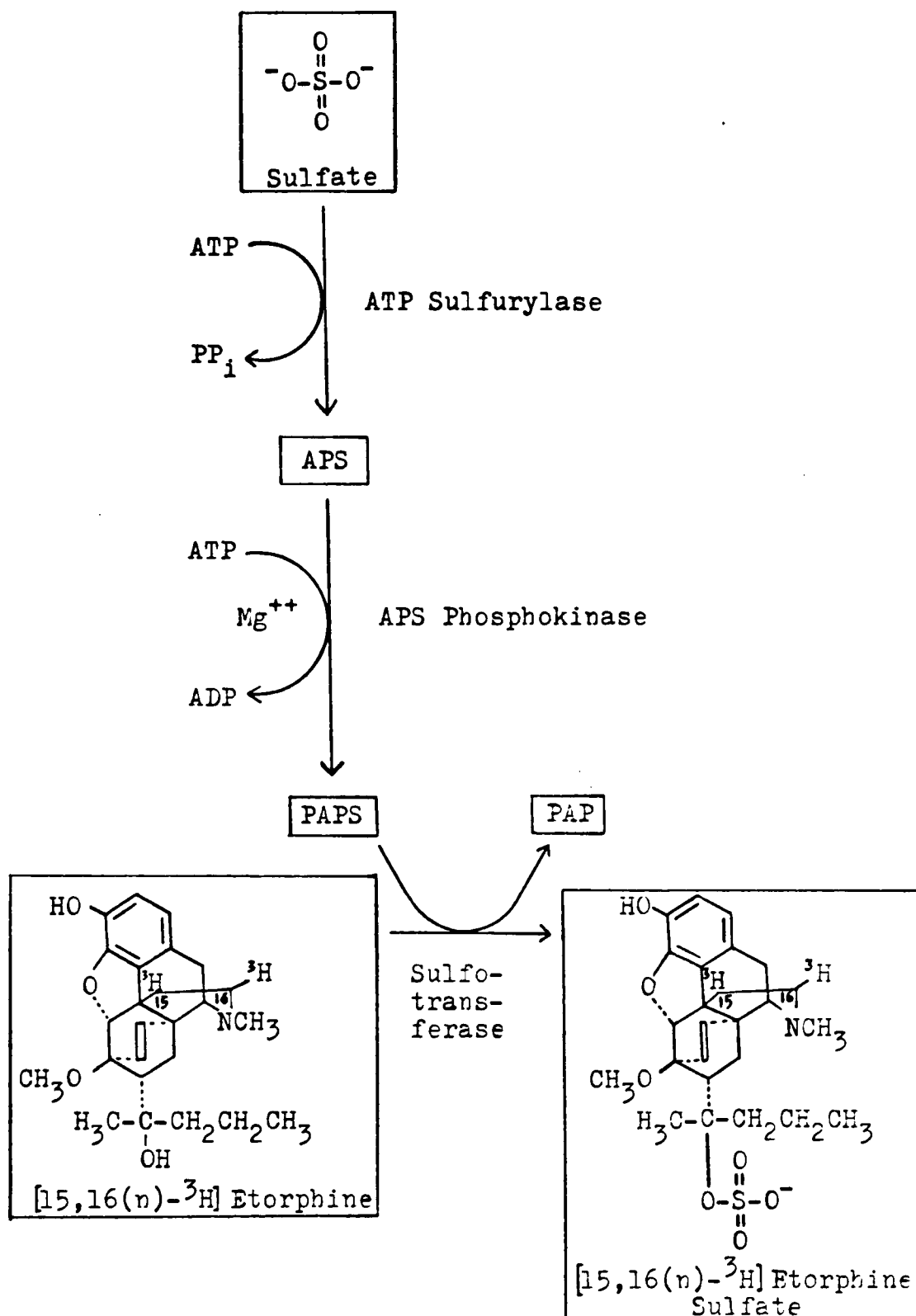


Figure 10. Conjugation of Etorphine with Sulfate as Adapted from Dutton (38).

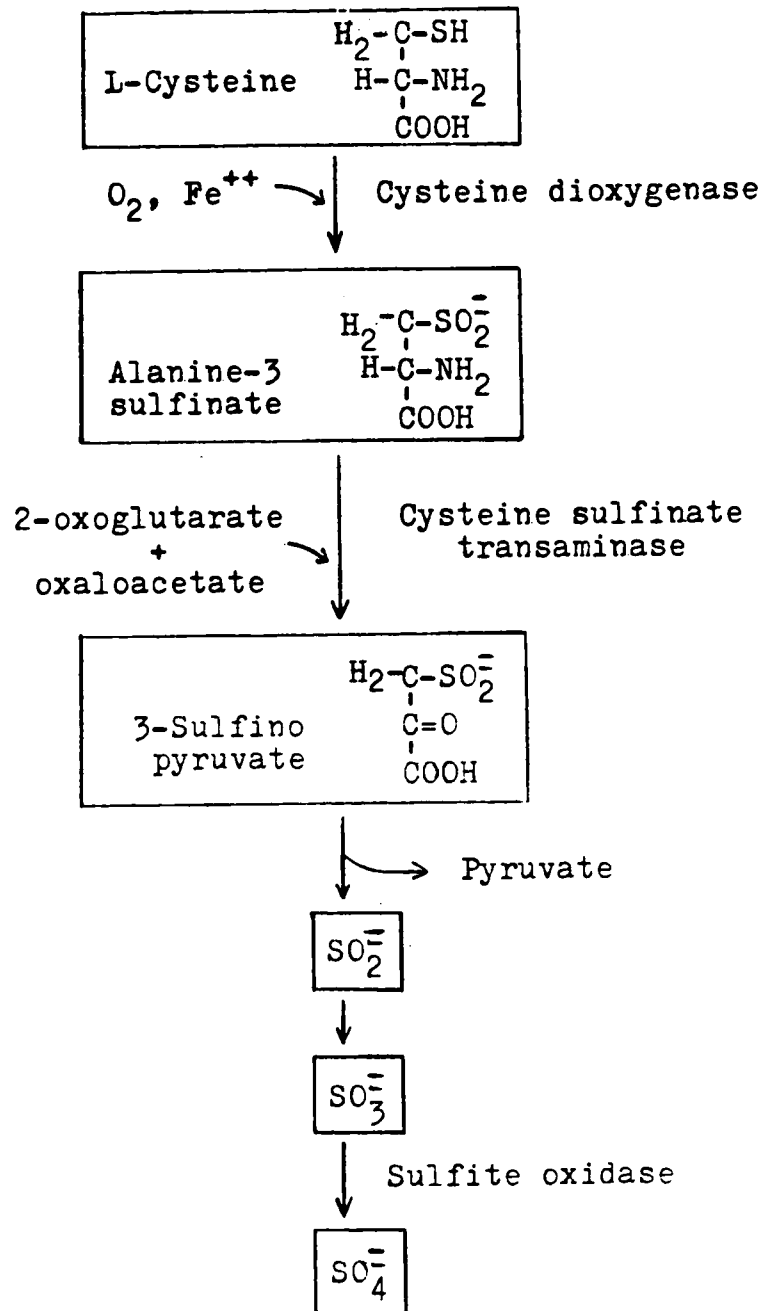


Figure 11. Sulfoxidation of Cysteine to Inorganic Sulfate as Adapted from Mulder (35).

Increases in the amount of methionine resulted in increased conversion to inorganic sulfate, indicating that the body eliminated surplus methionine sulfur by sulfoxidation.

However, when Lakshmanon et al. (40) eliminated threonine from the diet of man, a significant increase in urinary excretion of inorganic sulfate was noticed. This finding suggests that the increase may merely be an indicator of an unbalanced diet. The same researcher (41) found that after eight days of deleting sulfur-containing amino acids from the food in man, sulfate excretion was increased by approximately 65 percent. Traces of inorganic sulfate were still found, although the concentration of methionine in plasma had not yet decreased. Protein catabolism may have provided the cysteine and methionine. Organic sulfate may be released from tissue pools by catabolism of glycosaminoglycans.

The ratio of conjugates is determined by several factors: species differences in conjugation may be due to different amounts of the transferase. Dose-dependent changes are well-documented. In the rat, intravenous and intramuscular injections of phenol were conjugated increasingly with glucuronic acid as the dose increased (42). With 1-naphthol as substrate, the change was less pronounced. The percentage of sulfate conjugation decreased, but the amount of sulfate conjugation still increased. This indicates that sulfation was not saturated at the higher

dose. With the drugs acetaminophen, 4-methylumbelliferone, and harmol, Anderson et al. (43) found the primary metabolite was sulfate at low concentration. At higher concentration, the more glucuronidated metabolites were excreted. The opposite was true for phenolphthalein and 2-naphthol. Phenobarbital treatment increased glucuronidation of harmol while salicylamide inhibited its conjugation with sulfate (44). These findings illustrate drug specificity of conjugating systems and influence by drug interactions. Mulder (45) produced only small increases in the rate of sulfation in rats injected with a high dose of phenol by infusion of large amounts of sodium sulfate, indicating that depletion of sulfate plays a minor role in the decreased sulfation of phenol. A similar shift was noted when the drug paracetamol was injected into rats. The kinetic properties of the transferases play a role in these dose-dependent changes. Thor et al. (46) found variation in the K_m for sulfation of a group of substrates. Acetaminophen had the highest K_m for sulfation, but the apparent K_m for glucuronidation was still higher. In general, glucuronidation appeared to be low affinity and high capacity. Sulfation, the reverse, demonstrates high affinity and low capacity. One example of higher sulfation was with the substrate 7-hydroxycoumarin (47). Substrate specificity for the transferases, as well as cosubstrate availability, contribute to the determination of ratio of

conjugation. Changes in uptake by tissues as the dose increases could also be a factor. Conjugation pattern of the liver may be observed at low dose while at a high dose the kidney may contribute a different conjugation pattern.

Alterations in conjugation ratios can also be effected by dietary sulfur. Kurzynske (48) reported salicylamide conjugation with sulfur increased as the level of inorganic sulfate in the diet increased. Morris (49) investigated the effect of dietary sulfur on hepatic sulfotransferase and UDP-glucuronyltransferase (UDP-GT) with acetaminophen treatment. Neither enzyme appeared to be affected by either level of inorganic sulfate or supplementation with organic sulfur. The excretion of glucuronide conjugate increased significantly with low sulfate levels and cysteine supplementation. Also, the excretion of the mercapturic acid conjugate and the base drug decreased as dietary inorganic sulfate increased.

Quantifying Binding Sites

Scatchard (50) developed a formula for determining interaction of small molecules and ions on the surface of proteins. He stated that the function of such binding could only be fully understood after the simpler questions "how many?" and "how tightly bound?" were answered. The ratio of concentration of bound ligand $[L]_b$ to the concentration of total protein $[P]_t$ is represented by V . The limits of V are 0 (no ligand bound) to 100 percent

saturation of binding sites. Determination of bound ligand at various concentrations of drug with constant levels of protein provide the data necessary to determine number of binding sites per monomer unit (51).

Loh et al. (13) incorporated this analysis in their investigation of the inhibition of opiate binding by the enzyme cerebroside sulfatase. By comparing ^3H -naloxone binding to control and enzyme-treated synaptic membranes, they concluded that a highly significant decrease in the number of binding sites had resulted from hydrolysis of sulfatides. This inhibition was mediated by hydrolysis of less than two percent of the total sulfatide moieties. They postulated that since 100 percent inhibition was not obtained, two classes of receptors could be involved in opiate binding--one not sensitive to sulfatide hydrolysis, as well as the sensitive one exhibited in their work. Scatchard analysis was also employed by Klee (52) in comparing morphine-dependent rats with controls. A reduction in the number of binding sites did not parallel the development of morphine dependence. Experiments by Fischel and Medzihradsky (53, 54) revealed that experimental conditions of the opiate receptor assay markedly influenced the Scatchard analysis. Differential effects on binding of Na on agonists and antagonists is well established. Other researchers (55, 56, 57) have examined temperature effects in opiate binding studies. The opiate receptor is inherently

complex; assays, therefore, require specific considerations.

Multiple Receptors

The heterogeneity of pharmacological effects of opiates is of special interest. It would be extremely beneficial to be able to develop a drug with analgesic effects that did not produce addiction. The work of many researchers has produced evidence that questions whether or not a single class of receptor could mediate the diverse pharmacological responses to the various types of narcotic analgesics.

Martin et al. (58) provided evidence for three receptor types in the dog central nervous system (CNS). They found that different types of narcotic analgesics elicited strikingly different pharmacological responses and demonstrated lack of cross tolerance. Morphine-like drugs preferentially interacted with the mu (μ) receptor. Kappa (κ) receptors interact with benzomorphans and sigma (σ) with N-allylnorcyclozocine. A fourth type of receptor delta (δ), was postulated by Kosterlitz (59) to be the site for interaction of the shorter enkephalin peptides, a group of endogenous opiates. Further evidence of this preferential binding of endogenous ligands was proposed by Chang (60). Met-enkephalin may be specifically targeted for mu receptors and leu-enkephalin the delta ligand. β -endorphin, a peptide that contains the amino acid sequence of the enkephalins, interacts equally well with both classes.

Developmental differences between high and low affinity opiate binding sites were reported by Pasternak (61). He suggested that analgesia and respiratory depression may be mediated by different sites. Two-day-old rats were markedly insensitive to analgesia and entry of morphine into the brain compared to 14-day-old rats. However, the respiratory rate was depressed in both groups. These results indicate a correlation between high-affinity binding and analgesia and between low-affinity binding and respiratory effects.

In comparing the binding of ^3H -naloxone, Cho et al. (62) found qualitative differences. Binding of ^3H -etorphine to rat brain was completely inhibited by most of the 23 drugs tested, while ^3H -dihydromorphine (^3H -DHM) and ^3H -naloxone binding were not. The binding of the former exhibited competitive inhibition while that of the latter two did not. The conclusion that ^3H -etorphine has a single binding site common to the three drugs used was drawn. This site corresponded to the high affinity site of ^3H -DHM and ^3H -naloxone. Based on differential in vitro binding affinities in the presence and absence of sodium, Childers (63) also classified etorphine into a group of agonists that differ from morphine.

An enhancement of brain etorphine concentrations followed Lange's (64) morphine pretreatment of mice. However, a change in the analgesic ED_{50} for etorphine did

not parallel this enhancement. A two-fold increase in the binding affinity for the antagonist naloxone was also noted in the single dose, morphine-pretreated group compared to an eight-fold increase in morphine-tolerant animals. It was postulated that these unparalleled changes indicated that the antagonist receptor site for naloxone was different from the morphine (agonist) site.

Small fractional occupancy (two percent at the ED₅₀ tail flick assay) was found by Perry et al. (65) to elicit analgesia. In testing antagonists, their effects were approximately proportional to the fractional occupancy. Agonists, however, required increased fractional occupancy. These findings describe a high receptor-effector coupling efficiency. The control appeared to be by guanine triphosphate since its addition to the binding medium in vitro mimicked in vivo dissociation rates.

CHAPTER III

EXPERIMENTAL PROCEDURE

1. BASIC DESIGN

Female Sprague-Dawley rats from the colony of The University of Tennessee, Knoxville, Department of Nutrition and Food Sciences, were randomly selected for each phase of the study. The animals were selected from those weighing between 80 and 120 grams and were housed individually in cages.

A total of nine diets was incorporated into the study. These diets were calculated for implementation of two variables at three different levels. The basal formulation was a 15 percent casein-based, semi-purified diet (Tables 1 and 2). The two variations were for inorganic sulfate content and sulfur-containing amino acid supplementation (Table 3). Food and distilled water were available for ad libitum feeding for a period of at least 17 days before any of the tests were begun.

For each of the three phases of the study, five rats from each of the nine diet groups were investigated. The first group of 45 rats underwent the tail withdrawal reflex test as described by Janssen et al. (66) to determine the analgesic effects of the narcotic etorphine. (A pretest of the reaction time on a group of animals that had not undergone drug treatment showed no obvious difference in their

TABLE 1
Basal diet composition

Component	Quantity per 100 grams
	<u>Grams</u>
Casein	15.00
Sucrose	30.00
Cornstarch	30.00
Cod liver oil	2.00
Vegetable shortening	6.00
Vitamin mixture ¹	2.00
Basal salt mix ²	1.34

¹Nutrition Biochemicals Corporation, Cleveland, Ohio. Vitamin diet fortification mixture was formulated to supply the following amounts of vitamins (g/kg vitamin premix): thiamin hydrochloride 1.0, riboflavin 1.0; niacin 4.5; p-aminobenzoic acid 5.0; calcium pantothenate 3.0; pyridoxine hydrochloride 1.0; ascorbic acid 45.0; inositol 5.0; choline chloride 75.0; menadione 2.25; biotin 0.020; folic acid 0.090; vitamin B₁₂ 0.00135; α -tocopherol 5.0; vitamin A, 9×10^5 units; vitamin D, 1×10^5 units; and sufficient glucose to make 1 kg.

²Table 2.

TABLE 2
Composition of salt mix

Component	Quantity per 500 grams
	<u>Grams</u>
MgCO_3	33.700
NaCl	77.400
KCl	125.400
KH_2PO_4	237.800
$\text{FePO}_4 \cdot 2(\text{H}_2\text{O})$ or $(\text{FeC}_6\text{H}_5\text{O}_7)$	23.000
KI	0.090
NaF	0.100
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.450
$\text{AlK} \cdot \text{SO}_4 \cdot 12 \text{H}_2\text{O}$	0.019
$\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$	0.810
ZnCO_3	4.800

TABLE 3
Diet variables

	Level of sulfate		
<u>Variable diet constituents</u>	<u>0.0002%</u>	<u>0.02%</u>	<u>0.42%</u>
	<u>Grams/2,000 grams</u>		
No sulfur amino acid			
CaCO ₃	26.8	26.4	18.2
CaSO ₄	-	0.8	15.0
Alphacel ¹	246.4	246.0	240.0
Methionine-supplemented			
CaCO ₃	26.8	26.4	18.2
CaSO ₄	-	0.8	15.0
(D,L) Methionine	12.4	12.4	12.4
Alphacel	234.0	233.6	227.6
Cysteine-supplemented			
CaCO ₃	26.8	26.4	18.2
CaSO ₄	-	0.8	15.0
(L) Cysteine	10.0	10.0	10.0
Alphacel	236.4	236.0	230.0

¹Alphacel, Nutrition Biochemical Corporation, Cleveland Ohio.

reaction rates.) The time required for each animal to awaken after administration of the drug was recorded, and the animal was returned to its cage. A preliminary test indicated that cytochrome P₄₅₀ was induced by the use of the drug etorphine. Therefore, after initial injection, the animals were allowed a three-week recovery period, so that the enzyme would return to normal levels. A second injection of etorphine was then made, and the animal sacrificed by decapitation. Blood was collected so that circulating levels of the drug could be evaluated, and the brain was removed for isolation of the cerebroside sulfate.

The second phase of the study was performed on a similar group of animals. After injection and the test of their analgesic response, they were placed in metabolism cages for 24-hour urine collection. The glucuronidated and sulfated conjugates of the drug were evaluated to determine the effect of varying levels of sulfate on drug excretion.

The third phase of the study involved analysis of the effect of diet on the number of binding sites for the drug. Scatchard analysis (50) was used to plot the bound versus bound/free drug.

2. MEASUREMENT OF ANALGESIA

Injections of Opiate

Animals receiving injections were given 0.3 milliliters of etorphine (M99, purchased from Dr. Michael Pelton,

Department of Wildlife and Fisheries, The University of Tennessee, Knoxville) in a saline solution as vehicle. Tritiated etorphine (^3H -etorphine) purchased from Amersham International Limited, Amersham, England, was added to the injection for some of the tests ($\sim 2.6 \times 10^6$ CPM per injection).

Tail Withdrawal Reflex

Five animals were tested simultaneously, so their injections were spaced four minutes apart to allow for handling and recording. Immediately following the injection, each animal was placed in its restraining holder (Fisher Scientific Company, Atlanta, GA) with its tail extended from the holder. The end of the tail of the rat was submerged in 55° water at test time. The 4-minute timing sequence was signaled by an automatic timer, and the reaction time for each animal was evaluated with the aid of a stop watch. The time required for each rat to react was, therefore, measured every 20 minutes in second units with a cutoff time of 15 seconds to prevent injury if no response had been obtained. When an animal flicked its tail with a 15-second or less reaction time in two successive tests, the total sleep time of the animal was recorded.

Cytochrome P₄₅₀ Determination

Five animals receiving etorphine injections were paired with five untreated animals for measurement of cytochrome P₄₅₀. The animals were stunned by a blow to the head, decapitated, and their livers removed and placed in cold isotonic KCl (1.15 percent). Based on weight of the liver, a 90 percent homogenate was made using a Thomas homogenizer with a teflon pestle (Thomas C homogenizer, Thomas Company, Philadelphia, PA). Centrifugation (Lourdes Betafuge Model A, Lourdes Instrument Corporation, Brooklyn, New York) at 4° and 9,000 x g for 20 minutes followed. The supernatant was decanted and poured into a cold graduated cylinder. A second centrifugation at 90,000 x g (Beckman Model L5-50, Beckman Industries Inc., Scientific Instruments Division, Ervin, CA) for 40 minutes followed. The supernatant was discarded, and any lipid was removed with a cotton swab. The pellet was resuspended in isotonic KCl, and 0.1 M phosphate buffer pH 7.0 was added to adjust the absorbance reading between 0.3 and 0.5. Duplicate 3-milliliter samples were pipetted into cuvettes and the absorbance was determined using the Beckman spectrophotometer (Beckman quartz spectrophotometer, Model DU, Scientific Instruments Division, Ervine, CA) at 450 millimicrons. Carbon monoxide was bubbled into the cuvette for 20 seconds and then absorbance was determined. Forty-five milligrams of sodium

dithionate ($\text{Na}_2\text{S}_2\text{O}_4$) were added and contents of tube thoroughly mixed. Another absorbance determination was then made within 1 minute. The results were converted into nanomoles of cytochrome P₄₅₀ by dividing the differences in the absorbance by the molar extinction coefficient $91 \text{ c}^{-1} \text{ mM}^{-1}$.

Determination of Circulating Levels of Etorphine

Blood was collected from the animals at the time of sacrifice and kept on ice until centrifuged at $10,000 \times g$ for 10 minutes. The serum was then removed and a 0.5-milliliter portion pipetted into a scintillation vial for counting in the Beckman LS100C (Beckman Instruments Inc., Scientific Instruments Division, Ervin, CA) using 6 milliliters of methoxyethanol and 10 milliliters of 2,5-diphenyloxazole (twelve grams in one liter toluene) were added to the vial. A 0.5-milliliter sample of the brain homogenate prepared in a blender (Eberback monel metal semi-micro blender cup) with 90 percent phosphate buffer, pH 7.4, was similarly prepared for counting.

Isolation of Cerebroside Sulfate

A brain homogenate as prepared above was centrifuged under refrigeration at a force of $1,200 \times g$ for 15 minutes. The supernatant was then decanted and 10 milliliters of chloroform:methanol (1:1 v/v) were added and thoroughly blended, and poured into an Erlenmeyer flask. This was

placed in a boiling water bath for 1 hour. The mixture was transferred into centrifuge tubes and again subjected to a speed of $1,200 \times g$ for 15 minutes. The supernatant was decanted and saved, and 10 milliliters of chloroform:methanol (2:1 v/v) were added. Another incubation in a boiling water bath for 1 hour followed. The supernatant obtained after centrifugation was then combined with the other supernatant and placed in a freezer to be kept at -20° until the second phase of isolation.

The above brain supernatants of all of the animals fed the same diet were thawed and combined. The chloroform:methanol solvent was evaporated before adding the samples to a florisil (Sigma Chemical Company, St. Louis, MO) packed column activated at 1250°F , for chromatography. A slurry was made of florisil and chloroform, and the column was packed to a height of 10 centimeters (cm). Care was taken so that it was of consistent density. The column was 20 cm in height and one-inch in diameter. According to the method of Rouser et al. (67), a series of three different solvents were prepared. All contain 5 percent by volume 2,2-dimethoxypropane (water scavenger).

- Solvent 1: Chloroform and water scavenger (Sigma Chemical Company, St. Louis, MO)
- Solvent 2: Chloroform:methanol 19:1 and water scavenger
- Solvent 3: Chloroform:methanol 70:30 and water scavenger

A preliminary test of the procedures for cerebroside sulfate isolation with a labeled sulfur injection provided a standard for evaluating this technique. A test of the elutions produced by solvents 1 and 2 for radioactivity was done as a check. The automatic fraction collector (Research Specialities Company, Richmond, CA) was utilized in collecting the elutions from solvent 3. The solvents were then evaporated. Samples from four test tubes in sequence were compiled and added to scintillation vials and prepared for counting.

3. DRUG CONJUGATE DETERMINATIONS

Following injection, this group of animals also underwent the test for analgesia. Upon awakening, they were immediately placed in metabolism cages, and timing of a 24-hour urine collection began. To preserve the sample of urine, 1 milliliter of 0.1 N HCl was added to each collection bottle. The collection period was for 24 hours, and the rats received food and water ad libitum during this time. Each urine sample was filtered and then diluted to 25 milliliters with distilled water. A preliminary experiment had demonstrated that dichloromethane extracted ^3H -etorphine from an aqueous solution. Each urine sample was then treated with 30 milliliters of dichloromethane and frozen at -20° until further testing could be done. The hydrolysis of etorphine followed a method modified from

Levy and Matsuzawa (44). Two milliliters of glucuronidase phosphate solution made from glucuronidase of type IX bacterial powder 3,400 units (Sigma Chemical Company, St. Louis, MO) showed no sulfatase activity. To a 5-milliliter aliquot of the 25-milliliter prepared samples, 0.07 M phosphate at a pH of 7.0 was added. Three drops of CHCl_3 were added, and the samples were then incubated for 16 hours at 37°. To evaluate the amount of etorphine that was freed by the glucuronidase, the sample was placed in a separatory funnel and washed with 30 milliliters of dichloromethane three different times, and the freed drug was extracted. A sample of this extract was prepared for counting by evaporation of the dichloromethane and addition of the scintillator.

Hydrolysis of urine with sulfatase type V from limpets (Sigma Chemical Company, St. Louis, MO) in 0.1 M acetate buffer at pH 5.0 with a final concentration of 3.75 units of sulfate per 5-milliliter sample of urine followed. Samples were incubated at 37° for 96 hours, and then the freed etorphine was extracted by use of dichloromethane and separatory funnel. A sample of this extract was also prepared for counting.

4. BINDING SITE DETERMINATION

Animals in this phase of the study received no injection. Following a modified method of Fischel and

Medzihradsky (53) the animal was sacrificed by decapitation; its brain was removed and placed in cold 0.05 M Tris buffer at pH 7.4. A Thomas homogenizer with a teflon pestle was used to make a 10 percent homogenate. Ice was used to keep the brain homogenate cold, and the centrifuge head was chilled. An L5-50 ultracentrifuge was used at a speed of $20,000 \times g$ for 15 minutes to obtain a pellet. The supernatant was decanted and discarded, and the pellet was resuspended in the original volume of buffer. A five-fold dilution followed, and 0.2 milliliter samples (corresponding to 200 micrograms of protein) were pipetted into each of six tubes. The tubes also contained 0.15 milliliters of various concentrations of etorphine in 0.23 M sodium chloride with consistent levels of radiation. The range and concentration of the drug were from 1×10^{-3} M to 1×10^{-5} M. Approximately 0.05 μ Ci of ^3H -etorphine was present in each incubation tube. Incubation in a 45° water bath for 40 minutes followed. The unbound radioactive material was removed by centrifuging at $30,000 \times g$ for 10 minutes. The supernatant was removed and placed in a counting vial. Ten milliliters of the supernatant in 100 milliliters of distilled water were prepared for counting to serve as reference for total radioactivity. A cotton swab was used to be sure that all of the supernatant was removed from the tube. The tube and pellet were then washed with 1 milliliter 0.32 M sucrose. The pellet was stirred slightly with

a glass stirring rod and then centrifuged at $15,000 \times g$ for 10 minutes. This procedure was repeated once more. Each time the total amount of fluid was removed from the tube with cotton swabs. The pellet was then solubilized by the addition of 0.5 milliliters of soluene. This was incubated for 30 minutes in a 42° oven. The material was then washed with scintillation solution into vials and prepared for counting. This entire procedure was repeated with the antagonist of etorphine, naloxone (donated by J. W. Oliver, D.V.M, Ph.D, Department of Environmental Practice, The University of Tennessee, Knoxville). The protein content of the brain homogenates was determined by the method of Lowry et al. (68).

Statistics

One-way analysis of variance (ANOVA) was used to determine whether any significant differences were produced in serum levels of ^3H -etorphine and in the uptake of ^3H -etorphine by the brain. Differences in tail flick latencies were also tested by one-way ANOVA. An F_{max} test (69) was incorporated to determine whether the variances were homoscedastic. Transformation of the data to common logarithms was made to correct the inequality of variance among samples (heteroscedasticity).

The brains used to isolate cerebroside sulfate by chromatography were pooled within diet groups. This

pooling eliminated the use of statistical analysis of the data.

An arcsine transformation was made of the urine conjugation ratios. Analysis of difference between diet group means was evaluated by Duncan's new multiple range test for unequal sample size. Each difference was considered significant if it exceeded the corresponding least significant range ($P < 0.5$). Paired T analysis was utilized in comparing the effect of cysteine versus methionine supplementation on S:G ratios (70).

Linear regression was computed from the means of $\bar{X}$ (bound ligand per milligram of protein) and $\bar{Y}$ (bound/free) variables. The standard error of the estimate of the line was also determined. The data do not follow a linear function. Linear regression was used to determine the "apparent" number of binding sites rather than the constrictions of a model equation.

CHAPTER IV

RESULTS

Effect of Inorganic Sulfate and Methionine and Cysteine
Supplementation on Serum Levels of Etorphine

The data showing serum levels of ^3H -etorphine as a percent of dose administered subcutaneously are shown in Table 4. No statistically significant differences due either to level of inorganic sulfate or supplementation with methionine or cysteine were detected by ANOVA of these data. A wider variation of circulating levels of ^3H -etorphine resulted with intraperitoneal injection (Table 5).

Effect of Inorganic Sulfate and Methionine and Cysteine
Supplementation on Brain Uptake of Etorphine

Table 6 gives the CPM of ^3H -etorphine in brain homogenates expressed as a percent of brain weight for animals injected subcutaneously. Analysis of variance indicated no significant differences resulted from either dietary variable. Although not significant, the animals consuming the lowest level of inorganic sulfate and cysteine supplementation had the highest radioactivity levels. However, in comparing methionine and cysteine supplementation at the optimal level of inorganic sulfate (0.02 percent), methionine treated animals had the higher level. Intraperitoneal injection produced inconsistent levels of ^3H -etorphine to

TABLE 4

Effect of inorganic sulfate and methionine and
cysteine supplementation on ^3H -etorphine
serum levels as percent dose

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	CPM $\times 10^3$		
0	3.68 $\pm$ 0.11 ¹ (4) ²	4.26 $\pm$ 0.28 (4)	3.98 $\pm$ 0.22 (4)
Methionine (0.625%)	3.52 $\pm$ 0.17 (5)	3.16 $\pm$ 0.12 (5)	4.18 $\pm$ 0.25 (3)
Cysteine (0.505%)	4.36 $\pm$ 0.33 (4)	4.30 $\pm$ 0.54 (4)	4.18 $\pm$ 0.25 (4)

¹Values are means $\pm$ SEM.

²Numbers in parentheses indicate number of animals.

³Values in a line followed by different superscripts are significantly different ($\underline{P} < 0.05$).

⁴Values in column 0.02 followed by superscript symbol * indicate significant difference between methionine and cysteine supplementation ($\underline{P} < 0.05$).

TABLE 5

Results of intraperitoneal administration of
 ^3H -etorphine on serum levels as percent dose

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	CPM x 10^4		
0	$1.97 \pm 0.46^1 (5)^2$	$0.85 \pm 0.09 (5)$	$1.00 \pm 0.31 (4)$
Methionine (0.625%)	$0.74 \pm 0.08 (5)$	$0.40 \pm 0.14 (4)$	$1.24 \pm 0.61 (4)$
Cysteine (0.505%)	$1.47 \pm 0.41 (6)$	$1.35 \pm 0.42 (6)$	$0.77 \pm 0.09 (5)$

¹Values are means $\pm$ SEM.

²Numbers in parentheses indicate number of animals.

TABLE 6

Effect of inorganic sulfate and methionine and
cysteine supplementation on brain uptake of
etorphine as percent brain weight

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	CPM x 10 ³		
0	8.02±0.51 ¹ (4) ²	8.04±0.75 (4)	8.01±0.97 (5)
Methionine (0.625%)	7.60±0.51 (4)	9.06±1.01 (5)	8.94±1.38 (5)
Cysteine (0.505%)	10.39±1.47 (4)	7.39±0.71 (4)	9.08±0.68 (4)

¹Values are means ± SEM.

²Numbers in parentheses indicate number of animals.

³Values in a line followed by different superscripts are significantly different ($\underline{P} < 0.05$).

⁴Values in column 0.02 followed by superscript symbol * indicate significant difference between methionine and cysteine supplementation ($\underline{P} < 0.05$).

the brain (Table 7).

Effect of Inorganic Sulfate on Cerebroside Sulfate Levels

Table 8 shows the data for radiation associated with ^3H -etorphine. The highest levels of radiation were found in the cerebroside sulfates eluted from the brains of rats fed the diets with the lowest levels of inorganic sulfate. The effect appears to be greater in rats fed the diet with no organic sulfur supplementation than in those fed diets with organic supplementation. The results indicate the presence of inconsistent levels.

Effect of Methionine and Cysteine Supplementation on Cerebroside Sulfate Levels

In comparing the diets with optimum inorganic sulfate (0.02 percent) an assessment can be made of the effect of organic sulfur supplementation. From examination of the data in Table 8, it would appear that methionine supplementation greatly enhanced the levels of radiation associated with cerebroside sulfate fraction.

Analgesia Analysis of Variance

To determine if the level of inorganic sulfate or form of amino acid supplementation would alter the analgesic response of rats treated with an opiate, the tail flick latencies were determined. Since the F_{max} test demonstrated inequality of variance among groups, the data were transformed to the common logarithm. However, the data

TABLE 7

Results of intraperitoneal administration of
³H-etorphine on brain uptake
 as percent brain weight

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	CPM x 10 ³		
0	4.22±0.86 ¹ (3) ²	5.67±1.10 (5)	5.43±1.89 (4)
Methionine (0.625%)	6.11±1.15 (4)	4.05±0.95 (3)	4.18±1.04 (4)
Cysteine (0.505%)	5.39±1.14 (5)	6.16±1.49 (5)	4.37±0.56 (4)

¹Values are means ± SEM.

²Numbers in parentheses indicate number of animals.

TABLE 8

Effect of inorganic sulfate and methionine and
cysteine supplementation on radiation levels
associated with cerebroside sulfate

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	CPM per gram brain weight		
0	314.45 ¹ (4) ²	120.51 (4)	126.45 (5)
Methionine (0.625%)	168.44 (4)	221.01 (5)	178.89 (5)
Cysteine (0.505%)	217.72 (4)	154.44 (4)	124.15 (5)

¹Values are pooled within diets.

²Numbers in parentheses indicate number of animals.

shown in Table 9 indicate neither dietary inorganic sulfate level nor supplementation with cysteine or methionine produced alteration in the analgesia elicited by etorphine.

Effect of Inorganic Sulfate Levels on Conjugation of ^3H -etorphine

The arcsine of the ratio of urine conjugates of ^3H -etorphine (sulfated:glucuronidated) for each diet are presented in Table 10. Comparison of inorganic sulfate levels by Duncan's new multiple range test within a type of supplementation showed cysteine-supplemented animals had a significantly higher ratio of sulfate conjugation with dietary inorganic sulfate at 0.02 percent than at either the higher or lower sulfate level. A pattern of increased S:G conjugation was produced by the high levels of inorganic sulfur when compared with rats fed diets with optimal inorganic sulfate.

Effect of Methionine and Cysteine Supplementation on Conjugation of ^3H -etorphine

Cysteine supplementation significantly enhanced the conjugation of etorphine with sulfate in comparison to the effect of methionine supplementation in diets with optimal inorganic sulfate. The decrease in inorganic sulfate to 0.0002 percent had little effect on the conjugation ratio with methionine-supplemented animals, but as shown in Table 10 the ratio was decreased 50 percent in those animals fed cysteine-supplemented diets. This reduced

TABLE 9

Analgesia in rats injected with etorphine when
fed varying levels of inorganic sulfate
and methionine and cysteine

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	20 minute periods of analgesia		
0	2.75 $\pm$ 1.49 ¹ (8) ²	3.25 $\pm$ 1.39 (8)	3.80 $\pm$ 1.03 (10)
Methionine (0.625%)	3.60 $\pm$ 1.21 (11)	4.00 $\pm$ 1.05 (11)	3.20 $\pm$ 0.92 (10)
Cysteine (0.505%)	3.50 $\pm$ 1.42 (9)	3.30 $\pm$ 0.87 (9)	3.80 $\pm$ 1.23 (10)

¹Reported values are pooled data from two groups.

²Values are means $\pm$ SD.

³Numbers in parentheses indicate number of animals.

⁴Values in a line followed by the different superscripts are significantly different ($P < 0.05$).

⁵Values in column 0.02 followed by superscript symbol * indicate significant difference between methionine and cysteine supplementation ($P < 0.05$).

TABLE 10

Effect of inorganic sulfate level and methionine
and cysteine supplementation on urine
conjugates of etorphine

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	Arcsine of S:G		
0	62.74 $\pm$ 12.29 ¹ (5) ²	55.44 $\pm$ 2.54 (5)	64.65 $\pm$ 24.71(5)
Methionine (0.625%)	50.99 $\pm$ 2.28 (5)	50.04 $\pm$ 6.64* ³ (5)	59.16 $\pm$ 7.77(5)
Cysteine (0.505%)	65.37 $\pm$ 22.02 ^a ⁴ (5)	128.11 $\pm$ 24.89 ^b (4)	50.16 $\pm$ 4.54 ^a (5)

¹Values are means $\pm$ SEM.

²Numbers in parentheses indicate number of animals.

³Values in column 0.02 followed by superscript symbol * indicate significant difference between methionine and cysteine supplementation ($\underline{P}$ < 0.05).

⁴Values in a line followed by the different superscript are significantly different ($\underline{P}$ < 0.05).

level compared closely to the ratio of sulfate conjugation obtained without organic supplementation at high and low inorganic sulfate levels. The G:S ratios from which the transformations were made are in Table 11.

Effect of Inorganic Sulfate on Binding Sites for Etorphine

Incubation of brain homogenate samples with varying molar concentrations of ^3H -etorphine and ^3H -naloxone produced the values necessary for Scatchard analysis. The amount of ^3H -etorphine bound per milligram protein (as determined by CPM of the pellet) was converted to CPM per milligram per mole and plotted as $\underline{Y}$ variable. The unbound ^3H -etorphine was displaced from the pellet by centrifugation. The CPM of a sample of this supernatant was divided into the CPM for $\underline{X}$ variable. The mean values for three animals were used to calculate the slope and intercept of the straight line of regression that determined the number of binding sites for each of the nine diets.

Figure 12 is a Scatchard determination of etorphine binding sites in animals fed a low (0.0002 percent) inorganic sulfate diet. Figure 13 depicts the Scatchard analysis for animals fed the optimum level of inorganic sulfate (0.02 percent). The number of sites was quite elevated in the brains of animals fed the diet with optimal inorganic sulfate.

Table 12 lists the femto (f) mole per milligram protein for each of the diets used in this study. Rats fed

TABLE 11

Conjugated drug in the urine expressed as ratio
of sulfated ^3H -etorphine:glucuronidated
 ^3H -etorphine

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	S:G		
0	0.77 $\pm$ 0.09 ¹ (5) ²	0.67 $\pm$ 0.02 (5)	0.83 $\pm$ 0.11 (5)
Methionine (0.625%)	0.60 $\pm$ 0.02 (5)	0.58 $\pm$ 0.05* ³ (5)	0.73 $\pm$ 0.05 (5)
Cysteine (0.505%)	0.82 $\pm$ 0.12 ^a ⁴ (5)	1.23 $\pm$ 0.23 ^b (4)	0.58 $\pm$ 0.04 ^a (5)

¹Values are means $\pm$ SEM.

²Numbers in parentheses indicate number of animals.

³Values in column 0.02 followed by superscript symbol * indicate significant difference between methionine and cysteine supplementation ($\underline{P} < 0.05$).

⁴Values in a line followed by the different superscripts are significantly different ($\underline{P} < 0.05$).

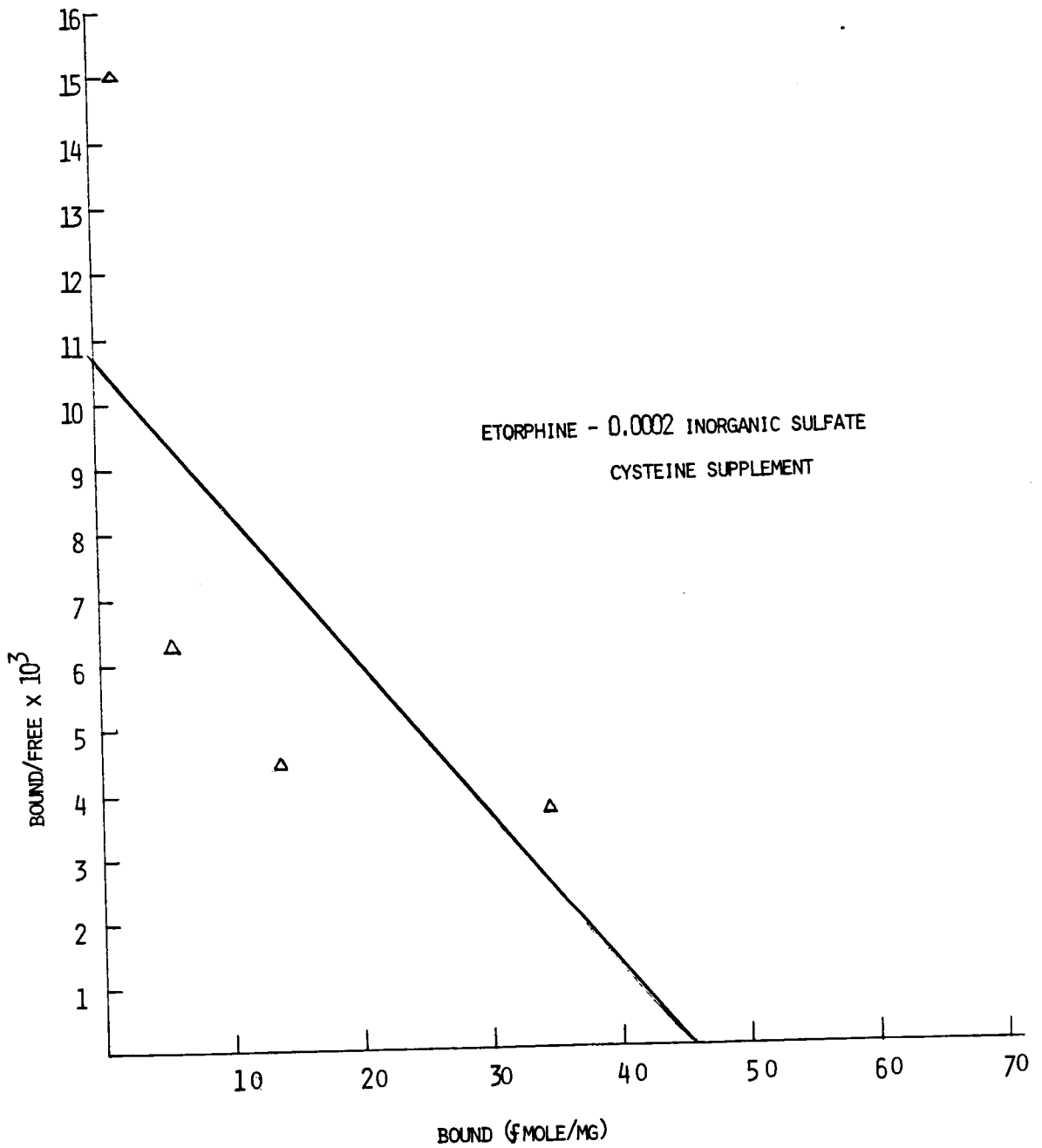


Figure 12. Scatchard Analysis for Etorphine in Animals fed 0.0002% Inorganic Sulfate and Cysteine Supplement.

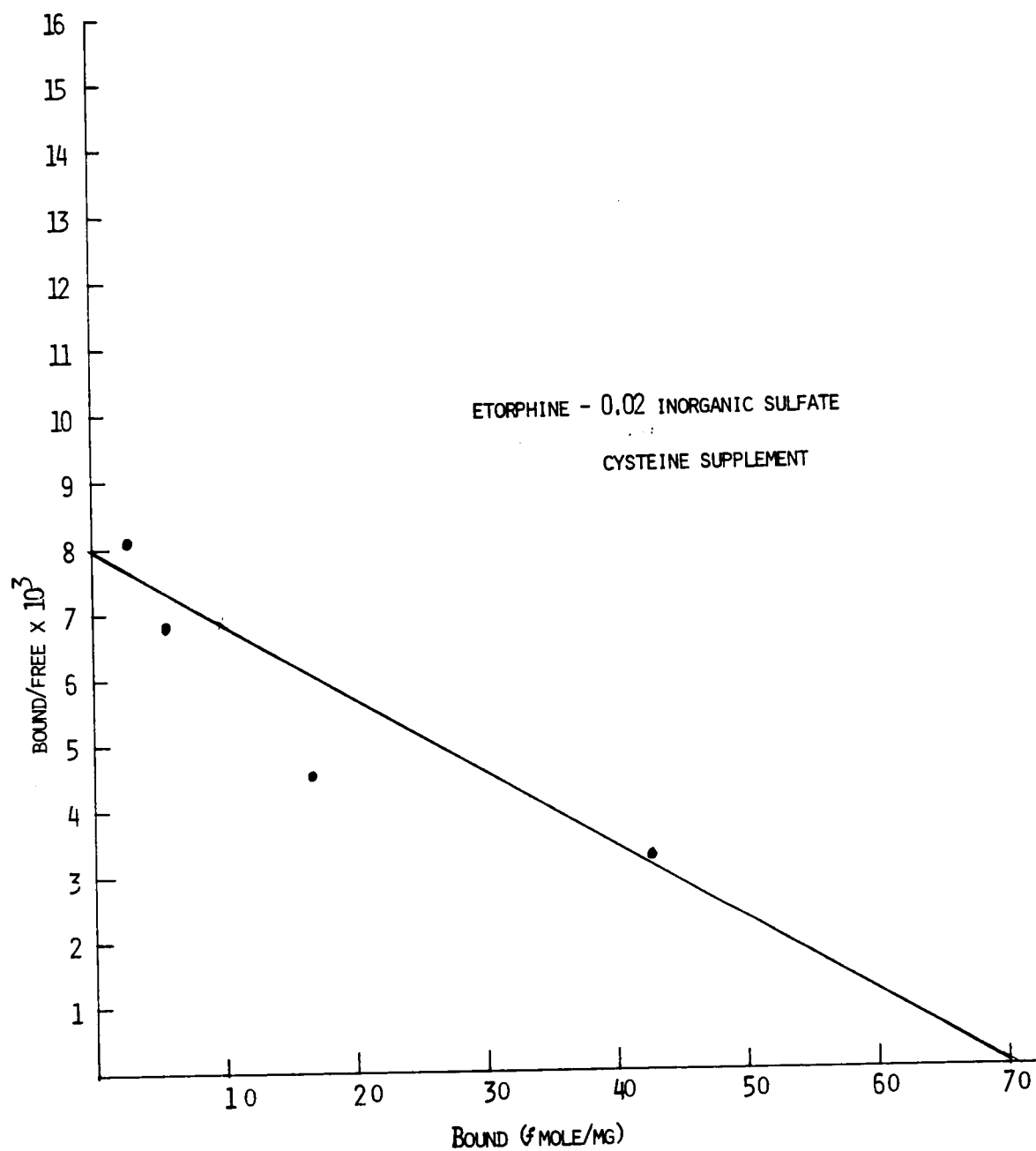


Figure 13. Scatchard Analysis for Etorphine in Animals fed 0.02% Inorganic Sulfate and Cysteine Supplement.

TABLE 12

Effect of inorganic sulfate and methionine and
cysteine on binding sites for etorphine

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	Bound ^3H -etorphine (f mole/mg protein)		
0	44	34	44
Methionine (0.625%)	59	109	59
Cysteine (0.505%)	44	71	42

¹Values are results of a Scatchard analysis based on the means of three animals.

methionine-supplemented diets at the 0.02 level of inorganic sulfate demonstrated nearly a two-fold increase in binding sites when compared to the number of binding sites for rats fed the other levels of inorganic sulfur. In cysteine-supplemented diets, the optimal level of inorganic sulfate produced 30 percent increase in binding sites over the lower level (0.0002 percent). From examination of these data, it appeared that the level of inorganic sulfate had an effect on the apparent number of opiate binding sites available as receptors for the drug etorphine, but that an increase to 0.42 percent inorganic sulfate did not further enhance binding site number.

Effect of Methionine and Cysteine Supplementation on Binding Sites for Etorphine

Table 12 also indicates that methionine supplementation at the optimal level of inorganic sulfate supplementation produced almost a 35 percent higher number of binding sites for etorphine than did supplementation with cysteine. On the basis of this comparison, it appeared that the form of dietary organic sulfur supplementation affects the apparent number of binding sites for the opiate etorphine.

Effect of Inorganic Sulfate on Binding Sites for Naloxone

The Scatchard determination of the number of binding sites for the antagonist naloxone in rats fed methionine

supplementation at the optimal level of inorganic sulfate is shown in Figure 14. This figure can be compared to Figure 15, which illustrates the number of binding sites determined for the animals consuming diets with the same level of inorganic sulfate but no organic sulfur supplementation. The f moles of bound naloxone per milligram protein are given for rats fed the various diets in Table 13. Examination of the table reveals the higher level of inorganic sulfur supplementation elevated the apparent number of binding sites. The increase in binding sites of animals fed diets with no organic supplementation was nearly two-fold. From these findings it would appear that the level of inorganic sulfate has an effect on the number of binding sites for the opiate antagonist naloxone.

Effect of Methionine and Cysteine Supplementation on Binding Sites for Naloxone

Comparison of the values in Table 13 for methionine supplementation and cysteine supplementation at the optimal level of inorganic sulfate indicate that this dietary manipulation did not alter the number of binding sites for the antagonist naloxone.

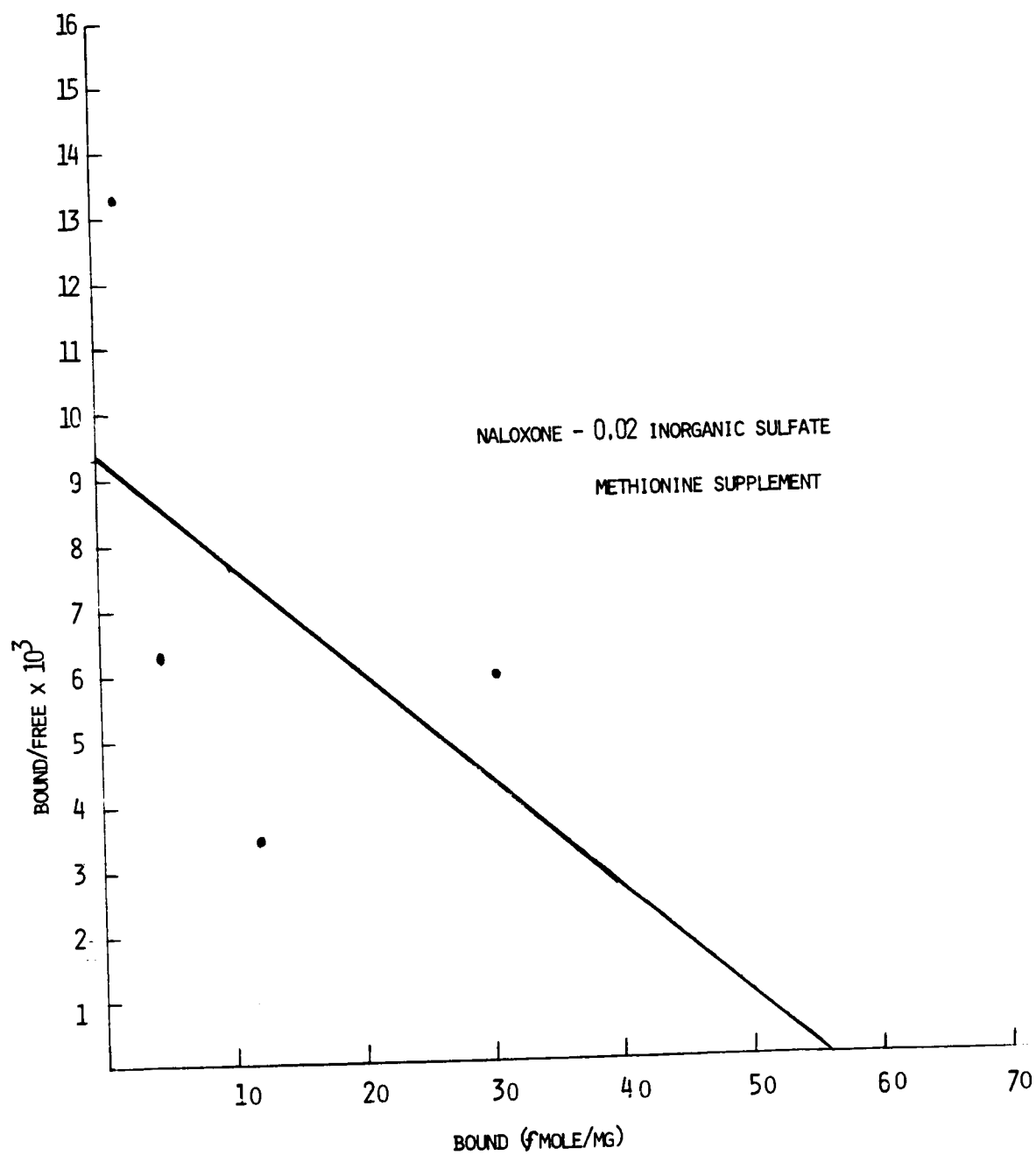


Figure 14. Scatchard Analysis for Naloxone in Animals fed 0.02% Inorganic Sulfate and Methionine Supplement.

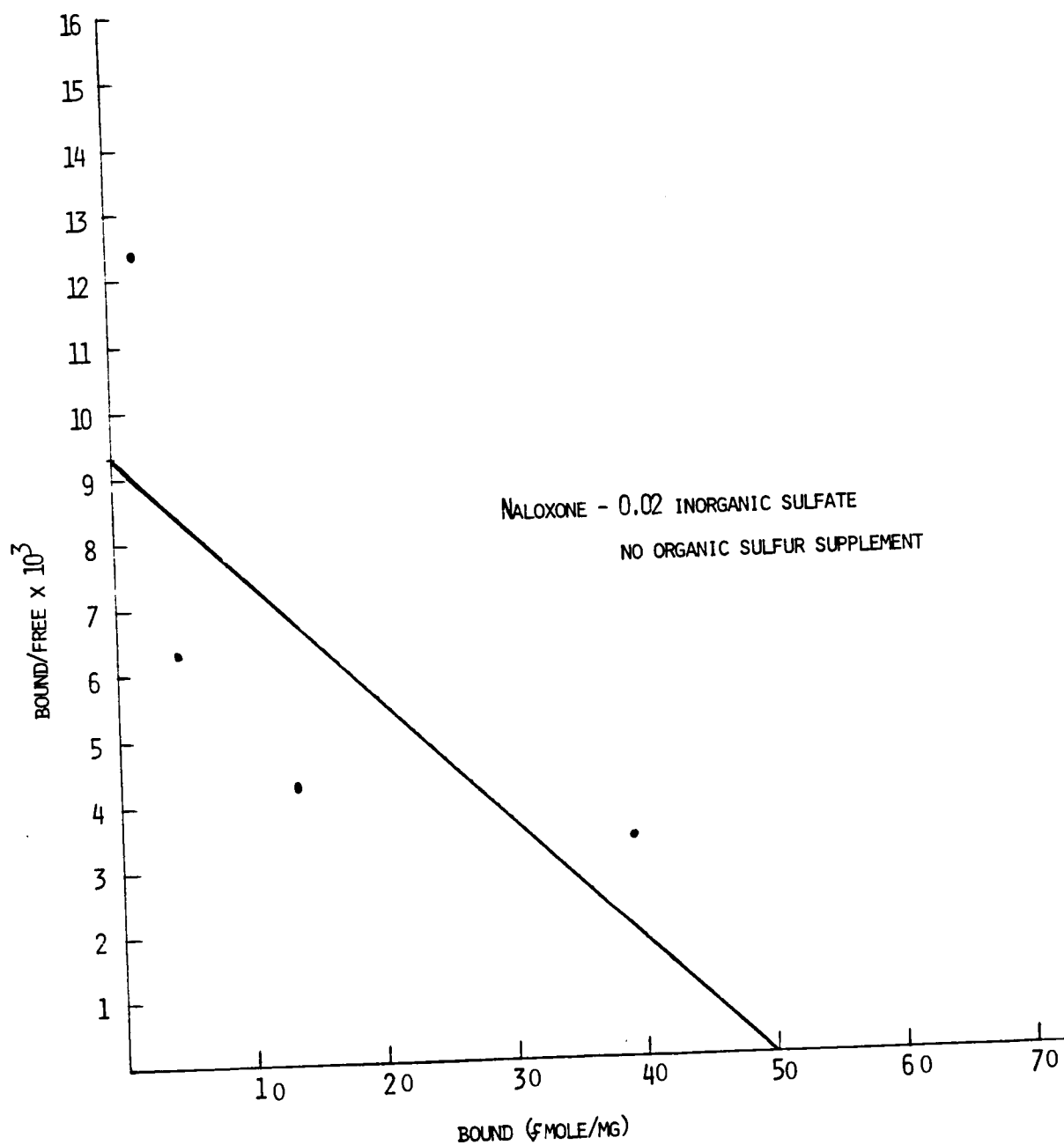


Figure 15. Scatchard Analysis for Naloxone in Animals fed 0.02% Inorganic Sulfate and no Organic Sulfur Supplement.

TABLE 13

Effect of inorganic sulfate and methionine and
cysteine on binding sites for naloxone

Supplement	Dietary inorganic sulfate %		
	0.0002	0.02	0.42
	Bound ^3H -naloxone (f mole/mg protein)		
0	74	49	102
Methionine (0.625%)	50	57	61
Cysteine (0.505%)	57	59	75

¹Values are results of a Scatchard analysis based on the means of three animals.

CHAPTER V

DISCUSSION

A role for cerebroside sulfate as a receptor for opiates was proposed by Loh (12). In support of his proposal, extensive research (11, 13, 14) has been conducted with several forms of inhibitors of sulfatides. Such inhibition resulted in modification of opiate binding. In view of these considerations, this study was designed to investigate the effect of dietary sulfur, inorganic and organic, on the number of receptors for opiates. If dietary alterations in the level of cerebroside sulfate could be linked to alterations in the effects such as analgesic response elicited by opiates, the theory of cerebroside sulfate as the cellular recognition site for opiates could be further substantiated. Quantification of binding sites in brain samples from animals subjected to various dietary sulfur treatments would not only provide evidence for a role for cerebroside sulfate as a binding site, but would also supply information for evaluating the effects of sulfur status on opiate administration.

The importance of inorganic sulfate and sulfur amino acids to drug metabolism and toxicity has been studied by other researchers in this laboratory (26, 33, 49). The analysis of the serum, brain homogenate, and urine levels

of the labeled drug etorphine in the animals after injection provided insight into distribution and metabolism of opiates. Comparison will be made not only between treatment effects within this study, but also with the results reported for other drugs.

Serum levels of ^3H -etorphine were not affected by the various dietary treatments. Circulating levels of the drug are determined by the route of administration. To ensure a more constant level of drug available for binding to the brain receptors, the method of injection was changed from intraperitoneal to subcutaneous, eliminating any differences due to first-pass metabolism. With subcutaneous administration, alterations produced by hepatic metabolism resulting from dietary sulfur availability did not influence circulating levels of the drug. The results from subcutaneous and intraperitoneal administration are presented in Tables 4 and 5 respectively (pages 51, 52). The levels of radiation were lower by intraperitoneal administration than subcutaneous; they were also more widely varied among dietary groups.

Etorphine was selected as the test opiate since Boura and Fitzgerald (36) reported it had a high potency due to its lipophilic nature. Dobbs (34) noted a 10-fold increase in entry into the brain when compared to morphine for this opiate.

The data on brain uptake as a percent of brain weight, therefore, provided evidence that a relatively equal amount of drug was present for binding to available receptors when the change was made from intraperitoneal to subcutaneous injection.

No significant differences in the uptake of etorphine by the brain were observed between rats fed the various diet groups. The radioactivity of a brain homogenate sample was a reflection of the nonsaturable interactions of the drug with subcellular fractions and not the amount of etorphine bound stereospecifically to the receptor sites (6).

Data have been presented that show enhanced binding of labeled opiate with cerebroside sulfate eluted from the brains of animals fed suboptimal dietary inorganic sulfate but not from those with organic sulfur supplementation. It would appear that these findings conflict with results submitted for publication by another researcher in this laboratory. Acuff found decreased sulfur in brain lipids from rats that had methionine-supplemented diets. In an attempt to explain these contradictions, it is postulated that the observed increase in radiation eluted is due to association with cerebroside rather than sulfatides specifically bound to a receptor.

The possibility that a lack of sulfate in cerebroside induces a conformational change that alters the

configuration of the receptor to one of nonsaturability for binding capacity is ruled out by data collected on the binding site number.

It was assumed that measurement of analgesic response would provide data to substantiate alterations in binding sites for opiates by dietary manipulation of sulfur. The increased ED₅₀ of morphine for the Jimpy strain of mice has been determined to be 11-fold higher than that for normals. This increased requirement is compatible with decreased binding site availability due to decreased formation of cerebroside (12). It has been reported by Acuff that an increase in the level of dietary inorganic sulfate from 0.0002 percent to 0.42 percent with either methionine or cysteine supplementation increased the sleep time of rats injected with pentobarbital. Because nonsignificant differences in tail flick latencies were found in the present study, it appeared there were no effects due to dietary sulfur manipulation. However, data have been presented (65) that showed the opiate etorphine was able to elicit analgesia with only 2 percent occupancy of binding sites. The high receptor-effector coupling efficiency of etorphine explains the consistency in the observed analgesic responses for all dietary groups. A high affinity receptor that elicited analgesia was reported by Pasternak to be distinct from the receptor for eliciting respiratory changes (61). These findings indicated that etorphine was

not a suitable opiate for this determination, and that injections of morphine would have been more effective in unmasking any dietary alterations in analgesic response.

Evidence that the urinary excretion pattern of etorphine is sensitive to the sulfur status of the animal was provided by this study. Previous work in this laboratory has shown that urinary excretion of the glucuronidated conjugates of the drug acetaminophen increased as the level of inorganic sulfate decreased (49). This finding was also reported by Kurzynske (48) for the drug salicylamide. These reported findings parallel the pattern of increased sulfate conjugations of etorphine for the rats fed diets with no organic sulfur supplementation and methionine supplementation.

In contrast, a decrease in the sulfated:glucuronidated ratio occurred when the inorganic sulfate level was increased from 0.02 percent to 0.42 percent with cysteine. This observed decrease in ratio is compatible with the activation of the glucuronidation pathway with cysteine that is reported by Kurzynske (48).

A significantly higher S:G ratio was noted in rats fed cysteine as compared to methionine-supplemented animals. This observed effect of organic sulfur supplementation supports data reported by other researchers that demonstrated dietary cysteine and methionine do not exert equivalent metabolic effects (27). Cysteine sulfur is more readily

available for activation by PAPS and consequent conjugation of drugs than methionine. Evidence to show that the urinary excretion pattern of etorphine is sensitive to the sulfur status of the animal was presented by this study. In contrast to acetaminophen, etorphine appears to be sensitive to both the presence and type of organic sulfur in the diet and to the level of inorganic sulfate. An investigation by Morris (49) of the hepatic activity of enzymes sulfotransferase and UDP-glucuronyl transferase determined that neither was affected by the level of dietary inorganic sulfate nor the form of organic sulfur supplementation. Therefore, the exact mechanism to which the differences should be attributed remains to be defined.

The data presented for the last phase of this study make a convincing argument for the role of cerebroside sulfate as a binding site or receptor for opiates, but more specifically for etorphine. The high number of binding sites (as determined by the Scatchard analysis) for those animals fed diets optimal in inorganic sulfate is in agreement with increased ^3H -etorphine associated with cerebroside sulfate.

The data presented for the agonist etorphine reflect increased binding sites in animals fed those diets with optimal cysteine. Since increasing the level of inorganic sulfate in diets of rats did not increase the binding site number, it would appear that inorganic sulfate has no

effect on etorphine receptors. The same conclusion could not be drawn from examination of the data for the antagonist naloxone. In this case, the highest number of binding sites were determined to be in the animals fed diets containing the highest level of inorganic sulfate.

These differing effects for agonist and antagonist can be viewed as supporting evidence of two types of receptors. Loh (13) speculated that more than one type of receptor existed for the antagonist naloxone when sulfatide hydrolysis resulted in only partial inhibition of binding. The Scatchard analysis determined a reduction in binding sites had occurred.

For the agonist etorphine, Loh (62) reported a single high affinity site to which binding was attributable. In contrast, the antagonist naloxone was shown to bind to multiple sites. Fischel et al. (53) also demonstrated two binding sites for the antagonist naltrexone.

These findings suggest there is a requirement for sulfate in both types of receptors. Since the increase in inorganic sulfate beyond the optimal level did not result in increased etorphine binding sites, this observation could indicate that the single high affinity receptor sites were saturated. Because no increased binding sites for naloxone were associated with the brains of animals fed the highest level of inorganic sulfate, the statement can be made that the sites for the antagonist did not appear to be

saturated at optimal inorganic sulfate level. Since naloxone has two types of receptors with which to interact, it is reasonable to assume their total available sites would outnumber the single high affinity sites for etorphine.

CHAPTER VI

SUMMARY

In view of a proposed role for cerebroside sulfate as receptor for opiates, the importance of dietary sulfur to opiate analgesia, conjugation and binding was investigated. It was found that when rats were fed dietary inorganic sulfate at levels greater than optimal and injected subcutaneously with etorphine, there was an increased conjugation of etorphine with sulfate. Cysteine supplementation further enhanced the conjugation of the drug with sulfate, indicating the sulfation pathway was not saturated by the administered dose.

Alterations in the apparent number of opiate binding sites in rat brain were produced for both etorphine and naloxone. Scatchard analysis determined that the greatest number of receptors for etorphine was associated with feeding methionine-supplemented diets. The highest number of apparent binding sites for naloxone were evident in the brains of rats fed the highest level of inorganic sulfate. These distinctions further substantiated previous reports of a single high affinity site for etorphine and multiple receptors for naloxone.

This study has demonstrated the significance of adequate dietary sulfur for drug elimination and binding. The physiological adjustment to chronic use of opiates, known as

development of tolerance, could possibly be explained as a compensatory protective mechanism by which sulfate availability for receptor formation is decreased with increased drug use.

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