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Kinetics of drug-drug interactions

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KINETICS OF DRUG-DRUG INTERACTIONS: TOLBUTAMIDE AND SULFADIMETHOXINE

by

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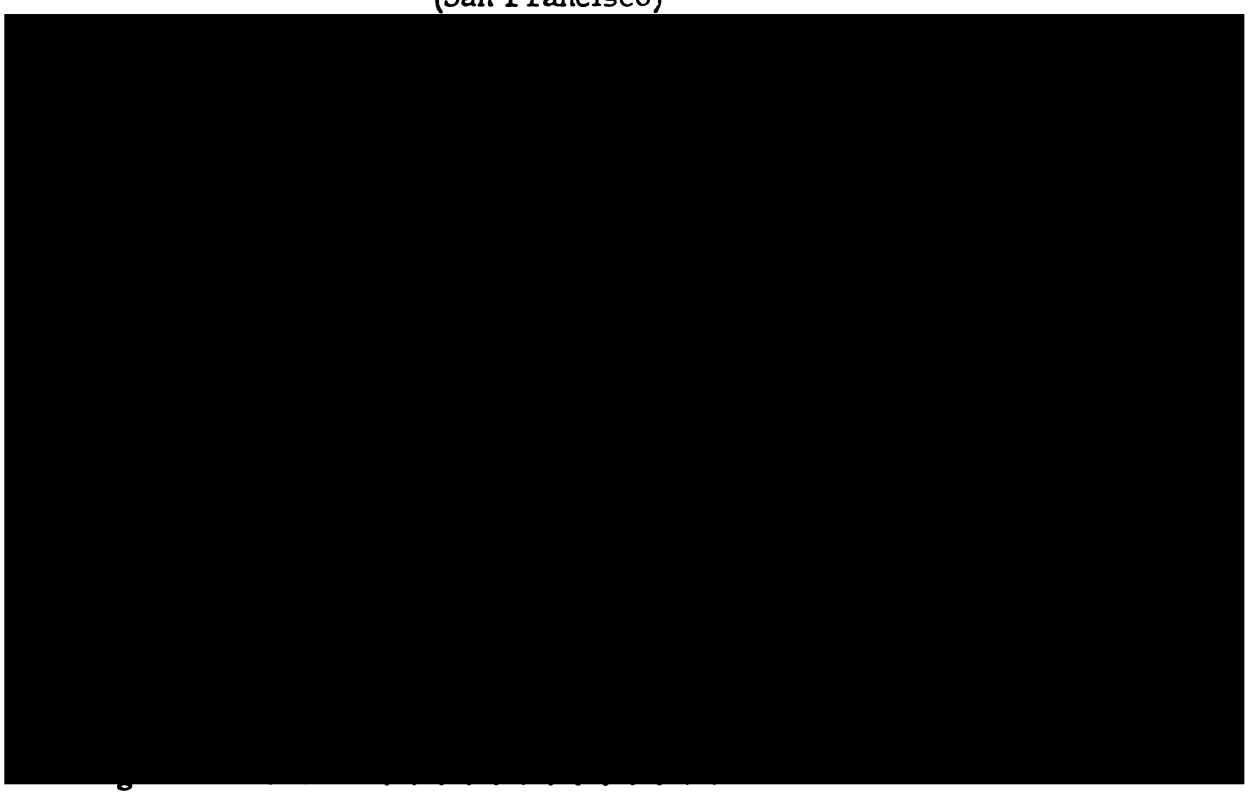

Trust in the Lord with all thine heart;

and lean not unto thine own understanding.

In all thy ways acknowledge Him,

and He shall direct thy paths.

Proverbs 3: 5-6.

Dedicated

to

my

wife

Marlene

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Finally, I wish to express my deep gratitude to my wife for her continual encouragement, thoughtfulness and assistance while completing this project.

ABSTRACT

About 75% of an I.V. administered dose of tolbutamide in the sheep could be accounted for in terms of urinary excretion of parent compound (13%) and metabolites (62%). Of the accountable metabolites, 90% represented hydroxytolbutamide and the balance carboxytolbutamide. The plasma disposition kinetics of an I.V. bolus of tolbutamide in the sheep could be described by a one compartment model, or a two compartment model with a rapid ($t_{\frac{1}{2}}$ of 0.5 to 1 hr) initial decay phase. The normal terminal log linear half-life in plasma ranged from 3 to 5.5 hr. The apparent volume of distribution had a range of 18.5 to 24.5% considering an average body weight of 50 Kg, while the total body clearance ranged from 12.7 to 39.1 ml/min based upon total plasma concentration. A 0.9 Gm I.V. bolus of tolbutamide caused a pronounced hypoglycemia followed by recovery to baseline values in 2 hr.

A two compartment model characterized the plasma concentration-time curve of hydroxytolbutamide after its I.V. administration in the sheep. In 2 experiments its terminal log linear half-lives were 13.5 and 26 min. While most of the dose (91.8 to 99.9%) was recovered in the urine, 90% of the recovered product represented the parent compound. The plasma clearance determined during an I.V. infusion (738 ml/min) appeared to approximate renal plasma flow. A 250 mg I.V. bolus of hydroxytolbutamide had no apparent hypoglycemic activity, but rather a decided diuretic activity described by a positive free water production.

A 4 Gm I.V. bolus of sulfadimethoxine in the sheep resulted in a plasma concentration-time curve appearing to be approximately bi-exponential. Kinetic analysis suggested a distribution (α) half-life of 1 hr, terminal half-life of 7.4 hr and a plasma clearance based upon total concentration of 31.8 ml/min. This dose had an insignificant hypoglycemic effect. Log unbound plasma concentration (C_f)- and log total plasma concentration (C_t)- time curves after most I.V. doses of sulfadimethoxine in the sheep exhibited continual curvature. Evaluation of Dose vs $\int_0^\infty C_f dt$ or $\int_0^\infty C_t dt$ suggested that the unbound plasma concentration of sulfadimethoxine dictated the total body clearance of sulfadimethoxine (235 ml/min).

Tolbutamide (100 mcg/ml) in sheep plasma was bound 85%. The principle binding site for tolbutamide had an apparent association constant of $1.1 \times 10^4 M^{-1}$ with this binding site concentration ($7.6 \times 10^{-4} M$) approximating twice the albumin concentration. Addition of sulfadimethoxine (100 and 200 mcg/ml) to sheep plasma containing tolbutamide (50 mcg/ml) caused a 50 and 100% increase respectively in the unbound tolbutamide concentration in vitro.

Sulfadimethoxine zero-order infusion (2 Gm/hr x 3 hr) in the sheep, in the presence of a steady-state tolbutamide plasma concentration (61 mcg/ml) caused a prolonged increase (100 to 220%) in the unbound tolbutamide concentration. The extent of the changes suggested that tolbutamide was protein bound extravascularly and that its distribution might be limited to the extracellular fluid. The renal clearance of neither tolbutamide nor hydroxytolbutamide were inhibited by sulfadimethoxine. Steady-state administration of tolbutamide (95 mg/hr)

followed subsequently by steady-state sulfadimethoxine (285 or 400 mg/hr) indicated that the unbound tolbutamide plasma concentration dictated its renal clearance (16.5 ml/min). This co-administration of agents in the sheep also revealed: 1) the total body clearance of tolbutamide might be dictated by the unbound tolbutamide plasma concentration (215 ml/min); 2) this total body clearance based upon unbound tolbutamide plasma concentration changed to 86 and 70 ml/min at steady-state plasma concentrations of sulfadimethoxine of 120 and 168 mcg/ml respectively; 3) the interaction could adequately be described as metabolic inhibition and protein displacement of tolbutamide by sulfadimethoxine; 4) calculation of metabolic inhibition constants (K_I (mcg/ml)) allowed a reasonable description of the interaction of sulfadimethoxine and tolbutamide in the sheep at steady state.

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CHAPTER I

I. Introduction

Many drugs, both of natural origin or the result of man's synthetic achievements, have taken their place in the physician's armament against a host of disease states. Drugs have in fact become the first and often the only weapon to be employed in many diseases. The co-administration of various drugs has been a common practise, especially when a patient is observed to bear concomitant illnesses. This simultaneous administration of drugs may occur even with a single pathological condition when one drug alone is known not to provide the necessary action. Self-medication by a patient provides yet another source of possible ingestion of more than one drug. Of course the term "drug" should be broadened beyond the regular physician-prescribed pharmaceutical entities to include materials such as alcohol, some foods, insecticides, possibly food additives and other chemical agents.

The mere presence of a drug in the human body may elicit a single or series of reactions of various types. Many chemical reactions are the result of a simple mutual presence of two chemicals and thus it should not be too surprising that reactions occur in the body which cannot be accounted for by the presence of each single entity when two drugs enter the body concomitantly. Such unaccountable drug interactions are known to exist in various forms. Stockley[1] has classified drug interactions as occurring within particular phases of drug activity, namely (1) the pharmaceutical phase (involving the release of the drug from the pharmaceutical preparation and its possible chemical incompatability with other ingredients), (2) the pharmacokinetic phase (where interactions are mediated by changes in drug absorption, binding, metabolism and excretion), and (3) the pharmacodynamic

phase (interactions principally mediated at specific sites of drug action). It should be noted however that any classification is rather arbitrary since a given drug interaction may occur in a combination or even all of the 3 phases of activity cited.

Interest in drug interactions has blossomed in recent years as evidenced by the numerous publications related to this area. There is little doubt that investigators have only scratched the surface with regard to adverse drug interactions which either cause deleterious effects or lead to ineffective therapy. The most information is known about those interactions in which the effects of the interaction are readily measurable (e.g., oral anticoagulants - prothrombin time; antidiabetic drugs - blood glucose). Lasagna[2] has commented:

> "It is apparent not only that a problem exists but also that, despite the fairly high frequency of reported trouble from drugs, the publicized cases constitute merely the floating tip of an iceberg, with much of the difficulty remaining hidden beneath the surface of our awareness."

As the awareness of possible drug interactions increases, it is felt that more cases will be reported.

Many articles have been written concerning drug interactions, which are useful, but little more than topographical maps with little weighting as to the quantitative significance of any cited interaction. Table I gives a brief list of some of the contributions in the field.

Table I: Some drug interaction publications

A. Reviews

Title	Author(s)	Year
Mechanisms of drug interactions 1. Basic principles	Stockley, I.H.[3]	1971
Drug interactions. III. Classes of drugs Interaction of cardiac drugs	Hartshorn, E.A.[4]	1970
Alcohol-drug interactions	Parker, W.J.[5]	1970
Hypoglycemic agents. Their interactions	Hussar, D.A.[6]	1970
Drug interaction	King, T.M. and Burgard, J.K.[7]	1967
Phenobarbital. Interactions with other drugs.	Cucinell, S.A.[8]	1972
Drug interactions. Principles and problems in relation to drugs of abuse.	Mannering, G.J.[9]	1971
Drug interactions in clinical medicine	Rosenoer, V.M. and Gill, G.M.[10]	1972
Interactions of drugs and foreign compounds	Brown, S.S.[11]	1972

B. Books

Hazards of medication	Martin, E.W.[12]	1971
Drug interactions	Hansten, P.D.[13]	1973

The many interactions which occur may easily be classified, according to Hansten,[13] in the following way: (1) ADME Interactions, (2) Pharmacologic Interactions, and (3) Miscellaneous Interactions. Although this division is again somewhat arbitrary due to a possibility of combinations, yet it will be followed in order to give a brief overview, illustrating some of the various mechanisms which have been reported in the literature.

A. "ADME" Interactions:

Drugs may affect the absorption, distribution, metabolism, or excretion ("ADME") of other drugs. This category then includes those interactions in which gastrointestinal absorption of a drug is affected, plasma protein binding is affected, drug metabolism is altered, or urinary excretion is enhanced or inhibited.

1. Absorption:

Many drugs are administered orally for absorption along the gastrointestinal tract. A wide range of conditions may reduce or increase the rate and extent of drug availability. In a classical example, there is experimental evidence that tetracyclines complex with di- and trivalent ions which reduces their absorption through membranes. Antacids containing Ca^{++}, Mg^{++} or Al^{+++} may therefore possibly lower the absorption of the tetracyclines by this mechanism.[14] Some of the saline purgatives like magnesium and sodium sulfate cause both dilution of the gut contents as well as "intestinal hurry" which can reduce the absorption of some materials.[15] Theoretically one might expect changes in the pH of the gut to alter the ionization of many acidic and basic drugs to such an extent that gross changes would be seen in drug absorption, but there is little evidence that such changes are of real clinical significane. There has

also been a suggestion that anticholinergics and ganglionic blockers, which reduce the peristaltic movement of the gut, could affect the absorption of compounds.[3]

2. Distribution:

Some compounds which enter the body's general circulation are carried solely in solution in plasma water, but a large number become bound to a greater or lesser degree to the plasma proteins. Drugs may interact by competing with one another for the same binding sites, the drug with the higher affinity for the area displacing the other drug. This competition is governed by the law of mass action and therefore also depends upon the concentration of the competing species. The displaced drug, denoted "free" drug, may thus rise in the presence of another agent, leading to an exaggerated pharmacologic response or more rapid elimination. The assumption here is that the free drug is responsible for the therapeutic action as well as its eventual elimination. The protein-displacement concept has been forwarded to account for exaggerated responses in the co-administration of a considerable number of compounds which are highly bound in the blood. These compounds include the sulfonylureas, anticoagulants, some sulfonamides, some anti-inflammatory compounds and other agents. The enhanced anticoagulant response is marked and occurs in nearly all patients treated with phenylbutazone and warfarin.[16] Various agents have been shown to enhance the bacterial activity of sulfonamides, presumably due to protein-displacement.[17]

3. Metabolism:

Many drugs are chemically changed by the body into less lipid-soluble derivatives so as to aid in their excretion. This biotransformation is carried out predominantly by the liver. In response to the presence of

some drugs over a period of time, an increased rate of metabolism is observed. Enzyme induction has been demonstrated quite substantially with the barbiturates. Chronic administration of phenobarbital has resulted in an accelerated metabolism of a variety of drugs such as bishydroxycoumarin, diphenylhydantoin and antipyrine.[18] Although enzyme induction is important, drug interactions which result in enzyme inhibition are of greater concern since drug activity is increased due to the accumulation upon prolonged administration, which results from the decrease in metabolism. Of the enzyme inhibitory drugs, the monoamine oxidese inhibitors are probably the most notorious, causing a rise in the levels of several biologically active amines within many tissues. Concomitant administration of agents such as sympathomimetics (amphetamine, ephedrine, etc.) cause a number of greatly exaggerated responses such as high blood pressure.[1] The administration of disulfiram in therapeutic doses to adult males who had received treatment with phenytoin (diphenylhydantoin) over a period of years resulted in a 100-500% rise in the concentration of serum phenytoin in the course of 9 days, apparently due to a decrease in the rate of degradation of phenytoin.[19] The administration of dicumarol to diphenylhydantoin-treated volunteers caused an increase in the serum-diphenylhydantoin level and a very considerable increase in the half-life (9→36 hr) of diphenylhydantoin evidently due to a metabolic block.[20]

4. Excretion

Most drugs are excreted through the kidneys. Since most drugs are either weak acids or bases, they will be present in solution in the tubular filtrate in both ionized and unionized forms, only the latter being able to pass through the tubule because of its lipid solubility. The proportion of molecules in each form depends upon the drug and the pH of the filtrate.

It is quite apparent then that a change in tubular pH which increases the number of lipid insoluble molecules, may increase the loss of drug in the urine. However changing pH influences drug elimination significantly only when the fraction of drug renaly cleared becomes large under the conditions of interest. Alkalinization of the urine with sodium bicarbonate decreases, while acidification with ammonium chloride increases the extent of urinary excretion of unchanged amphetamine after its administration.[21] Elimination of potentially toxic drugs may be expedited by appropriate adjustment of urinary pH. This has already proved useful in phenobarbital and salicylate intoxications.[22] In addition to the above mentioned method of excretion, many drugs and their metabolites are actively secreted by the proximal tubular active transport mechanism and interactions may arise from competition for this system. The hypoglycemic action of acetohexamide is enhanced by the simultaneous administration of phenylbutazone.[23] This is apparently due to inhibition of the renal excretion of the active hydroxy metabolite. One might say that there have been some sweet uses in adversity involving drug combinations. The acidic drug probenecid which blocks the renal active transport and thus the excretion of a number of compounds has been used beneficially to prolong the sojourn of ampicillin in the body thus making the antibiotic more effective in the treatment of gonorrhoea.[24]

B. Pharmacologic Interations:

Drugs may have additive or synergistic pharmacologic effects resulting in untoward reactions. On the other hand antagonistic pharmacologic effects may exist causing a reduction in action. In either of the above cases, the action may be mediated at the same or different receptor sites. Most pharmacology texts will give an in depth treatment of various receptor

sites at which certain drugs may act. An example would be the drugs that act at synaptic and neuroeffector junctional sites such as all preganglionic autonomic, all postganglionic parasympathetic, and a few postganglionic sympathetic fibers at which acetylcholine is the neurohumoral transmitter.[25(a)] It would then be unadvisable to co-administer a parasympathomimetic and an antimuscarinic agent. The adrenergic fibers which comprise the majority of the postganglionic sympathetic fibers have norepinephrine as the neurohumoral transmitter. Beta-adrenergic stimulation by isoproterenol is specifically and competitively antagonized by appropriate doses of propranolol. The phenothiazines are extremely useful in psychiatric disorders when patients require tranquilization. Yet, because phenothiazines can block alpha-adrenergic receptors, the choice of drugs for the treatment of hypotension is limited. If the pressor used elicits both alpha- and beta- adrenergic responses, the net effect during alpha receptor blockade could be beta-mediated arterial dilation and, paradoxically, exaggerated hypotension.[26] Possible additive action, or potentiation in untoward effects is another area of pharmacologic interactions which requires caution during the co-administration of drugs. Ethacrynic acid should be used only with extreme caution in patients receiving aminoglycoside antibiotics such as kanamycin[27] since the diuretic can produce ototoxicity which may add to or potentiate the ototoxicity of kanamycin.

C. Miscellaneous Interactions:

This group is conveniently comprised of those interactions which cannot be assigned to the above classifications. It would be tedious to attempt an in depth overview in such a classification as this. Only a few examples will be given. In some patients, drug toxicity is not produced

by simple additive effects of drugs given simultaneously. Toxicity appears as a result of drug-induced changes in the patient. For example, thiazide diuretics, by producing potassium loss may predispose patients to digitalis toxicity.[27] Likewise, reserpine, which depletes myocardial stores of norepinephrine, may cause undue bradycardia when digitalis is given.[28] Antibiotic therapy which alters intestinal flora that normally synthesize vitamin K, reduces the prothrombinopenic does of warfarin.[27] It has been suggested that an interaction might exist between dextroamphetamine and the penicillins, for Mukherjee[29] found an increase in the bound fraction of penicillin G in patients pretreated with the amphetamine. The results indicated that increased serum protein-being of the antibiotic was due to a rise in plasma free fatty acid induced by dextroamphetamine. Free fatty acid in vitro caused an increase in the binding of penicillin G which the author suggested would decrease the antimicrobial action of the antibiotic. A pharmacokinetic drug interaction between propranolol and lidocaine has been found in which the administration of dl-propranolol prolonged the half-life of lidocaine.[30] The drug interaction occurs in which beta-adrenergic blockade due to the dl-propranolol decreases cardiac output and liver blood flow, thus reducing the rate of delivery of lidocaine, whose elimination is blood-flow limited, to its major site of elimination in the liver.

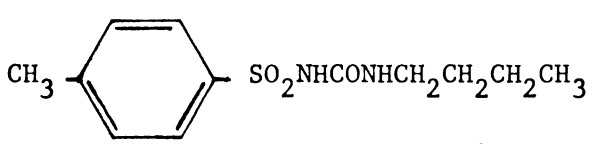
The previous sections have thus provided a brief overview of some of the mechanisms of drug interactions. The volume of publications related to the area attest to its importance. In a study of 1000 consecutive patients in two medical wards, Sotaniemi and Palva[34] pointed out that about 70% of the patients received one to four drugs regularly and 21% received five or more drugs. Not only does the co-administration of drugs present a potential for problems, but a number of reviews have been written recently listing drugs which interfere with clinical laboratory

tests.[31, 32, 33] The general drug co-administration practise along with the interference by drugs in laboratory tests has placed an extraordinary strain upon the health professions. Many major health centers are increasingly employing the computer to screen drug-drug interactions and drug modifications of laboratory tests. Mercy Hospital in Pittsburgh, Pa.[35] has such a computerized system. The data base for the system consists of approximately 7000 individual drug-laboratory test interferences and 10,000 drug-drug interactions reported in the literature. This system has been used successfully to avert many potential problems.

II A Drug: Tolbutamide

A quick perusal of the literature on drug-drug interations will reveal that the majority if not all of the reports are merely qualitative. As the number of these interactions reported grows, it becomes of paramount importance to have quantitative information concerning a drug interaction. Information of this type could provide a dosage schedule for interacting drugs which might reduce or eliminate the untoward reaction without substantially reducing the therapeutic effect of each agent. However, before quantitation of an interaction can be achieved, the exact nature of the interation must be determined.

Any of a number of drugs could be selected for further study. One drug which has received wide attention is the oral hypoglycemic agent, tolbutamide:



N-(4-methylbenzenesulfonyl)-N^1-n-butylurea

It is a drug widely used, generally on a chronic basis for many years, particularly by elderly diabetics who are prone to other pathological conditions which require additional medication. The co-administration of drugs which then ensues has lead to many reported complications as will be noted shortly. The next few sections will then deal with the diabetic condition and the involvement by tolbutamide in the therapy of it, the metabolism of tolbutamide, and finally drug interactions which have been reported with tolbutamide.

A. Diabetes and Tolbutamide:

Diabetes mellitus has been recognized as a medical problem for more than 2000 years and many interesting facets encompass its history.[35] The discovery of insulin by Banting and Best[36] initiated an active therapy program for diabetes. After the finding by Janbon and co-workers[25(b)] that a sulfonamide (p-amino-benzenesulfonamido-isopropylthiadiazole) induced hypoglycemia, many potential hypoglycemic agents have been investigated and a few have survived years of rigorous testing to become agents frequently used in the management of diabetes. Tolbutamide is one of these agents.

Diabetes mellitus which has been commonly called diabetes, is a chronic systemic disease which is characterized by disorders in (1) synthesis, release or utilization of insulin, (2) metabolism of carbohydrate, fat and protein and (3) the structure and function of blood vessels.[37] The connection between the pancreas and spontaneous diabetes was placed on a firm experimental basis by Mering and Minkowski[38] who showed that pancreatectomy in the dog was followed by a permanent and severe diabetic condition which closely resembled severe diabetes in man. Since the work

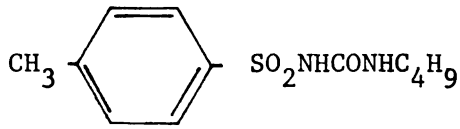
of these researchers, in 1889, the pathology of diabetes mellitus has been greatly investigated and the role of insulin in the disease has become a well-documented part of books written concerning the disease.[37] Recent investigations into pancreatic function by Grodsky and co-workers[39] has lead them to propose a two-compartmental system for insulin secretion. It consists of a small compartment of stored insulin which is particularly labile to stimulating agents and a large compartment containing most of the stored insulin which is relatively stabile, the release from which occurs at a rate comparatively slower than that from the labile compartment. The work of this group involved an in vitro perfusion of a functioning rat pancreas. They have shown under constant glucose infusion, a multiphasic response exists - an initial prompt release of insulin, then a near loss in insulin release followed by a secondary progressive rise in insulin secretion. Constant tolbutamide perfusion causes the immediate release of insulin but, in contrast to glucose, does not produce the secondary rise in the secretion rate. However it was noted that after the initial "spike" of insulin release, the rate fell to a level of about three times the baseline throughout the stimulatory period. Although it is common knowledge that tolbutamide administration to man or other animals results in insulin release and subsequent depression of blood glucose,very little has been reported on the quantitative aspects of this relationship. In rabbits, Hasselblatt and Bludau[41] have shown that the hypoglycemia, as measured by area under the blood glucose depression curve, increases linearly with the logarithm of the infusion does (mg/kg/hour). At most however these experiments were carried out for four hours. Baird and Duncan[42] carried out some experiments on the hypoglycemic response to tolbutamide in which a dose up to 6 grams was given orally. They found it difficult to define the relationship of

dose or plasma level of tolbutamide to the hypoglycemic response. They found that the plasma levels of tolbutamide resulting from the administration of the same dose to different patients varied considerably, as did the sensitivity to the hypoglycemic action. Any attempt to relate the dose of tolbutamide either to the magnitude or the duration of the response in the group studied as a whole merely served to indicate trends and to suggest approximate values which would apply in the majority of cases, rather than to define absolute values. It was clear also that the same response in terms of absolute fall in blood glucose concentrations occurred at widely different levels, not only in different patients, but also in the same patient. The variation between individuals could be accounted for by differences in their sensitivity to the hypoglycemic action of the drug. In any one patient it could be explained by the fact that there appeared to be an optimum therapeutic plasma concentration of tolbutamide. Above the optimum level no increase in the intensity of the hypoglycemic response would result, and in fact a diminished response might occur. It thus becomes quite difficult to quantitate the tolbutamide generated hypoglycemic response.

In addition to its action on the pancreas, tolbutamide is known to elicit extrapancreatic effects which have been reviewed recently.[40] These actions are reported to be inhibition of insulinase, regulation of the ratio of unbound insulin to insulin bound to plasma proteins, increased uptake and oxidation of glucose by adipose tissue, antilipolytic effect on adipose tissue, antiketogenic effect on liver, alteration in the rate of amino acid incorporation into protein, inhibition of transaminase activity, potentiation of insulin stimulation of muscle carbohydrate transport, inhibition of glucose output by the liver, and potentiation of the action of insulin on the liver.

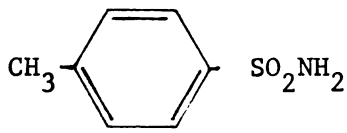
B. Metabolism of Tolbutamide:

The metabolism of tolbutamide (I) has been studied fairly extensively. The literature, although occasionally in conflict, indicates

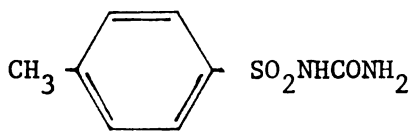


I

some species differences. Wittenhagen, Mohnike and Langenbeek have reported that the dog excretes only p-tolylsulfonamide (II) and p-tolylsulfonylurea (III).[43, 44] However, no mention is made of the half-life. Although there

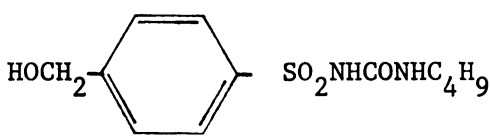


II



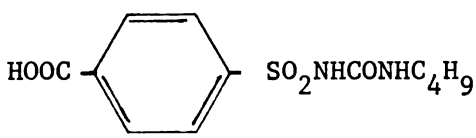
III

is some difference of opinion regarding the metabolism in the cat, Larsen and Madsen[47] have indicated that the primary elimination product is N-(4-hydroxymethylbenzolsulfonyl)-N^1-n-butylurea (IV). In rats, guinea pigs,



IV

rabbits and man, tolbutamide is converted to hydroxytolbutamide (IV) and/or carboxytolbutamide (V).[43, 45, 46.]



V

In man[48], tolbutamide (I) is converted almost quantitatively to hydroxytolbutamide (IV) apparently by NADPH-linked microsomal enzymes. Not more than 2% of the unchanged drug appears in urine. Hydroxytolbutamide is further metabolized to carboxytolbutamide (V), presumably by way of enzymes in the cytoplasmic soluble fraction. All the tolbutamide admistered can be found as metabolites IV and V in the urine of man. The ratio between urinary hydroxy-to carboxytolbutamide varies between 1/3 to 1/9. Whereas carboxytolbutamide is devoid of hypoglycemic activity, hydroxytobutamide is reported to have marked activity when the same dose as tolbutamide is administered as an intravenous bolus.[49] The half-life of tolbutamide is normally between 4 to 8 hours[48] and the oxidation of tolbutamide is the rate limiting step in elimination of this drug and its metabolites from the body. Subsequent oxidation steps are very rapid and when administered separately the half-lives of the hydroxy- and carboxy- metabolites are 40 minutes and 20 minutes respectively.[48]

C. Tolbutamide-Drug Interactions:

Together with the anticoagulants, the interactions with the oral hypoglycemic agents constitute some of the most adverse clinical cases of drug interactions. Hypoglycemic crises have been reported when patients, stabilized on tolbutamide, have added certain other drugs to their drug therapy. Some of the reviews[6, 13] deal with many of the interactions. Ethanol[50, 51, 52], methandrostenolone[53], chloramphenicol[54], bishydroxy-coumarin[55, 56, 57, 58], phenylbutazone[59, 60], phenyramidol[56], salicylate[61,62], sulfaphenazole[59], dicoumarol[55], isocarboxazid[63] and phenprocoumon[63] interacting with tolbutamide, have reportedly caused a greater than normal hypoglycemia. Whereas most of the compounds caused a slowing of tolbutamide

elimination, alcoholics, due to the ingestion of large quantities of ethanol, exhibited an increased rate of tolbutamide metabolism.

The most well studied tolbutamide interaction is that between the sulfonamide sulfaphenazole and tolbutamide. Various authors[58, 49, 64] have reported that sulfaphenazole increases the half-life of tolbutamide from the normal 4 to 8 hours to values ranging from 24 to 70 hours. Clinical crises are reported to arise during an unaltered regimen when tolbutamide accumulates excessively upon chronic administration in the presence of sulfaphenazole. Matin *et al*[64] were able to quantitate this interaction. It was found that as a first approximation the elimination kinetics of tolbutamide in the presence of the inhibitor, sulfaphenazole could be described as shown in Eq.1,

$$\text{Rate of tolbutamide elimination} = \left(\frac{-dT}{dt}\right) = \frac{k_T\, T}{1 + \frac{I}{K_I}} \qquad \text{Eq.1}$$

where T and I were the amounts of tolbutamide and sulfaphenazole in the body, k_T was the rate constant for decay of tolbutamide in the absence of inhibitor, and K_I was defined as the amount of inhibitor which diminished the effective k_T by one-half (or prolonged the half-life two-fold). It was found that k_I has a value of 200 mg sulfaphenazole, which was small compared to the 1-2 Gm daily dose often recommended for this sulfonamide. Usually, 0.5 Gm tolbutamide and 1 Gm sulfaphenazole are given orally twice daily. Substituting the appropriate values, they were able to show that upon chronic co-administration of the two agents in the usual dosage regimen, the new half-life of tolbutamide would be 7 to 8 times longer than normal, and more significantly, the new plateau level of tolbutamide in the body

would be 7 to 8 times the average plateau level in the absence of the inhibitor. If it were necessary to give these two drugs in combination it was apparent that the dosage schedule of tolbutamide would have to be reduced eight fold to maintain essentially the same amount of tolbutamide in the body. In any individual the degree of interaction would depend upon the dosage regimen and half-life for each drug, and the k_I in that individual.

There is evidence that certain compounds interact with tolbutamide in an indirect way. The reversal of the hypoglycemic effect of tolbutamide by diphenylhydantoin has been reported.[65] This interaction is thought to be mediated via the adrenal glands. These type of interactions however appear to be of minor importance.

Of particular interest is the paper by Wishinsky and co-workers[61]. They determined the binding of several sulfonylureas including tolbutamide to albumins of various species by means of equilibrium dialysis or ultrafiltration. In addition they investigated the effect of salicylate upon the protein binding of the sulfonylurea drugs under study. They found a decreased protein binding of tolbutamide to bovine albumin in the presence of salicylate. It was then suggested that this protein displacement might account for the increased hypoglycemic effect of salicylate upon the sulfonylureas. This protein-displacement phenomenon has been suggested as the mechanism for various drug interactions, although no <u>in vivo</u> evidence adequately supports this hypothesis. It was then proposed to investigate the tolbutamide protein-displacement phenomenon further, as will be indicated in the next section.

III. Birth of a Research Project

Tolbutamide, an acid, is significantly bound to plasma proteins[61] and probably to tissue proteins. Its hypoglycemic activity presumably is a function of the unbound drug concentration in the plasma and tissue waters.[66] Sulfaphenazole, dicoumarol and phenylbutazone are also acids, highly bound to plasma proteins, and are capable of displacing tolbutamide and one another from albumin in vitro.[67] Because of these associations, protein binding displacement has been intimated as a contributory cause of the enhanced hypoglycemia experienced when these drugs are used in combination with tolbutamide. It is worthwhile then to consider when displacement will significantly influence the phamacologic effect of a drug. To answer this question, one must know whether displacement is from both plasma and tissue proteins, whether drug clearance depends on the total (bound and unbound) or unbound plasma concentrations, and whether the drug is given on a single occasion or continually.

Appreciable drug displacement occurs when a major portion of the same binding sites are occupied by the displacing agent. Consequently, to displace drugs with a 1:1 molar binding ratio from albumin, requires that the plasma concentration of the displacer approaches or exceeds 0.6 millimolar, the concentration of plasma albumin. This would correspond to a concentration of 180 mcg/ml. for a substance of molecular weight of 300. These plasma concentrations are seen with sulfonamides and salicylates. Plasma concentrations exceeding 0.6 millimolar are also probably achieved following the rapid intravenous bolus ($>$10 sec.) of quite modest doses. These events are likely fleeting, however, as displacer mixes with the vascular system and distributes extravascularly. The rapid injection of even larger doses ($>$14 mg/kg) of drugs which reside primarily in plasma,

may still only produce transient significant rises in the unbound concentration since displaced drug moves down the newly created concentration gradient out into the large tissue water space.[68]

The apparent volume of distribution (Vd) represents that volume which accounts for an amount of drug in the body (Ab) when the concentration in the plasma (Cp) is known, i.e. $Vd = \frac{Ab}{Cp}$. If β is used to designate the fraction of a drug bound in the plasma, whose volume is 3 liters, then the fraction of the drug bound to plasma proteins is $3\beta/Vd$. For a drug like tolbutamide which is fairly highly bound ($\beta = 0.95$) and with a small Vd, around 10L, this means that as much as 29% in the body resides on the plasma proteins. If this entire bound portion were displaced ($\beta \longrightarrow 0$), this 29% added to the remaining 71% could only increase the unbound concentration in the body maximally by 40%. Changes larger than this may be seen normally in the administration and elimination of the drug. Any substantial increase in the unbound concentration above that anticipated from plasma displacement implies that tissue binding is significant and that displacement from these sites must also have occurred. As a result, drug displacement is unlikely to be clinically significant unless the drug is substantially bound and displaced from both plasma and tissue binding sites.

The preceding discussion relates to a situation where the drug is given only once in the absence or presence of the suspected displacer. However, in normal drug therapy, it is more common to give both drug and displacer on a multiple dosage regimen. Upon a constant drug intake (R^o), the steady state plasma concentration (Cp_{ss}) is defined by Eq.2,

$$R^o = CLCp_{ss} \qquad \text{Eq.2}$$

where CL is the clearance of the drug. For a compound whose clearance

(CL) is dependent upon the total concentration of the drug, the final steady state total plasma concentration (Cp_{ss}) should be the same before or after the administration of a constant multiple dosage regiment of displacer. However, the value of $\propto$ (unbound drug in plasma) would be higher, dictated by the concentration of displacer in the plasma at steady state. The situation is different in a case where the clearance of the drug is dependent upon the unbound concentration (Cf). Since at steady state

$$\propto = \frac{Cf_{ss}}{Cp_{ss}} \qquad \text{Eq. 3}$$

substitution of Eq.3 into Eq.2 yields:

$$R^{o} = CL. \frac{Cf_{ss}}{\propto} \qquad \text{Eq. 4}$$

If clearance is dependent on the unbound pasmas concentration then

$$CL = CL_{max} \propto \qquad \text{Eq. 5}$$

where CL_{max} could be either glomerular filtration rate or the maximum metabolic clearance when $\propto = 1$. Displacement of a drug by increasing $\propto$, would increase clearance. But substitution of Eq.5 into Eq.4

$$R^{o} = CL_{max}\ Cf_{ss} \qquad \text{Eq. 6}$$

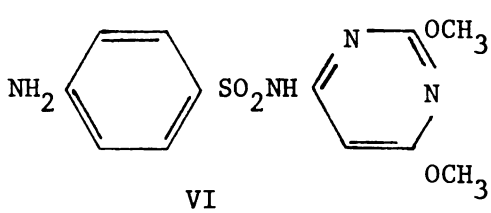
indicates that at steady state, when rate out balances rate in, the unbound concentration should be constant and independent of the degree of protein binding. Between steady states, the unbound concentration may be greater or less than the Cf_{ss}, depending upon whether the concentration of the displacing agent is rising or falling. It should also be noted that the total steady state drug concentration (Cp_{ss}) would be $\frac{Cf_{ss}}{\propto}$ from Eq.3.

Since α would be greater in the presence of displacer, Cp_{ss} would be lower at the steady state when displacer had been co-administered.

The foregoing discussion indicates that when clearance of a compound is dependent upon the unbound concentration, the steady state co-administration of a drug and its displacers should not likely cause an interaction. Since it appears that this phenomenon has never been demonstrated, this project was initiated to investigate more closely the kinetics involved in protein-displacement by an agent and tolbutamide. The aim was to possibly demonstrate that at steady state the unbound concentration should be constant and independent of the degree of protein binding for a drug such as tolbutamide whose clearance appears to be dependent upon the unbound concentration.[69] At the same time one was to be aware of and have at one's disposal the needed methods to explain an interaction if it proved more complicated than that envisioned during the preceding discussion.

IV. A Drug Combination: Tolbutamide and Sulfadimethoxine

In the search for a displacing agent initially, any compound was considered as long as it was highly bound to plasma proteins. As a result compounds such as indocyanine green and rose bengal were initially investigated *in vitro*. However, subsequently it was found that sulfadimethoxine (VI)



VI

might be more suited as a displacing agent. Anton[70] reported that sulfadimethoxine was highly bound (>90%) to plasma proteins. Christensen *et al*[58]

reported that when the sulfonamide was given to several tolbutamide-treated patients, no significant hypoglycemic effect was observed in any of the patients. Yet, Kristensen and Christensen[67] reported that sulfadimethoxine caused a profound increase in the unbound concentration of tolbutamide in serum. This information then suggested that the tolbutamide-sulfadimethoxine combination could represent a useful choice to investigate the protein-displacement phenomenon. As will be seen in Chapter II, Experimental Methods, it was necessary to develop an animal model which would permit a vigorous investigation of this drug combination. In addition, appropriate assays and protein binding methods were required and these will be dealt with as well in Chapter II.

CHAPTER II

EXPERIMENTAL METHODS

Throughout this chapter various designations will be used to indicate the method of delivering volumes of materials: P = pipette, D = disposable Pasteur pipette, G = Biopette automatic pipette, M = microcap, R = repipette delivery system and E = Eppendorf microliter pipette.

I PHYSICAL

A. Protein Binding Methods:

In 1949, Goldstein[81] published his classic review of interactions between drugs and proteins which surveyed and summarized a rather large literature and also clearly emphasized the potential importance of protein binding with respect to the behavior of drugs. The next major review was published by Meyer and Guttman[82] in 1968. Their review covers some of the experimental techniques which have been employed to investigate protein-small molecule interactions, and the methods used to present and to treat experimental data. Three classical methods used to detect, determine, and study binding characteristics are equilibrium dialysis, ultrafiltration and ultracentrifugation. These methods have been employed in the present study and the technique and equipment used will now be dealt with.

1. Equilibrium Dialysis:

In recent years, equilibrium dialysis has gained a measure of sophistication with the development of the Dianorm Equilibrium Dialysis System (Inno Med, Zurich, Switzerland). The Dianorm system has been especially

designed to meet the requirements for research in any field of binding studies and possesses the following: 1) ability to standardize equilibrium dialysis experiments with the 20 cells which may be run simultaneously; 2) short dialyzing times; 3) small dialyzing volumes of 1 ml or less may be used; 4) teflon cells are used to minimize adsorption to the cell wall; 5) the binding may be performed at any reasonable temperature between -10^{o} and 65^{o}C, and 6) the apparatus is easy to use. In the system a semi-permeable membrane separates a dialyzing teflon-cell into two halves. In the present study, 1 ml plasma was injected into one half-cell and 1 ml of an appropriate buffer into the other.

Method: a) Membranes:

The day prior to use, Visking dialysis tubing,13/16" flat width x 0.025 mm (V.W.R.), was cut to the required size and soaked for at least 15 min. in distilled water. Thereafter the tubing was unfolded and soaked for 30 min. in 30% ethanol. Then the membranes were repeatedly rinsed with distilled water, allowed to stand, rinsed, and finally stored in distilled water until used. Fresh Krebs-Henseleit buffer[83] pH 7.4 was prepared just before the start of the experiment. The membranes were then lightly blotted on tissue paper and soaked in the freshly prepared buffer for 30 minutes. After lightly blotting again, the membranes were inserted into the cells.

b) Procedure:

After assembly of the cells, membranes and supports, the materials were introduced into the cells. Two 1 ml tuberculin syringes and appropriately supplied stubs were used for each cell. One syringe was filled (1 ml) with plasma containing the drug while the other was filled (1 ml) with the freshly prepared Krebs-Henseleit buffer. These materials were then injected simultaneously into the labelled half-cells. After all the necessary cells were filled, the instrument was assembled and placed into

a 37°C constant temperature water bath. Unless otherwise noted, dialysis was allowed to proceed for 6 hrs. Prior to the removal of the apparatus from the water bath a syringe was prepared for each half-cell. This involved sharpening a 1 ml tuberculin syrings so that it would accommodate an Eppendorf pipette tip (100 mcl) and then cutting the tip so that it would fit into the hole in the half-cell. Banks of 5 cells were removed from the water bath and each half-cell was quickly emptied with the prepared syringe and the contents transferred to a labelled disposable culture tube, with the buffer side representing the plasma wtater half-cell.

c) Cleaning:

After use, the cells were disassembled and each half-cell thoroughly rinsed with water. After soaking in methanol overnight, the cells were rinsed with acetone and then allowed to air dry.

2. Ultrafiltration:

Ultrafiltration is a process of selective molecular separation. It employs membranes with pore diameters which retain solutes and particles of larger molecular dimensions while passing solvent and solutes of smaller size. The driving force is generally provided by hydraulic pressure. Although various techniques and devices have been developed to perform ultrafiltration, the Centriflo (Amicon) apparatus was utilized. The CF50A membrane cones were used which have a retention or cut off of 50,000 molecular weight and whose volume capacity is 7 ml when the centrifuge is fitted with a horizontal swing-head rotor.

Method: a) Sample:

The sample to be subjected to ultrafiltration was weighed (4 Gm) into a screw cap culture tube. Unless otherwise noted, the tube was placed in a 37°C water bath for 1 hr. prior to the ultrafiltration procedure.

b) Procedure:

The Centriflo cones, conical supports and centrifuge tubes fitted with a 12 x 75 mm disposable culture tube, were assembled and placed in an International Model UV centrifuge. Thereafter the centrifuge, fitted with a heating hair dryer and thermometer was run at 2000 rpm for 20 min. and the dryer adjusted so as to maintain a temperature of 37°C within the centrifuge. The centrifuge was stopped and the samples were added quickly to the cones, being careful not to pour the plasma beside the cone. After the inside of the centrifuge had again reached 37°C by use of the hair dryer, the centrifuge was started. After the speed had reached 2000 rpm, the samples were centrifuged an additional 4 min. (timed) after which the centrifuge was stopped, at all times maintaining the temperature at 37°C. The plasma water (about 0.4 ml, see page 53) collected in the disposable culture tube was then ready for analysis.

c) Cleaning:

Only the conical supports required cleaning. They were rinsed with distilled water, soaked in methanol overnight, rinsed with acetone and then allowed to air dry.

3. Ultracentrifugation:

Although the modern preparative ultracentrifuge is now used for a very wide range of different purposes, it has often become the reference method in determining the protein binding of many compounds. Gravitational force in excess of 300,000 x g. generated by centrifugal motion is used to separate large molecules such as proteins and protein-drug complexes from small molecules such as most drugs. Since the ultracentrifuge is quite widely used, the next section will only deal with the technique used in this study.

Method: The plasma samples were carefully introduced into 7/16 x 2 3/8" Beckman Polyallomar centrifuge tubes so as not to allow air bubbles to remain in the tubes, and allowing a space of 1/8 to 1/16" between the meniscus and the top of the tube. The centrifuge tubes were then placed in the SW-56 swinging bucket rotor and the complete assembly was placed in a 37°C constant temperature oven unless noted otherwise for 1 hr. prior to centrifugation. Thereafter the rotor was inserted into a Beckman L2-65B ultracentrifuge and subjected to centrifugation at 55,000 rpm (400,000 x g.) for 24 hrs. unless noted otherwise. After completion of the procedure, the centrifuge tubes were carefully removed from the buckets on the rotor and the sample(s) of 0.5-1.0 ml (G) were removed from the top of the liquid, keeping the tip of the biopette as close to the meniscus as possible at all times. Thereafter these plasma water samples (see page 53) were placed in disposable culture tubes and were now ready for further analysis.

B. Radioactivity Measurements:

Radioactivity measurements were made by scintillation counting in 10-15 ml Aquasol (NEN). Although two methods were employed, the basic preparation of ^{3}H or ^{14}C containing samples was identical.

Method: 1) Preparation of samples:

A volume of material equivalent to at least 1000 counts per minute (cpm) was introduced (M) into a glass scintillation vial containing 10-15 ml Aquasol (NEN). The microcap (M) was then rinsed 3 times with the Aquasol in the vial. When phase separation was encountered, 200-400 mcl(G) of water was added to the vial in order to produce a clear liquid. Thereafter the sample was counted 3 times for 5 - 10 min. on a Packard Tri-Carb scintillation counter employing the preset ^{3}H or ^{14}C channels. In order to

relate the observed counts to absolute disintegrations per minute (dpm), two methods were used. In all cases a blank accompanied the counting which contained either Aquasol alone, or Aquasol and water equivalent to the volume of radioactive material added to the vial (cpmblk). The ^{3}H blank had an average value of 23 cpm (a range of 17 to 27 cpm) while the average value for the ^{14}C blank was 38 cpm (a range of 32 to 43 cpm). The magnitude of the range reflected the use of different Tri-Carb scintillation counters.

2. Internal standard counting:

At the first counting (cpm^1), a known amount (dpmstd) of appropriate tracer (^{3}H- or ^{14}C- toluene) was added to each vial (M) using the same technique as in part 1). The samples were then recounted (cpm^2). The absolute radioactivity (dpm) present in the volume of unknown radioactive material introduced into the vial was then calculated according to Eq.7.

$$dpm = \frac{(cpm^1 - cpmblk)(dpmstd)}{cpm^2 - cpm^1} \qquad \text{Eq. 7}$$

3. AES ratio counting:

A series of vials containing known amounts (dpmstd) of appropriate tracer (^{3}H- or ^{14}C- toluene) in Aquasol were first counted along with a blank (cpmblk), to ensure that all vials contained about the same amount of radioactivity (±1%). Thereafter to duplicate vials was added an increasing volume of plasma/water free of radioactivity to act as a quencher. The vials were then recounted (cpm) and an AES ratio value was simultaneously obtained. The AES ratio feature of the Tri-Carb scintillation counter, representing a normalized automatic external standard counting procedure using an americium-241 and radium-226 compound source, indicates the degree

of quenching in the sample. Calibration curves (Fig. I and II) were then constructed for each tracer (^{3}H and ^{14}C) relating AES ratio to counting efficiency, where counting efficiency was found according to Eq.8.

$$\text{Efficiency} = \frac{\text{cpm} - \text{cpmblk}}{\text{dpmstd}} \qquad \text{Eq. 8.}$$

All subsequent countings were then performed in the instrument which had been used for the construction of the calibration curves. Absolute radioactivity (dpm) in an unknown sample of known tracer could then be calculated easily after obtaining its AES ratio and cpm. The AES ration was used to determine the efficiency from the appropriate calibration curve and the dpm content were calculated using Eq.9.

$$\text{dpm} = \frac{\text{cpm} - \text{cpmblk}}{\text{efficiency}} \qquad \text{Eq. 9.}$$

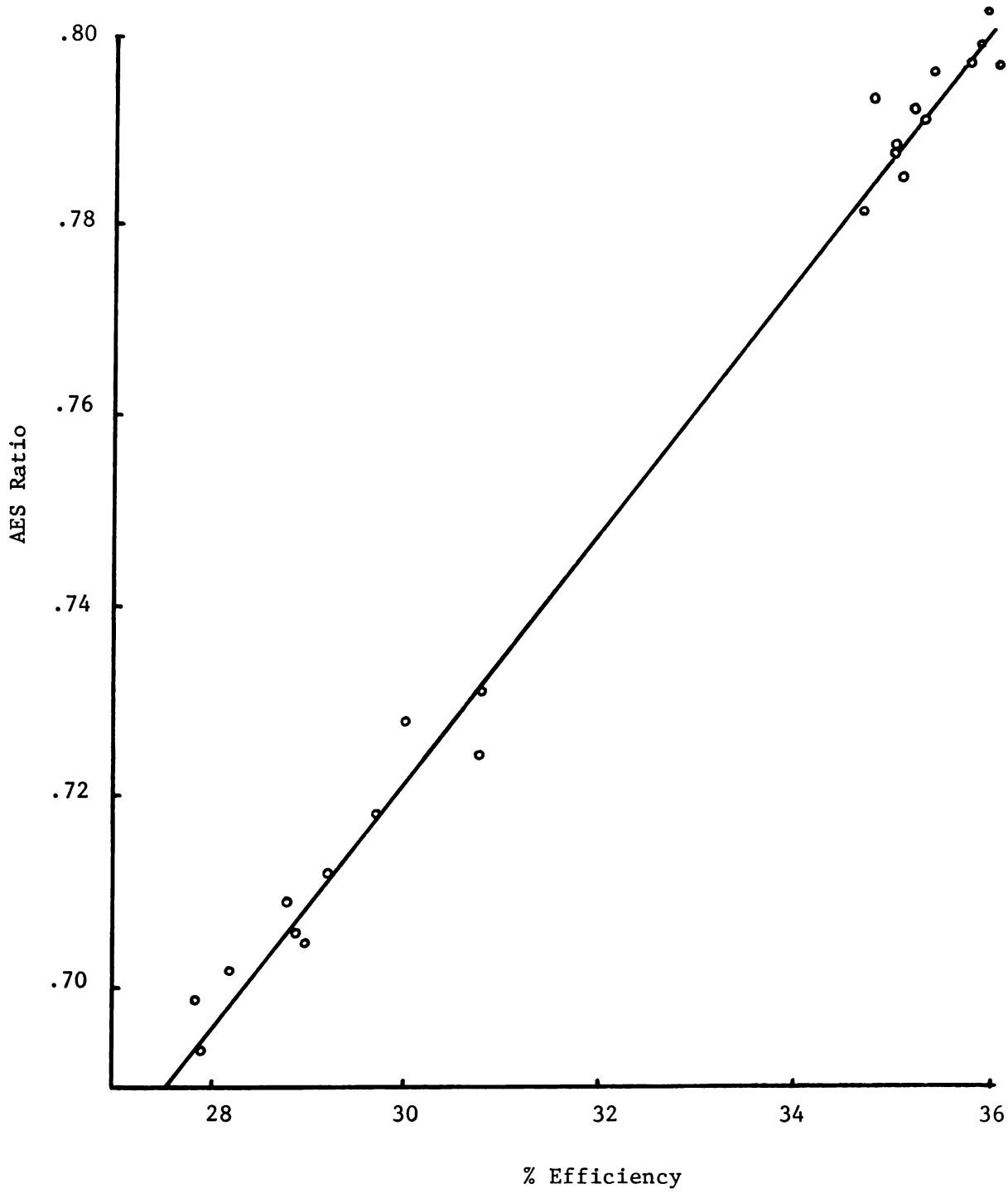


Fig.I: AES ratio and % efficiency calibration curve for ^{3}H- toluene (1.72×10^{4} dpm/vial) using sheep plasma as quencher.

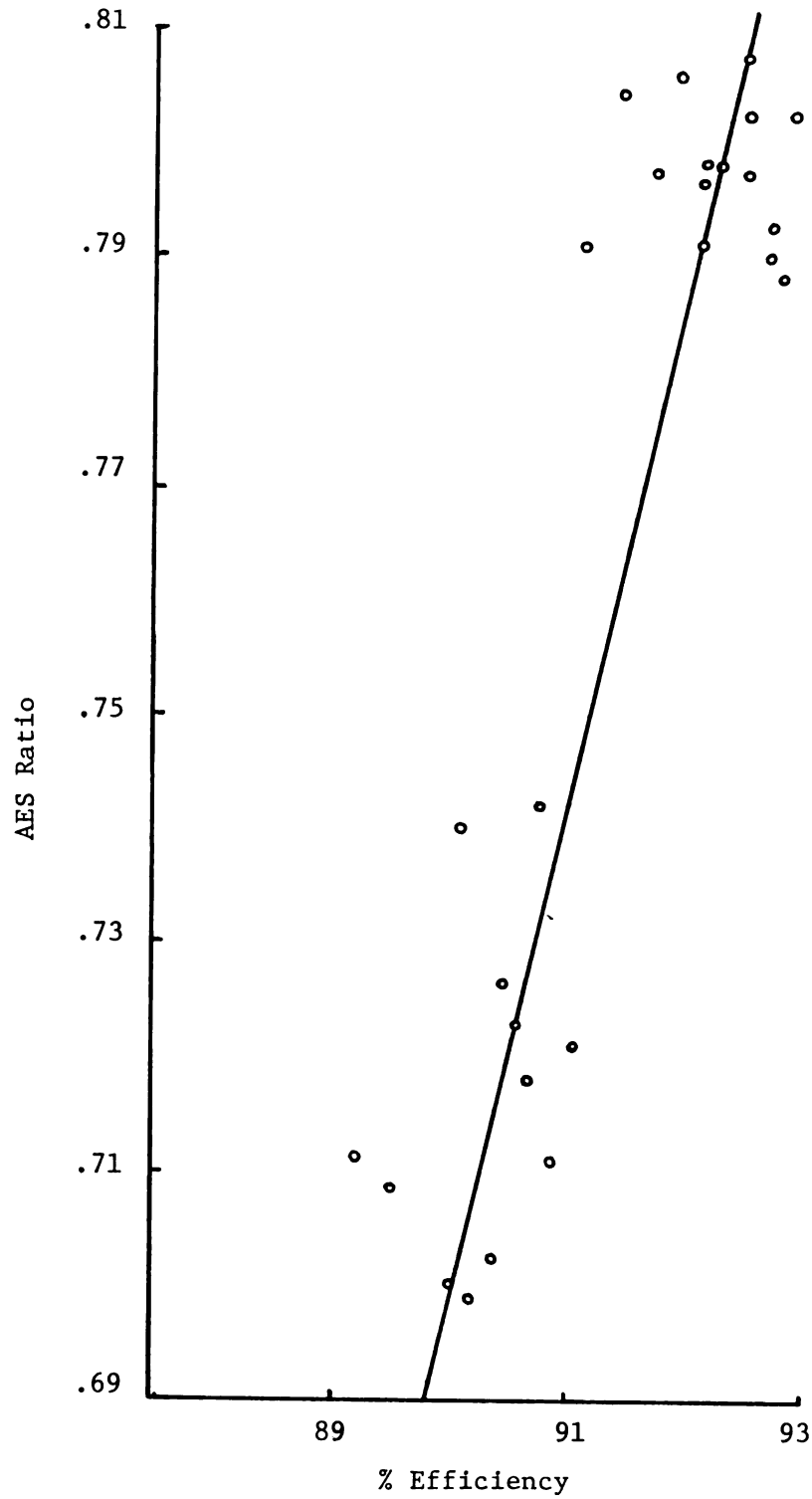


Fig.II: AES ratio and % efficiency calibration curve for ^{14}C- toluene (4.17×10^3 dpm/vial) using sheep plasma as quencher.

C. Thin Layer Chromatography:

Using the method of Thomas and Ikeda[45] thin layer chromatography was performed on the urine collected up to 9 hrs after dosing a sheep with ^{3}H- tolbutamide(exchange label, Dr. S. Matin, UCSF). 2 ml (G) of urine acidified with 0.6 ml (G) HCl were extracted for 1 hr on a test-tube shaker with 20 ml ether. The ether layer was removed (D), dried over anhydrous sodium sulfate and then evaporated under a stream of nitrogen. The resultant brown residue was dissolved in a minimum amount of methanol and spotted on a 20 cm x 20 cm x 2 mm silica gel plate (with F254, E.M. Reagents). In addition, 1 ml (G) urine was extracted in an identical fashion and the eventual methanolic residue divided into 2 parts. To one part was added known hydroxytolbutamide and to the other known carboxytolbutamide. These 2 parts were then spotted beside the largest methanol fraction on the plate. The plate was developed in a 1- butanol:piperidine:water (81:2:17) solvent system until the solvent front had progressed up the plate a distance of 15-20 cm. After removal from the chromatography tank, the solvent front was marked and the plate allowed to air dry. Subsequently the plate was examined under short wavelength ultraviolet light to locate standards as well as boundaries of the band of spotted urine by means of fluorescence quenching. Thereafter 1 cm strips of the silica gel were scraped from the plate and placed in scintillation counting vials along with 15 ml Aquasol. The vials were shaken, allowed to sit for 30 hrs, shaken again and then counted on a Packard Tri-Carb scintillation counter.

D. Mass Spectroscopy:

Chemical ionization mass spectroscopy was performed on the urine of a sheep dosed with tolbutamide in order to further confirm the metabolic

pattern. Two different methods were used in preparation of the sample for analysis. In each case the urinary specimen represented a sample collected 1-2 hours after the administration of tolbutamide.

1) Sample Preparation:

a) 5 ml urine acidified with 1.5 ml 6N HCl were shaken with 5 ml chloroform for 5 min. After centrifugation at 2000 rpm for 10 min., 4 ml of the chloroform extract were dried over anhydrous sodium sulfate and then analyzed.

b) 5 ml urine acidified with 1.5 ml 6N HCl were shaken 5 min. with 10 ml ether. After centrifugation at 2000 rpm for 10 min., the ether was removed (D) and dried over anhydrous sodium sulfate. The ethereal extract was then evaporated under a stream of nitrogen after which diazomethane prepared from Diazald (Aldrich) was added to the residue until a permanent yellow colour remained. Then the ether and excess diazomethane were removed with a stream of nitrogen, 0.5 ml chloroform added and the sample analyzed.

2) Sample Analysis:

The mass spectroscopy was performed on an AEI M.S.-9 instrument employing chemical ionization. The source temperature was 200°C, the reactant was isobutane and the pressure was 0.4 Torr.

E. Culture Tube Cleaning:

All the Kimax 16 x 125 mm culture tubes and teflon-line screwcaps were cleaned and reused.

1) Culture tubes:

Initially the tubes were soaked overnight in a Liqui-nox soap solution, scrubbed, rinsed with distilled water and then heated with

steaming 30% nitric acid for 3-4 hrs. After cooling, the tubes were removed, rinsed with distilled water and then dried in an oven. Although this method was generally satisfactory, occasionally suspected tube contamination was encountered in certain assays. Thereafter the preferred method of Kushinsky and Paul[84] was followed. The tubes were treated with soap solution as before, rinsed with tap water, air or oven dried and then cleaned 4-5 hrs. in the "cleaning cycle" of a self-cleaning oven. (Sears Kenmore model # 42320). A few grains of sugar were placed in one tube as a check on the cleaning cycle. No contamination problems were encountered with tubes after implementing this latter procedure.

2) Screwcaps:

After soaking in 30-50% ethanol for one day, the caps were thoroughly rinsed with distilled water and left overnight in Liqui-nox soap solution. Subsequently the caps were thoroughly rinsed, replaced in new soap solution, subjected to ultrasonic (Aerograph ultrasonic cleaner) treatment for 1 hr, rerinsed thoroughly, then ultrasonicated again in distilled water for 1 hr. The caps were finally rerinsed thoroughly and dried in an oven.

II CHEMICAL

A. Tolbutamide Assay:

1. Plasma:

a) Introduction:

A number of assays have been devised for the determination of tolbutamide with varying sensitivities. A method employing the ultraviolet spectrum of the intact molecule was used by Forist et al[72] but lacks

specificity and is generally unsuitable below 10 mcg/ml. Since the intact molecule is rather heat labile, certain workers have first methylated the molecule and then subjected the methylated tolbutamide to gas chromatography[73, 74]. These gas chromatographic methods however are rather tedious. Use has been made of the Spingler[71] assay by Matin and Rowland[75] to develop a suitable assay for tolbutamide. It basically involves the hydrolysis of tolbutamide to butylamine followed by reaction of the amine with 2,4-dinitrofluorobenzene. This method could be considered non-specific because tolbutamide, hydroxytolbutamide or carboxytolbutamide all yield butylamine on hydrolysis. Yet, appropriate solvent extractions separate tolbutamide from the two latter compounds as will be seen in the "Tolbutamide Metabolite Assay" section thus proving to be specific and also conveniently simple and rapid.

b) Preliminary work:

The plasma levels of tolbutamide encountered were most often less than 100 mcg/ml. Using the information of Matin and Rowland[75] that 1 mcg tolbutamide could readily be determined using their gas chromatographic procedure, an effort was made to prepare a calibration curve using 2-16 mcg tolbutamide per 100 mcl plasma with 8 mcg chlorpropamide as internal standard. Although a reasonable calibration curve was obtained, contamination as evidenced by additional inconsistent peaks created difficulties. This contamination was eliminated by cleaning the tubes in the assay in a self-cleaning oven (p.34), and using freshly prepared 2,4-dinitrofluorobenzene solution. Thereafter the assay proved very reliable.

c) Assay procedure:

Pipette 100 mcl (M) plasma containing 2-10 mcg tolbutamide into a 16 x 125 mm Kimax culture tube fitted with a teflon-lined screw cap.

Add 0.5 ml (G) water, 100 mcl (E) of aqueous internal standard solution (6 mcg chlopropamide), 1 ml 1NHCl (G) and 10 ml 1% isoamyl alcohol/hexane (R). Shake manually for 1 min., centrifuge (International HN-S) at 2000 rpm for 2 min. and transfer (D) about 90% of the top organic phase to a clean culture tube. Add 1 ml 0.5N NaOH (G), shake for 1 min. and then recentrifuge at 2000 rpm for 2 min. After aspirating off the top organic phase add 3 ml redistilled pentyl acetate (R) and 1 ml 1N HCl (G). Shake the tube for 1½ min. and then centrifuge at 2000 rpm for 10 min. Carefully transfer (D) about 90% of the pentyl acetate layer to a clean culture tube (avoid transfer of any aqueous phase). After adding 10 mcl (E) freshly prepared 1% 2,4-dinitrofluorobenzene in redistilled pentyl acetate, heat the tightly capped tube for 1 hr. at 120°C (oil bath). Upon cooling, inject 1-2 mcl of the pentyl acetate into a gas chromatograph equipped with an electron capture detector and a 3% OV-17 column.

Figure III illustrates gas-chromatographic recorder tracings obtained by taking 0.1 ml blank plasma (A) and 0.1 ml plasma containing chlorpropamide and tolbutamide (B) through the outlined procedure. A Varian 1200 model gas chromatograph was used equipped with a nickel-63 detector, containing a 6 ft. 1/8 inch o.d. glass column packed with 3% OV-17 on 100-120 mesh, acid washed DMCS chromosorb W. Injection port, column and detector temperatures were 245, 210 and 310°C respectively. Carrier gas (5% methane in argon) flow was maintained at 30 ml/min. Figure IV represents a calibration curve in which 2-10 mcg tolbutamide have been added to 0.1 ml plasma containing 6 mcg chlorpropamide. Peak height ratio of tolbutamide to chlorpropamide (T/C) has been used for quantitation purposes. It should be noted however that it is the butylamine derivative of tolbutamide and the propylamine derivative of chlorpropamide which are in fact measured.

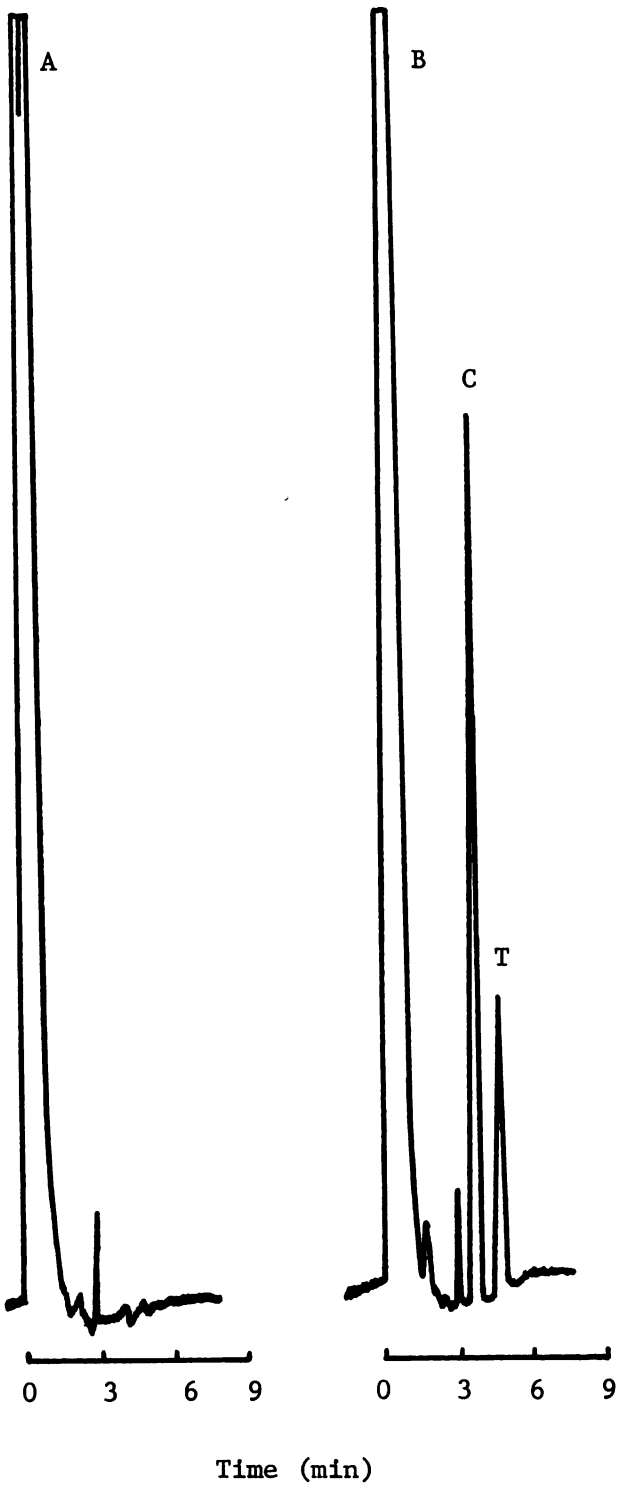


Fig.III Chromatogram of tolbutamide gas-chromatographic assay using 0.1 ml blank plasma (A) and 0.1 ml plasma (B) containing 6 mcg chlorpropamide (C) and 2 mcg tolbutamide (T). Injector, column, detector temperatures were 245, 210 and 310°C respectively. Attenuation was 4 and range 10^{-10} amps/mv.

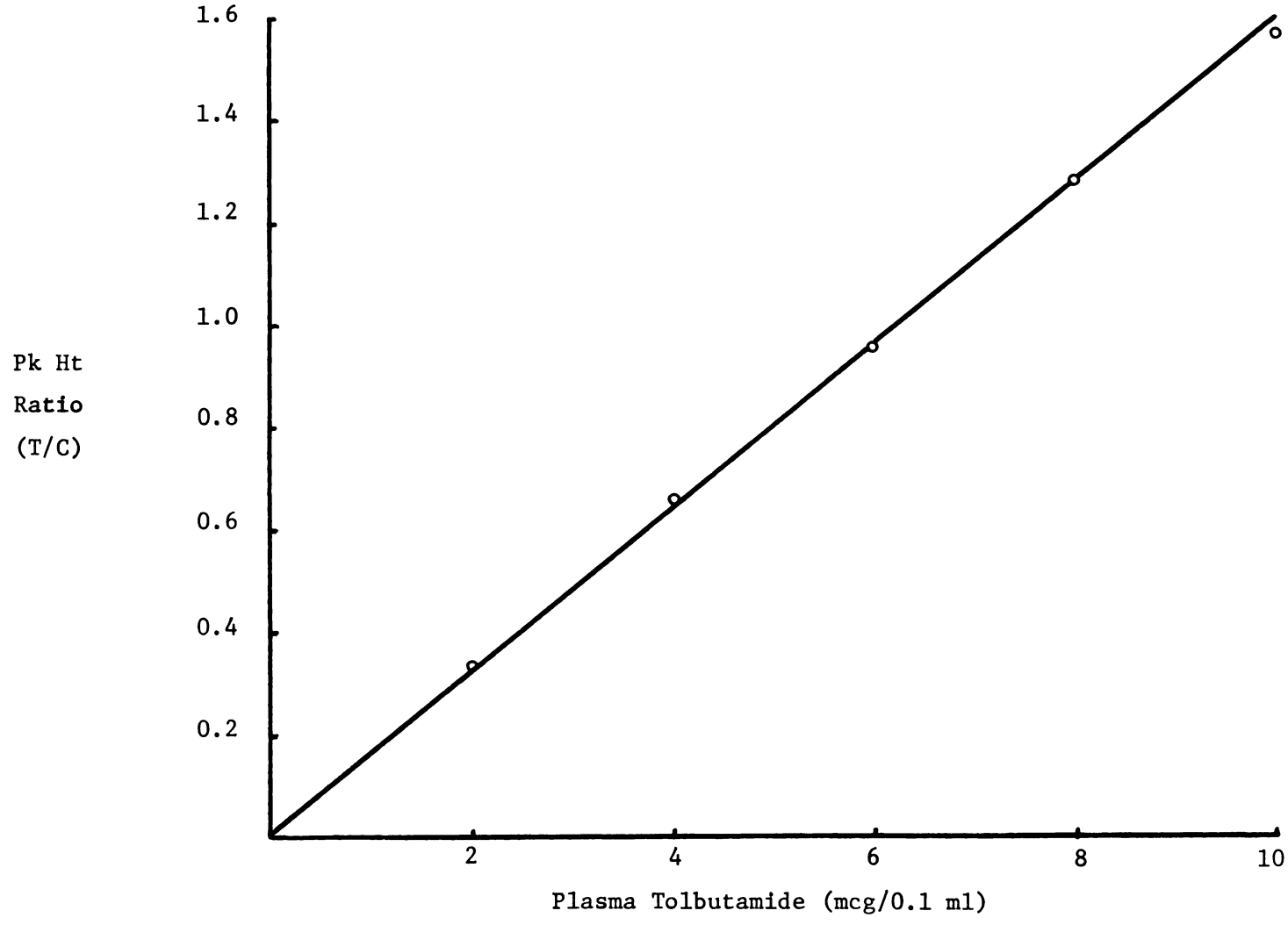


Fig.IV: Calibration curve for the gas-chromatographic assay of tolbutamide (T) in plasma using 6 mcg chlorpropamide (C) as internal standard.

d) Evaluation of assay:

As can be seen from Fig.III (A), blank plasma was devoid of any apparent chlorpropamide or tolbutamide peaks. When 6 mcg chlorpropamide was carried through blank plasma an apparent peak height ratio (T/C) greater than 0.03 was never obtained.

The calibration curves were linear over a 10 fold range. Thereafter deviation from linearity occurred possibly due to non-linearity of the electron capture detector response.

Although one sample was never reanalyzed numerous times for statistical information, some information may be reported on the variability at different concentrations during the preparation of the calibration curve on different days. At 2, 4, 6, 8 and 10 mcg tolbutamide the coefficients of variation were 5.2, 1.1, 2.6, 2.1 and 11% respectively.

This assay may be used when carboxytolbutamide is absent or present in insignificant amounts. Otherwise the method of Matin and Rowland[75] must be followed. The interference due to hydroxytolbutamide is small, only 1% of the amount present determined as tolbutamide. Sulfadimethoxine does not change the gas chromatographic recorder spectrum of plasma devoid of chlorpropamide or tolbutamide, nor alter the peak height ratio when tolbutamide is present at a concentration of 100 mcg/ml.

2. Plasma water:

Plasma water as obtained by ultracentrifugation, ultrafiltration or equilibrium dialysis was analyzed for tolbutamide content in the identical way as in part 1. "Plasma". Initially the following modifications were made: 2 mcg chlorpropamide was used as internal standard, 1.5 ml pentyl acetate was employed, and finally only 10 mcl of 0.2% dinitrofluorobenzene was added. A volume of plasma water corresponding to a level of 0.25 to

4 mcg tolbutamide was used. A calibration curve was prepared using 0.25 to 4 mcg tolbutamide in 100 mcl whole plasma as seen in Figure V. This method represents the lower limit to which the tolbutamide assay has been tested. However after the initial use of this plasma water assay, it was decided to rather employ the regular tolbutamide plasma assay (Part 1) but use 0.25 ml of the plasma water. This change in the assay method was made after the finding that $>16\%$ of the plasma tolbutamide was found in the plasma water. As a result the statistical aspects of the plasma water assay are those found in Part 1.

3. Urine:

a) Introduction:

The determination of tolbutamide in biological fluids as published by Matin and Rowland[75] provided for a colorimetric procedure if the plasma concentrations were greater than 25 mcg/ml. This assay procedure again involved the hydrolysis of tolbutamide to butylamine followed by reaction of the amine with 2,4-dinitrofluorobenzene. The assay was easily adaptable to urine and was employed in the present study.

b) Assay procedure:

Add 1 ml urine (P) corresponding to an absolute amount of tolbutamide of 25 to 150 mcg to a culture tube. Add 0.5 ml 6N HCl (G) and 10 ml 1% isoamyl alcohol/hexane (R). Shake manually 1½ min. and centrifuge 5 min. at 2000 rpm. Transfer (D) as much of the top organic layer as possible along with 2 x 1 ml, 1% isoamyl alcohol/hexane washings of the aqueous layer, to a new culture tube. Now add 1 ml 0.5N NaOH (G) to the organic phase, shake for 1½ min. and centrifuge at 2000 rpm for 5 min. Aspirate off the organic phase being careful not to remove any aqueous phase, then add 1 ml 1N HCl (G) and 3 ml pentyl acetate (R). Shake the tube for 1½ min., centrifuge 10 min., at 2000 rpm and then transfer (D) about

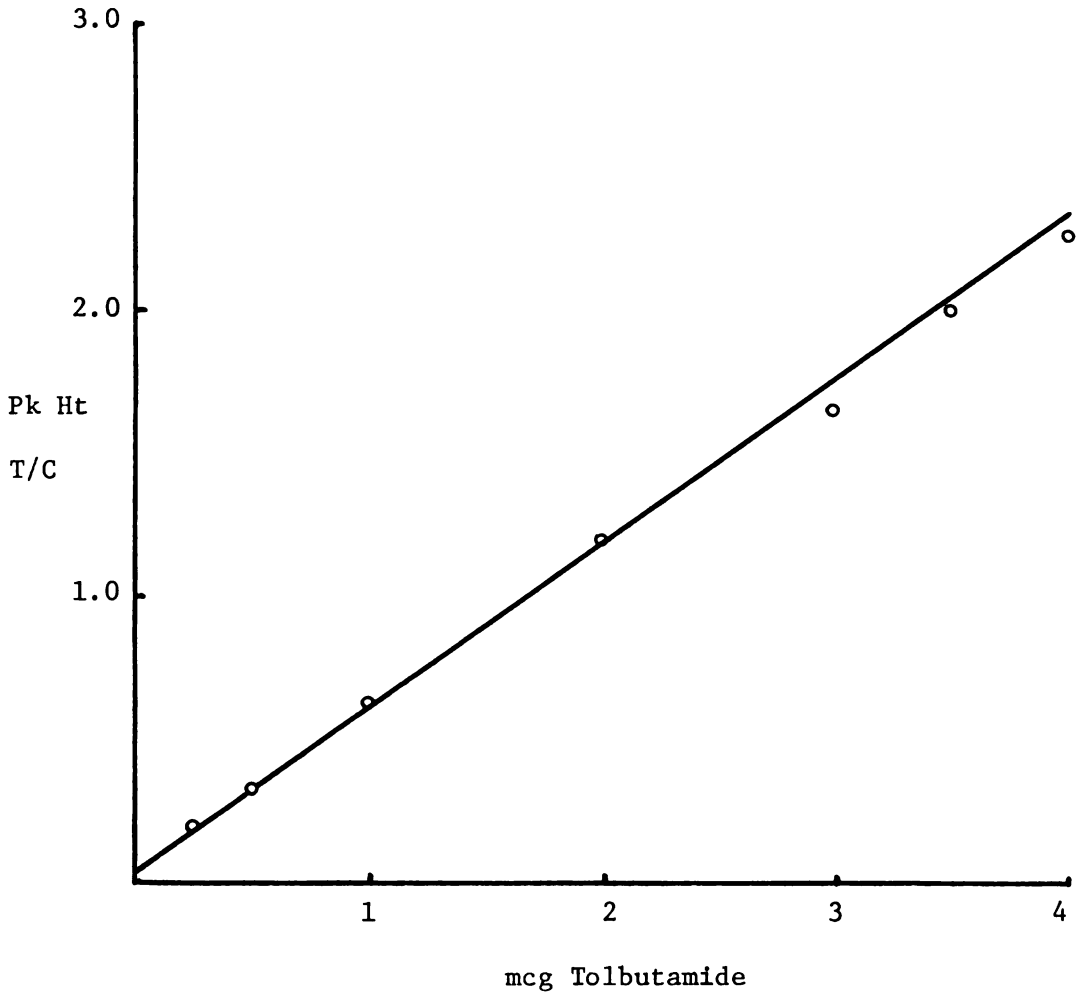


Fig.V: Calibration curve for the gas-chromatographic assay of tolbutamide (T) in plasma water using 2 mcg chlorpropamide (C) as internal standard.

2/3 of the pentyl acetate layer to a new culture tube. After adding 20 mcl (E) 5% dinitrofluorobenzene in pentyl acetate and heating 1 hr. at 120°C, a colorimetric measurement is made at 420 nm against an appropriate blank constituting an analogously heated tube containing 20 mcl (E) 5% dinitrofluorobenzene in 2 ml pentyl acetate (R).

A Cary-15 model spectrophotometer was used to make the colorimetric measurements. The concentration in an unknown urine sample was determined by reference to an absorbance vs tolbutamide concentration calibration curve obtained by taking 40 to 150 mcg tolbutamide in 1 ml blank urine through the procedure. Figure VI represents a calibration curve obtained during this study.

c) Evaluation of assay:

The assay proved very convenient and simple. The absorbance of blank urine taken through the procedure was never greater than 0.01. As can be seen from Figure VI, good linearity was achieved over a wide concentration range. At urinary tolbutamide concentrations of 40, 50, 100, 140 mcg/ml the coefficients of variation were 3.9, 14.2, 4.5 and 5.1% respectively. Again as in Part 1 these statistical values were obtained as a result of the preparation of the calibration curve on different days.

The assay procedure may be used when carboxytolbutamide is absent or present in insignificant amounts. Otherwise additional precautions must be taken. The interference due to hydroxytolbutamide is small, only 1% of the amount present determined as tolbutamide. The addition of 400 mcg sulfadimethoxine to 1 ml blank urine did not increase its absorbance nor did it change the absorbance when added to 1 ml blank urine containing 140 mcg tolbutamide.

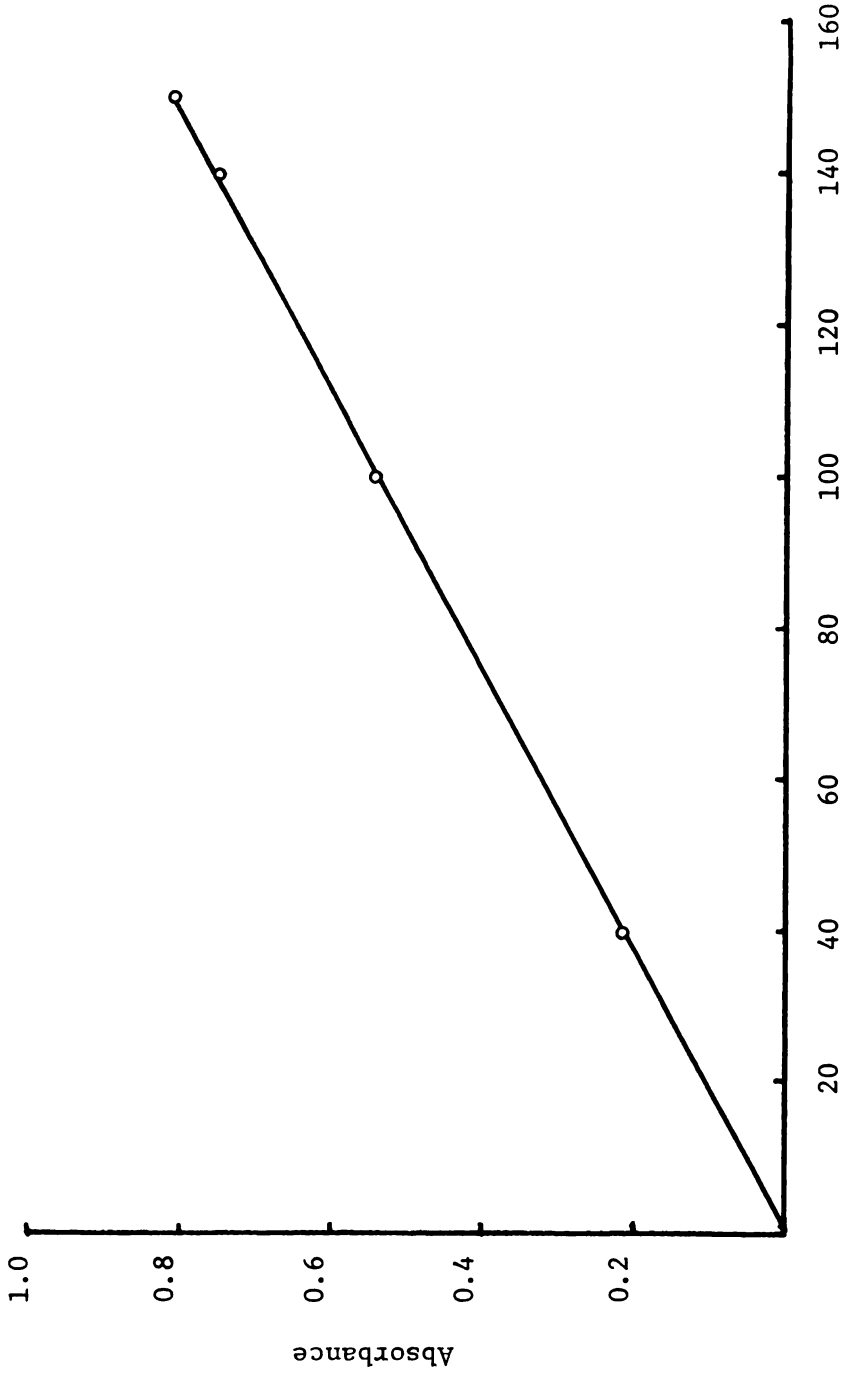


Fig.VI: Urinary tolbutamide calibration curve.

B. Tolbutamide Metabolite Assay:

1. Plasma:

a) Introduction:

As indicated in Chapter I, the metabolites of tolbutamide encountered in the majority of animals including man are hydroxy- and carboxytolbutamide. Although an assay for carboxytolbutamide has been reported[77], it would not differentiate the parent compound from its oxidation products. Prescott and Redman[74] mention the lack of interference of hydroxy- and carboxytolbutamide in their gas chromatographic assay of tolbutamide but mention no assay for the metabolites. Matin and Rowland[76] have developed an assay which will quantitatively estimate each of the oxidized metabolites when mutually present in plasma or urine. Their assay was modified because of the finding that the sheep produces very little carboxytolbutamide.

b) Assay procedure:

1 ml plasma (P) corresponding to an absolute amount of 0.5 to 2 mcg hydroxytolbutamide is addeed to a 16 x 125 mm Kimax culture tube fitted with a teflon-lined screwcap. Add 2 ml 1N HCl (G), 10 ml ether (R), cap tightly shake manually for 1½ min. and centrifuge at 2000 rpm for 10 min. Transfer (D) the entire ether layer as well as 2 x 0.5 ml ether (R) washings of the aqueous fraction to a clean culture tube. Add 1 ml 0.5N NaOH (G) to the ether fraction, shake 1½ min., centrifuge 5 min. at 2000 rpm and then carefully aspirate off the ether layer avoiding removal of the aqueous layer. Now add 1 ml 1N CHl (G) to the aqueous fraction followed by successive extractions with 10, 5 and 5 ml of 1% isoamyl alcohol/hexane (R), shaking 1 min., centrifuging 5 min. at 2000 rpm and then aspirating and discarding the organic phase, being careful not to remove any of the aqueous fraction each time. Thereafter add 100 mcl (E) of the aqueous internal standard

solution (1.5mcg chlorpropamide), 1 ml redistilled pentyl acetate (R), shake 1½ min. and centrifuge 10 min. at 2000 rpm. Carefully remove about 3/4 of the pentyl acetate fraction (D) and transfer to a clean culture tube. Add 20 mcl (E) of freshly prepared 0.25% 2,4-dinitrofluorobenzene in redistilled pentyl acetate and heat the tightly capped tube at 120°C (oil bath) for 1 hr. After cooling inject 1-2 mcl of the pentyl acetate into a gas chromatograph as indicated in the "Tolbutamide Assay in Plasma" using the identical instrument column and conditions.

Figure VII illustrates a chromatogram of 1 ml blank plasma containing 1.5 mcg chlorpropamide (A) and 1 ml blank plasma containing 1.5 mcg chlorpropamide and 0.5 mcg hydroxytolbutamide (B). Figure VIII exhibits a calibration curve for the assay employing 0.25 to 3 mcg hydroxy-tolbutamide in 1 ml plasma. The ordinate represents peak height ratio ($^{OH-T}/C$) of hydroxy tolbutamide to chlorpropamide. It should be noted again that the peaks represent the propylamine deriative of chlorpropamide and the butylamine deriative of hydroxytolbutamide.

c) Evaluation of assay:

As can be seen from Fig. VII (A), blank plasma gave a small apparent hydroxytolbutamide peak. This contamination usually resulted in an apparent peak height ration ($^{OH-T}/C$) of about 0.05. The calibration curves often appeared linear over a 10 fold range, but instances of considerable non-linearity were evident at times, with the curves linear only over a 4-5 fold range.

The coefficients of variation at 0.5, 1.0 and 2.0 mcg/ml hydroxytolbutamide were 26.7, 11 and 13.9% respectively in day to day assays.

This hydroxytolbutamide plasma assay is beset with certain difficulties. As will be noted, the internal standard is not added until the initial extractions which remove tolbutamide have been completed.

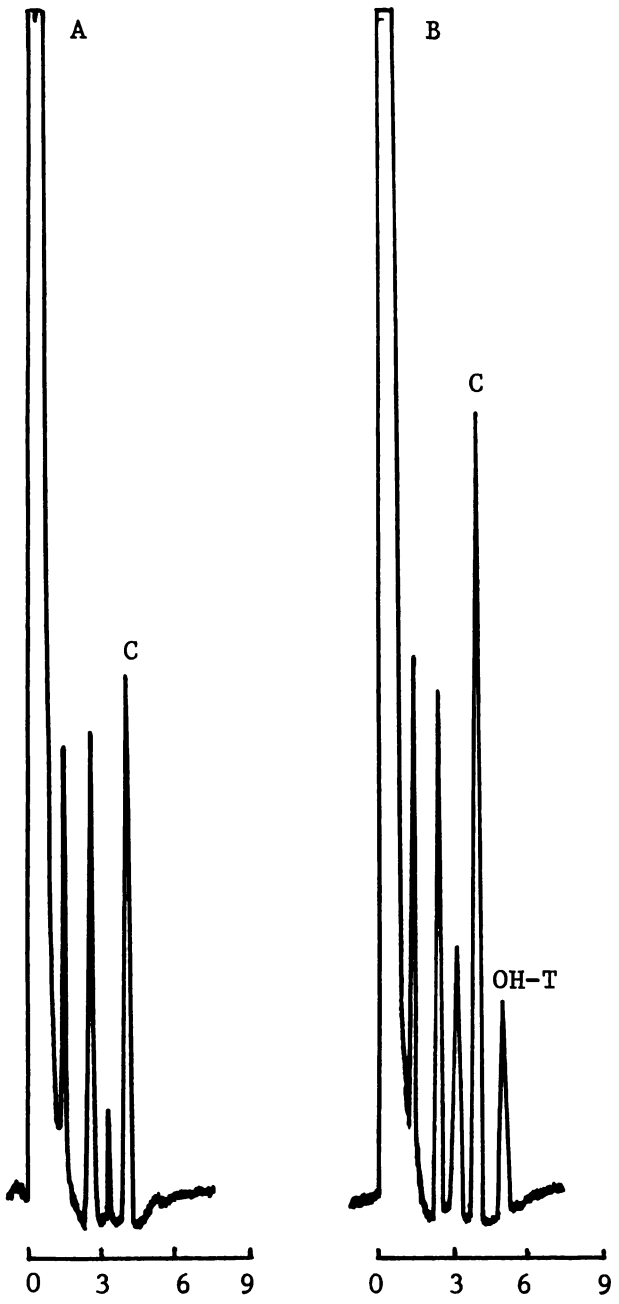


Fig.VII: Chromatogram of hydroxytolbutamide assay using 1.5 mcg chlorpropamide (C) in 1 ml blank plasma (A) and 1.5 mcg chlorpropamide plus 0.5 mcg hydroxytolbutamide (OH-T) in 1 ml blank plasma (B). Injector, column, detector temperatures were 245, 210 and 310° respectively. Attentuation was 4 and range 10^{-10} amps/mv.

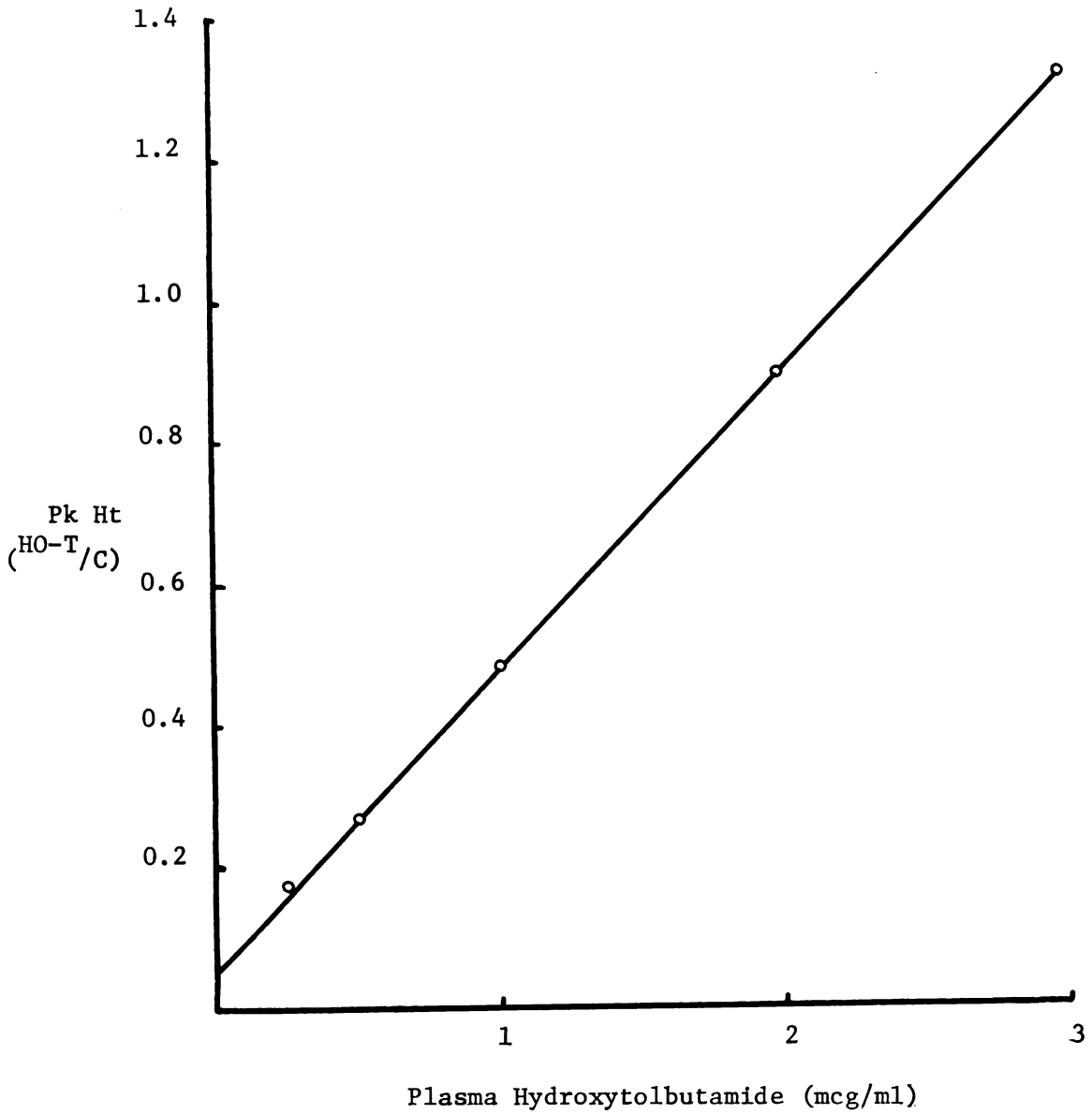


Fig.VIII: Calibration curve for the gas-chromatographic assay of hydroxy-tolbutamide (OH-T) in plasma using 1.5 mcg chlorpropamide (C) as internal standard.

The variable nature of these extractions in removing some of the hydroxytolbutamide is evident in the coefficients of variation. Also, any remaining tolbutamide after the intial extractions is then determined as hydroxytolbutamide. The addition of 100 mcg tolbutamide to 1 ml plasma and subsequent analysis for hydroxytolbutamide results in an apparent hydroxytolbutamide peak height ratio ($^{OH-T}/C$) of 0.12 to 0.18 which would correspond to about 0.25 mcg/ml hydroxytolbutamide. A considerable refinement could be made to the assay by the use of a more suitable internal standard.

The assay as used will measure all carboxytobutamide as hydroxytolbutamide. However since the sheep produces only small amounts of carboxytolbutamide, this aspect was disregarded.

2. Urine

a) Introduction:

The urinary assay of hydroxytolbutamide again followed the work done by Matin and Rowland[76]. Their assay involves a separation of hydroxy- and carboxytolbutamide as well as separation of these metabolites from tolbutamide. Since the sheep produces very little carboxytolbutamide, the assay was modified so that total hydroxy- and carboxytolbutamide were determined.

b) Preliminary work:

A difficulty encountered during the urinary hydroxy- tolbutamide assay was that the final organic extract (hydroxy- tolbutamide fraction) of blank urine absorbed considerably at 420 nm. Although this absorption could be reduced by using 0.5% isoamyl alcohol/dichloromethane instead of pentyl acetate as the final organic extraction solvent, it was still considerable, and varied for different urine samples. It was also found that the absorbance of a pentyl acetate extract did not change after the addition

of 2,4-dinitrofluorobenzene and heating for 1 hr. at 120°C. Therefore it was decided to maintain pentyl acetate as the organic solvent for extracting hydroxytolbutamide since use of this organic solvent shortened the assay. In general it was found that volumes of urine less than 200 mcl gave absorbance values less than 0.02 when devoid of hydroxytolbutamide.

c) Assay procedure:

100 to 200 mcl (M) urine corresponding to an absolute amount of hydroxytolbutamide of 25 to 150 mcg is added to a culture tube. Add 1 ml 1N HCl (G) and 10 ml 1% isoamyl alcohol/hexane (R). Cap tightly and shake for 1½ min. Centrifuge at 2000 rpm for 5 min and remove the organic layer via aspiration. Wash the aqueous layer with 2 x 1.0 ml 1% isoamyl alcohol/hexane (R) and also remove the organic layer by aspiration being careful not to remove any of the aqueous phase. Add 3 ml pentyl acetate (R), shake 1½ min. and centrifuge 10 min. at 200 rpm. Transfer (D) about 2/3 of the pentyl acetate layer to a clean culture tube and add 20 mcl (E) 5% 2,4-dinitrofluorobenzene in pentyl acetate. Heat the tightly capped tube for 1 hr. at 120°C (oil bath) and after cooling the absorbance is read at 420 nm. against an appropriate blank constituting an analogously heated tube containing 20 mcl (E) 5% 2,4-dinitrofluorobenzene in 2 ml pentyl acetate (R).

Appropriate standards in 100-200 mcl (M) blank urine must be carried through the procedure with the unknown samples. An absorbance vs hydroxytolbutamide concentration calibration curve is then constructed as seen in Figure IX.

d) Evaluation of assay:

The assay proved very convenient and simple. The absorbance of blank urine (100 to 200 mcl) taken through the procedure was usually less than 0.02. However, blank urine samples (1 ml) could result in an

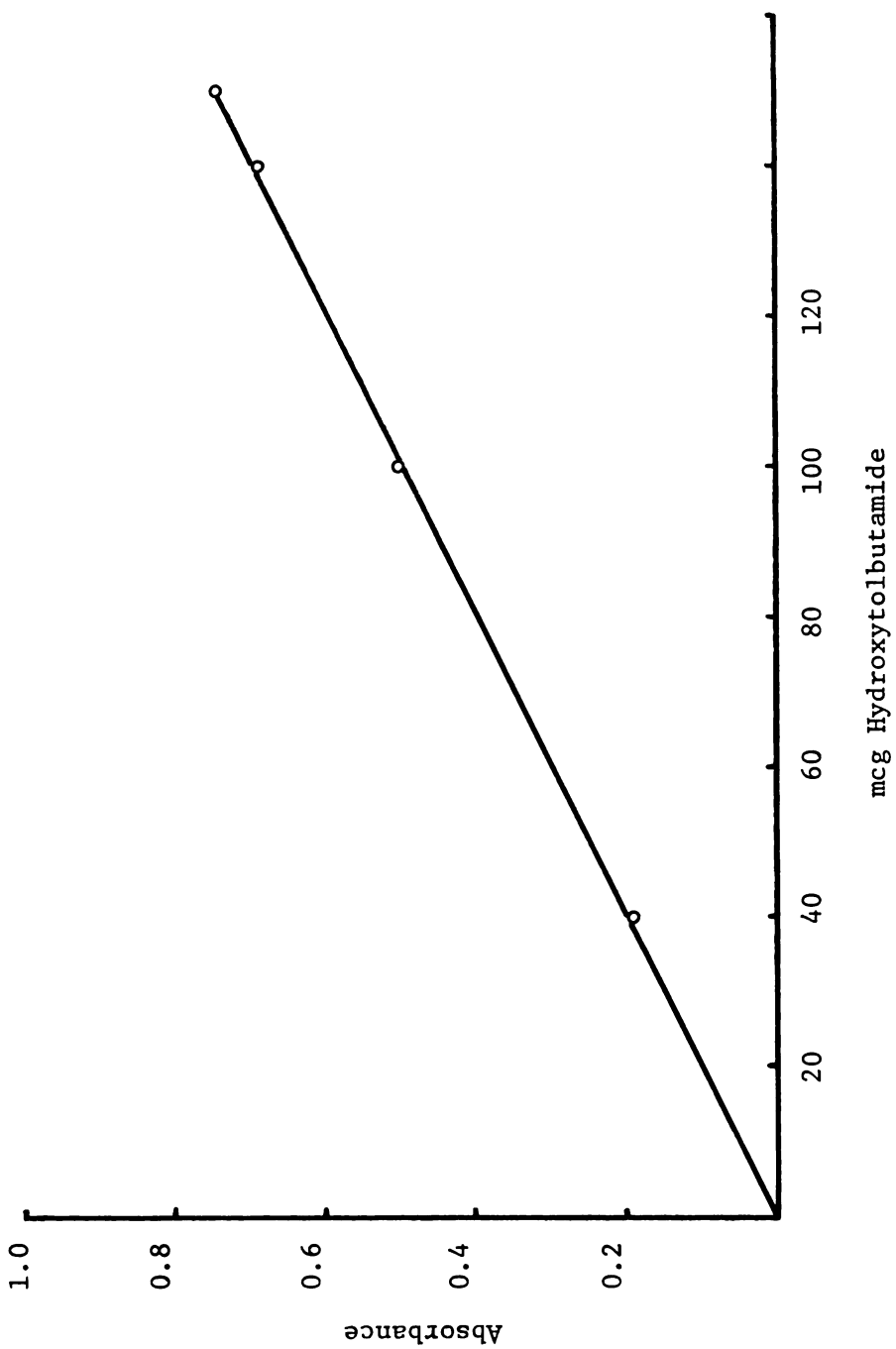


Fig.IX: Urinary hydroxytolbutamide calibration curve

absorbance as high as 0.1. As a result, when large (greater than 200 mcl) volumes of urine were used, the contribution of hydroxytolbutamide was taken as the difference in absorbance before and after heating of the pentyl acetate (see preliminary work). When volumes greater than 200 mcl are used a corresponding adjustment must be made in the hydrochloric acid initially used.

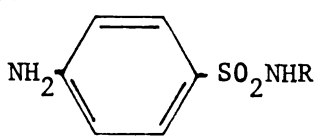
Statistically the assay had good day to day reproducibility for the coefficients of variation at 40 and 100 mcg hydroxytolbutamide were 7.2 and 5.4% respectively.

The assay procedure will read all of the carboxytolbutamide as hydroxytolbutamide. As a result, the term "hydroxytolbutamide equivalents" has been adopted to indicate all metabolites which according to this assay would be determined as hydroxytolbutamide. This would naturally include the small quantities of carboxytolbutamide which the sheep excretes after administration of tolbutamide. "Total tolbutamide equivalents" excreted would then include the unchanged tolbutamide plus the hydroxytolbutamide equivalents (corrected for molecular weight by multiplying by $\frac{270}{286}$).

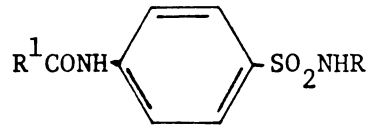
C. Sulfadimethoxine Assay:

1) Introduction:

Several procedures are available for the determination of sulfonamides. The most commonly employed method involves a colorimetric determination utilizing the well known Bratton-Marshall[78] assay which will differentiate between parent sulfonamide (VII) and its N^4 conjugated metabolite (VIII).



VII



VIII

Certain modifications were made in the procedure for ease of reproducibility. Both the urine and plasma were treated alike.

2. Assay Procedure in Plasma or Urine:

To a culture tube add a volume of plasma, urine or dilutions of either (M) which corresponds to 10-100 mcg of total sulfadimethoxine. Add 0.5 ml (G) water followed by 2 ml (P) of an acid solution: 120 ml 10% trichloracetic acid and 100 ml 1N HCl. Vortex well and then centrifuge at 2000 rpm until the solution is clear (10-20 min.). Transfer 1 ml (P) to a disposable culture tube [A: for parent sulfonamide; VII] while the balance of the solution is transferred (D) to a screw capped culture tube [B: for total sulfonamide; VII & VIII].

a) Tube [A: parent sulfonamide]:

Add 0.2 ml (G) freshly prepared 0.1% aqueous sodium nitrite, vortex well and allow to stand 5 min. Add 0.2 ml (G) freshly prepared aqueous 0.5% ammonium sulfamate, vortex and allow to stand 2 min. Add 0.6 ml (G) freshly prepared aqueous 0.1% N-(1-napthyl) ethylenediamine dihydrochloride, vortex and allow to stand 20 min. Thereafter determine absorbance at 545 nm against a 0.9% sodium chloride blank taken through the procedure.

b) Tube [B: total sulfonamide] :

Cap the tube loosely and heat in a water bath at 90°C for 1 hr. Cool, shake and transfer 1 ml (P) to a disposable culture tube. Thereafter the same reagents and methods are used as in part a). The absorbance is also taken at 545 nm against a 0.9% sodium chloride blank carried

through the procedure.

All spectrophotometric measurements were made on the Cary-15. The concentration in unknown samples is obtained from a calibration curve of absorbance vs sulfonamide concentration constructed by taking known concentrations (10-100 mcg) in blank plasma or urine through each assay procedure. A calibration curve for unconjugate sulfadimethoxine in plasma can be seen in Figure X.

3. Evaluation of Assay:

Since conjugated sulfadimethoxine was found in plasma initially, subsequent analysis was confined to part a). The assay proved very reliable and the interday coefficients of variation at 20, 40, 60, 80 and 100 mcg/ml sulfadimethoxine in plasma were 1.5, 1.3, 2, 2 and 2% respectively.

The urinary assay present a difficulty and the results and discussion will be presented in Chapter III.

D. Protein Assays:

Essentially three types of protein analysis were required: total protein, electrophoretic protein separation, and determination of protein in plasma water. Plasma water is defined as plasma from which the protein has been removed by the methods of ultracentrifugation, ultrafiltration or equilibrium dialysis. Use was made of the Clinical Labs, Moffitt Hospital, San Francisco to obtain the first two protein analyses: total protein and electrophoretic protein separation.

1) Total Protein Assay:

Total protein was determined on a Technicon, 2-channel auto-analyzer utilizing the Biuret[92] procedure. This procedure was performed by the Clinical Labs, Moffitt Hospital, UCSF.

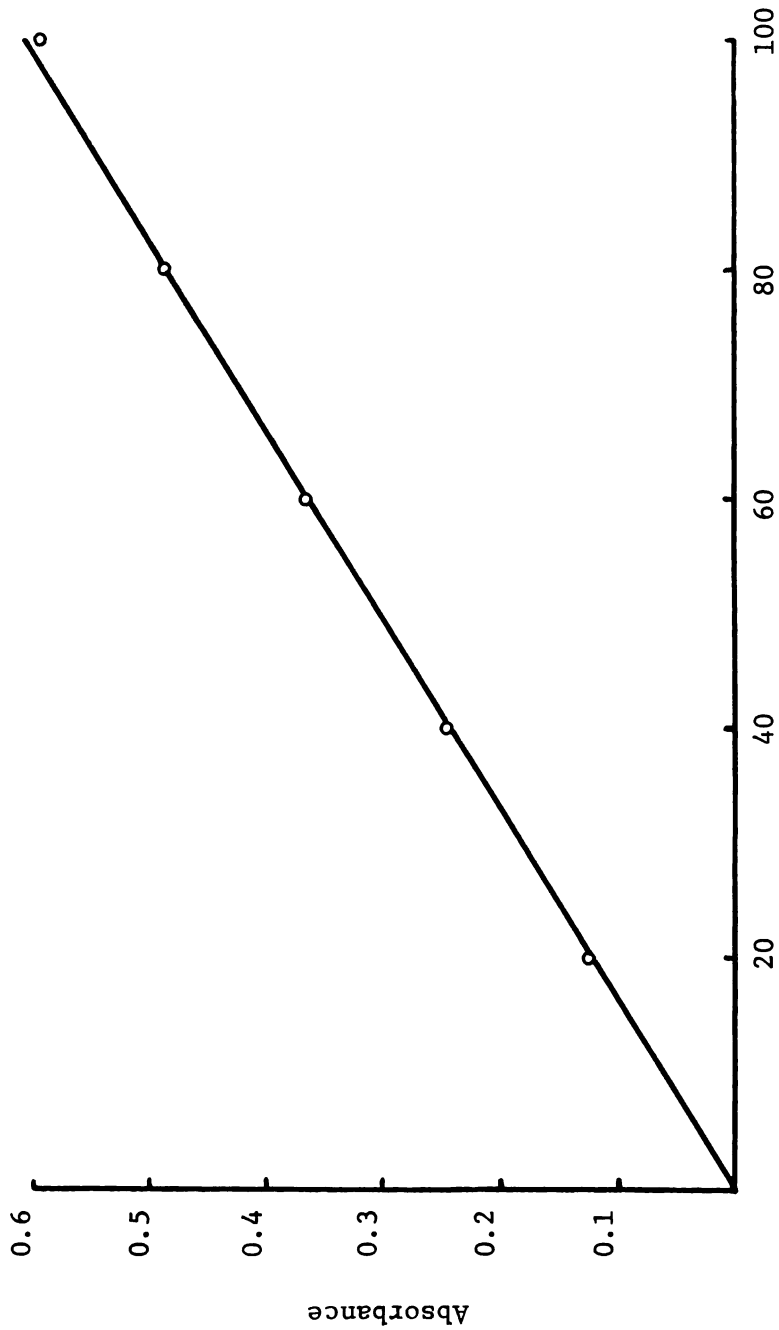


Fig.X: Plasma sulfadimethoxine calibration curve for unconjugated compound.

2. Electrophoresis:

Electrophoresis of plasma was performed on cellulose acetate using a Beckman microsome system. Subsequently the protein fractions were quantitated using a Clifford Densitometer. This procedure was performed by the Clinical Labs, Moffitt Hospital, UCSF.

3. Plasma Water Protein Assay:

a) Introduction:

Various methods have been reported to determine protein. A modification was made of the Lowry[79] method which proved very convenient. A trichloroacetic acid precipitation was used in the procedure so as to eliminate materials which might react with the Folin-Ciocalteau reagent.

b) Assay procedure:

Add 100 mcl (M) of the plasma water to a 400 mcl Beckman microfuge tube. Thereafter add 150 mcl (G) of 15% trichloroacetic acid. Vortex well, allow to stand 15 min., vortex well again and then centrifuge 5 min. in a Beckman Spinco Microfuge. Being careful not to remove any of the precipitate, withdraw as much of the aqueous phase as possible using a #23 x 2" B-D Special Needle attached to a 1 cc disposable tuberculin syringe. Cut the tips of the microfuge tubes containing the precipitate and remaing aqueous medium, and transfer to a 12 x 75 mm disposable culture tube. Wash 1 ml (G) reagent C (50 ml 2% sodium carbonate in 0.10N NaOH and 1 ml of 0.5% $CuSO_4.5H_2O$ in 1% sodium or potassium tartrate) through the microfuge tube into a 12 x 75 mm culture tube. Vortex lightly, allow to stand 10 min., vortex lightly and allow to stand an additional 10 min., in order to permit digestion of the precipitate. While vortexing lightly, add 100 mcl (G) of reagent D 2N Folin-Ciocalteau Reagent (Fisher Labs) mixed with equal parts water to the culture tube. Allow to stand 1 hr. and then determine the absorbance at 750 nm using an appropriate blank of

0.9% sodium chloride taken through the procedure.

Standards must be run with the unknown samples. A 0.01 dilution of the plasma used to obtain the plasma water, is made with 0.9% sodium chloride. This stock dilution is further diluted 0.8, 0.6, 0.4, 0.2, 0.1, 0.05 with the 0.9% sodium chloride. These samples along with the stock dilution then constitute the standards. The resultant calibration curve, an example of which can be seen in Fig.XI, then relates absorbance to a dilution factor of the original plasma sample. The protein determination in the plasma water samples will thus give protein content as a fraction of the original plasma sample.

c) Evaluation of assay:

The assay proved simple and the calibration curve (Fig.XI) exhibited good linearity. The disadvantage of the assay is that the absolute quantity of protein in the plasma water is not determined Yet it does provide information about the relative ratio of protein present in the plasma water to that originally in the plasma after ultracentrifugation, ultrafiltration or equilibrium dialysis. The ability of each of these methods to provide protein-free plasma water can be seen in Table XVII, p. 106.

E. Glucose Assay:

Glucose determinations were kindly performed by the Steroid Lab of the University of California Metabolic Laboratory, San Francisco. The determination was made on a Beckman Glucose Analyzer which employs the enzymatic glucose oxidase method.[93]

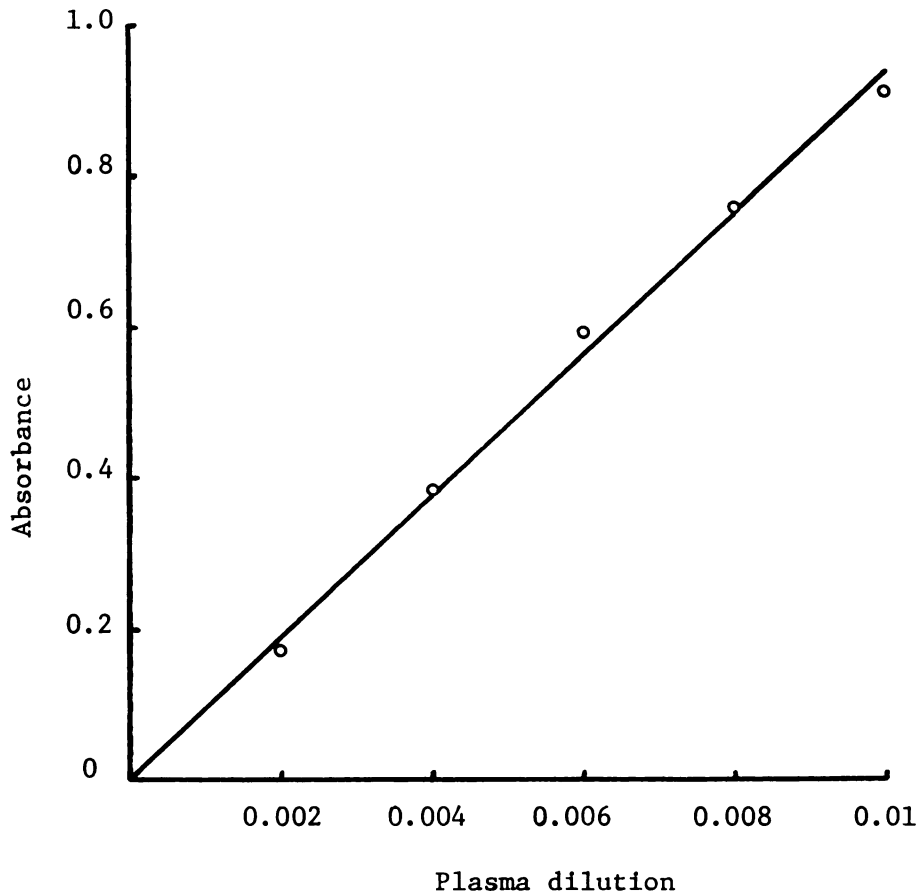


Fig. XI: Plasma water protein calibration curve.

F. Free Fatty Acid Assay:

Free fatty acid determinations were kindly performed by the laboratory personnel of Dr. R.J. Havel,Cardiovascular Research Institute, Moffitt Hospital, UCSF. The method followed was that of Dole[80] which measures non-esterified (free) fatty acid.

G. Urinary Electrolyte Assay:

Urinary sodium, potassium, chloride and osmolality determinations were made by the Clinical Labs, Moffitt Hospital, UCSF.

III BIOLOGICAL

A. Animal Model

Ideally it would be desirable to study all drug-drug interactions in man. Yet this is not always possible or permitted and as a result animals are often used. Since this project centered around the investigation of a concept which would require vigorous testing, it was decided to develop an animal model. Due to the multiple large blood samples required for plasma protein binding studies it was soon realized that an animal not much smaller than man himself, with a large blood volume was a necessity. Consequently it was decided to employ the sheep as the animal model. After assessment of the requirements for the study it was deemed necessary to have arterial and venous catheters as well as a bladder catheter, all chronically implanted to permit ready sampling. All surgeries were performed under sterile conditions.

1. Arterial and Venous Catheters:

The vasecular catheters consisting of 0,09" o.d. tygon flexible tubing (type S-54-HL, Port Plastics Inc.) were inserted into the saphenous vein and femoral artery, or the jugular vein and carotid artery of sheep weighing 40 - 50 Kg. When the saphenous vein and femoral artery of a hind limb were used, the animal was given spinal anesthesia. This involved injecting about 2 ml, 2% lidocaine (Zylocain, Astra) subcutaneously followed by 2.5 ml tetracaine (Pontocaine, Winthrop, 1 mg/ml) spinally. Both catheters were then inserted and passed along the blood vessels so that the end of the venous catheter opened in the inferior-vena cava while the arterial catheter in the abdominal aorta. After securing the catheters at the point of insertion, the other ends were passed under the skin and externalized on the side of the animal. Here

a patch pocket was sewn to the skin of the animal, using velcro stripping, in which the catheters were hidden when not in use.

A similar procedure was used for the jugular and carotid catheters, except that only 2% lidocaine was used subcutaneously at the site of insertion in the neck. The arterial catheter was passed in the direction of the aortic arch while the venous catheter in the direction of the superior vena cava. The other ends of the catheters were then passed under the skin and externalized on the dorsal side of the neck. Here the catheters were merely rolled in a knot and allowed to dangle free.

Points of incision were treated with Neosporin ointment(Winthrop) to promote healing and prevent infection. When necessary to combat infection, the animals were given I.M. injections of Streptillin (Trico Pharmaceutical Co.) or I.V. ampicillin (Polycillin, Bristol). The catheters were normally plugged with pieces of thick sterile wire. Every 3-4 days the catheters were flushed which involved removing the contents of the catheters, injection of sterile normal saline and then filling the catheters with 1000 units/ml heparin (Lipo-Hepin, Riker). All catheters were easily connected to sterile disposable syringes using a 15 gauge luer stub adapter (Clay Adams).

2. Bladder Catheter:

The insertion of a chronic bladder catheter was performed under general anaesthesia using injectable pentobarbital (Nembutal, Abbott) administered via the venous catheter. Entrance was made to the peritoneal cavity by means of a ventral mid-line abdominal incision. A small opening was made in the fundus of the bladder and a foley 20 FR catheter (Bardex, 165V) was inserted and secured using purse-string sutures after distending the 5 ml balloon. The free end of the catheter was then passed through

the peritoneal cavity and externalized at the side of the animal. Here again it was hidden in a pocket like the vascular catheters when not in use. The foley catheter was conveniently plugged with the rubber cap from a 7 ml vacutainer tube. When the bladder catheter was capped the animal was able to perform normal urinary elimination. Occasionally, the bladder was flushed with a solution of 1 ml Neosporin G.U. Irrigant (Burroughs-Wellcome) in 150 ml sterile normal saline to prevent possible infection.

3. General Comments:

The sheep proved to be a hardy animal and a preparation was retained as long as 8 months. Great care had to be taken to prevent infection from setting in at the point of externalization of the catheters. This infection occurred most readily when the vascular catheters were placed in the hind limb, resulting in considerable hind limb inflammation. It was considered more desirable to have the vascular catheters in the neck region for various reasons: 1) less potential for infection; 2) pockets sewn to the skin for hind limb catheters needed to be reattached every 3 weeks due to the growth of wool which caused the sutures to come out; 3) the animals were not able to walk as well with hind limb catheters.

4. Location and Care:

When not used for studies, the sheep were kept in 4' x 6' pens. At all times their diet consisted of alfalfa pellets and water. During studies the sheep were transferred to special mobile cages which restricted their movement and yet allowed them to be in a standing or lying position.

B. Collection of Samples:

Only arterial blood samples were collected using sterile disposable plastic syringes (Monoject).

The blood was then immediately transferred to either of two sterile vacutainer tubes. Blood for glucose assays was placed in 7 ml vacutainer tubes containing 14 mg potassium oxalate and 17.5 mg sodium fluoride. The remaining samples were placed in 10 ml vacutainer tubes containing 143 units sodium heparin. All blood samples were then centrifuged at 2000 rpm as soon as possible for 10-20 min, whereupon the plasma was removed (D), placed in appropriate screwcap containers and frozen until required for analysis.

All urine samples were collected in an appropriate container, their volumes determined, an aliquot transferred to plastic scintillation vials and then frozen until required for analysis.

C. Administration of Compounds:

1) Bolus:

Since all compounds used were acids, practically insoluble in water, they were administered as the corresponding sodium salt. Outside of tolbutamide (Orinase Diagnostic, Upjohn) which was supplied as the sodium salt requiring the mere addition of water, all compounds were dissolved in a minimum of 1-10N NaOH and then diluted with water. Solutions were made in volumetric flasks, aliquots (P) transferred to syringes and then administered I.V. through a Millipore filter (0.45μ, 13mm, Millipore Corp.) followed by 10-20 ml normal saline. All experiments were timed (minute timer, Precision Scientific) beginning at the point when one-half of the solution had been administered.

2. Infusion:

All compounds given by means of infusion were prepared as indicated in section 1). Solutions were millipore filtered into a 50-60 ml sterile plastic disposable syringe whereafter they were administered I.V. using a defined rate with an infusion pump (Harvard Apparatus Compact Infusion Pump). All experiments were timed from the first administration of compound.

IV MATERIALS AND EQUIPMENT

A brief list has been made in Table II and III of major materials and equipment used in this study together with their source.

Table II: Source of materials

Material	Source
Ampicillin	Polycillin, Bristol
Aquasol	New England Nuclear (NEN)
Carboxytolbutamide	Dr. S. Matin, UCSF
Chlorpropamide	A gift, Pfizer
Diazald	Aldrich
2, 4-Dinitrofluorobenzene	Pierce Chem. Co.
Foley catheters	Bardex 165 V, 20FR, 5cc Balloon
Folin-Ciocalteau Phenol Reagent	Fisher Labs
Hydroxytolbutamide	Farbwerke Hoechst (a gift)
^{3}H - Hydroxytolbutamide	Dr. S. Matin, UCSF
2% Lidocaine	Xylocaine, Astra
Neosporin	Burroughs-Wellcome
3% OV-17 column packing	Applied Sci.
Sulfadimethoxine	Hoffman La Roche (a gift)
Sulfadimethoxine - 4-^{14}C	Hoffman La Roche (a gift)
Tetracaine	Pontocaine, Winthrop
Tolbutamide	Orinase Diagnostic, Upjohn
Tolbutamide - Ureyl -^{14}C	Farbwerke Hoechst (a gift)
^{3}H - Tolbutamide	Dr. S. Matin, UCSF
^{14}C- or ^{3}H-Toluene	International Chemical and Nuclear Corp.
Ultracentrifuge Tubes	Polyallomar, Beckman #328874
Ultrafiltration CF50A cones & apparatus	Amicon
Vascular catheters	Port Plastics Inc., Type S-54-HL
Visking dialysis tubing	Van Waters & Rogers (VWR)

Table III: Type and/or source of equipment

Equipment	Source/Type
Balance	Mettler, H-10-T
Constant temperature waterbath control	Precision Scientific
Culture tube centrifuge	International, HN-S
Dianorm	Inno Med., Zurich, Switzerland
Electrobalance	Cahn Gram Electrobalance
Gas Chromatograph	Varian, Model 1200
Infusion pump	Harvard, compact infusion pump
Kimex culture tubes	Scientific Products
Mass Spectrometer	AEI M.S.-902
Microfuge	Beckman Spinco Microfuge
pH meter	Beckman Expandomatic
Scintillation counter	Packard Tri-Carb
Self-cleaning oven	Sears, Kenmore #42320
Spectrophotometer	Cary-15
Timer	Precision Scientific
Ultracentrifuge	Beckman, L2-65B with SW-56 head
Ultrafiltration centrifuge	International, Model UV
Ultrasonic cleaner	Aerograph
Vortex	Clay-Adams, Cyclo Mixer

CHAPTER III

RESULTS AND DISCUSSION

The Appendix found at the back of the thesis contains experimental data values used in the Figures as well as an explanation of designated abbreviations found in this Chapter.

I. INTRAVENOUS TOLBUTAMIDE BOLUS

All tolbutamide given to the sheep was administered intravenously. Unlike the oral route, the intravenous mode guarantees an accurate assessment of the dose reaching the vascular compartment. A three-fold purpose existed in intravenous (I.V.) bolus administrations. Firstly, the bolus was used to determine the metabolic fate of tolbutamide. Secondly, the bolus served to characterize the disposition kinetics of tolbutamide in the sheep. Finally, the bolus permitted an appraisal of the pharmacologic response by the sheep to a pulsed dose of tolbutamide. This delineation will be used to provide the results and discussion of this section.

A. Metabolic Fate of Tolbutamide in the Sheep:

After the administration of 100 mg tolbutamide containing 2.89 x 10^7 dpm ^{3}H-tolbutamide to sheep #368 and collection of urine for 9 hours, 1.96 x 10^7 dpm radioactivity was excreted in 430 ml urine. The radioactivity excreted over this period represented 67.8% of the administered dose. Using the method of Matin[76] the urinewas separated into fractions representing tolbutamide, hydroxytolbutamide and carboxytolbutamide, and radioactivity in each fraction was determined. This was achieved by adjusting the urine to pH 1, extracting with 0.5% isomylalcohol in dichloromethane and then separating the organic phase with appropriate buffers. See Table IV for the results.

Table IV Pattern of urinary radioactivity following I.V. 100 mg bolus of tolbutamide (^{3}H - tolbutamide, 2.89 x 10^{7} dpm) to sheep #368

Fraction	% dpm*1
Aqueous after extraction	12.6
Organic	86.5
Total*2	99.1
Carboxytolbutamide	7.7
Hydroxytolbutamide	73.5
Tolbutamide	1.8
Total organic	83.0

*1 all values represent % of total urinary dpm

*2 represents 67.8% of the administered radioactivity

Thin layer chromatography of an ether extract of acidified urine from the same sheep given the ^{3}H-tolbutamide provided additional information as seen in Figure XII.

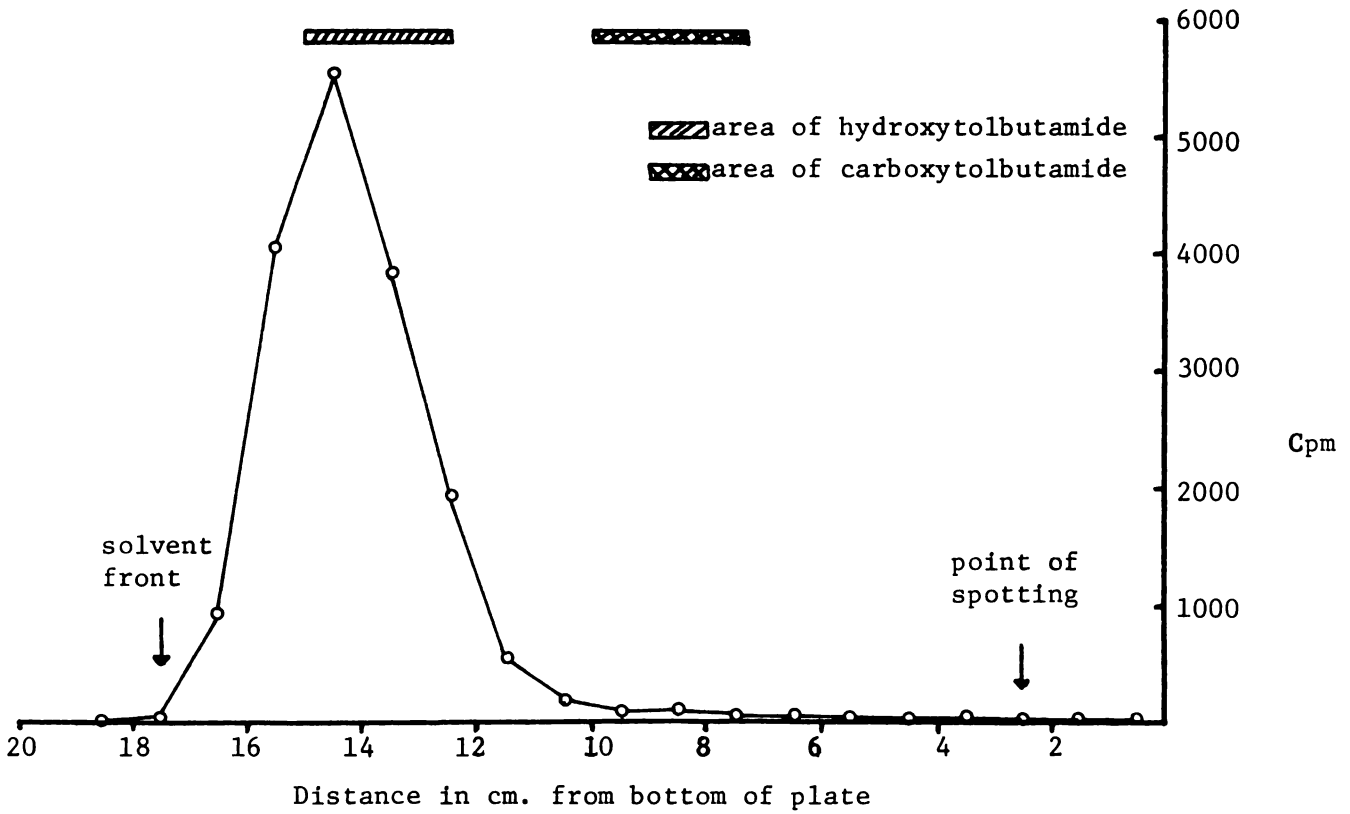


Fig. XII: TLC of ether fraction of acidified urine collected for 9 hrs following an I.V. bolus of 100 mg tolbutamide (^{3}H-tolbutamide, 2.89×10^{7} dpm)

Mass spectral information although not quantitative, yet provided qualitative information about the contents of either a chloroform or ether extract of 1 to 2 hr. acidified urine obtained from sheep #1494 after given a 0.9 Gm I.V. dose of tolbutamide. Only the residue after ether extraction was methylated with diazomethane. Table V summarizes those mass to charge ratios (m/e) related to tolbutamide or its potential metabolites as a result of oxidation. Only those peaks whose heights were double the baseline were noted.

Table V Mass spectral data of extracted acidified urine obtained from sheep #1494 which received 0,9 Gm tolbutamide I.V.

m/e	Extracting Solvent	Methylated	Potential Compound*
271	chloroform	no	tolbutamide
285	ether	yes	methylated tolbutamide
287	chloroform	no	hydroxytolbutamide
301	ether	yes	methylated hydroxy-tolbutamide
315	ether	yes	methylated carboxy-tolbutamide
329	ether	yes	dimethylated carboxy-tolbutamide

* m/e corresponds to the protonated compound

Note: The urine used was that collected in the 1 to 2 hour interval after the I.V. dose of tolbutamide.

In addition, the regular urinary assay for tolbutamide, and "hydroxytolbutamide equivalents" (p. 51) was performed after the I.V. administration of tolbutamide. These results are summarized in Table VI.

Table VI: Urinary data after I.V. tolbutamide bolus in sheep #1494

Experiment	Date	Dose (mg)	Urine Collection (hr)	$t_{\frac{1}{2}}$ (hr)*1	Tolbutamide (mg)	Hydroxy-Tolbutamide*4 Equivalents (mg)	Total (mg)	% Dose	% Dose (Corrected)*2
II	2/21/73	865	30	3	119	567	686	79.4	79.6
IV	6/6/73	860	28.8	4	128	465	593	69.0	66.0
III	4/26/73	900	10.5	10.5	59	271	330	73.4*3	70.0*3

*1 half-life of terminal linear phase of log rate of excretion versus mid-point time plot

*2 hydroxytolbutamide equivalent converted to tolbutamide equivalents

*3 based upon estimate that 1/2 the dose would be excreted after one half-life

*4 see p. 51.

Discussion:

Administration of tolbutamide to most species results in the oxidative metabolism to hydroxy- and/or carboxytolbutamide as was indicated in Chapter I. In many cases the quantitative aspect is missing. A previous report of the metabolism of tolbutamide in sheep has not been found in the literature. A knowledge of the fate of tolbutamide aids in a more complete understanding of the tolbutamide-sulfadimethoxine interaction. The radioactive work suggests the major metabolite to be hydroxytolbutamide with small amounts of carboxytolbutamide. Yet it should be noted that p-hydroxymethylbenzenesulfonamide was found to possess the same Rf value as hydroxytolbutamide in the TLC solvent system used. The sulfonamide could therefore be present. A considerable mass spectral peak of m/e = 188 corresponding to the protonated sulfaonamide is in evidence. However, a number of major peaks exist at m/e values less than 200 in the urine of sheep not receiving tolbutamide making it questionable whether the amide is present. The same may be said for protonated p-methylbenzenesulfonamide, m/e = 172. With regards to the mass spectral data, an m/e ratio corresponding to carboxytolbutamide was not found in the chloroform extract while it did appear when the urinary extract was methylated. This illustrates the difference in volatility of the metabolites and points out the necessity of forming volatile derivatives.

A major contribution of the two sulfonamides mentioned can be refuted by means of the urinary colorimetric assay. The assay, which requires the intact alkylurea portion of the sulfonylurea molecule for detection purposes, thus confirms the major metabolite to be either carboxy- or hydroxytolbutamide. However, the TLC experiment indicates

that it must by hydroxytolbutamide. Due to the small amount of carboxytolbutamide present which must have resulted from the oxidative metabolism of a minor portion of hydroxytolbutamide, no attempt was made to assay for carboxytolbutamide specifically. Rather the urinary assay was designed to measure total oxidized products (hydroxy- and carboxytolbutamide) representative of the p-methyloxidation as a route of tolbutamide degradation.

The absence of kinetic information and the short collection time (9 hrs) precludes a statement as to the extent of excretion of unchanged drug and metabolism to hydroxy- and carboxytolbutamide from the preliminary experiment with radioactive tolbutamide. The longer collection time and kinetic information with unlabelled drug (Table VI) permits a more definitive statement. It appears that of the recoverable tolbutamide (about 75%) approximately 20% exists as unchanged drug and 80% as hydroxytolbutamide equivalents with the majority (72%) as hydroxytolbutamide itself.

Since only about 75% of a tolbutamide dose can be accounted for, one might speculate about the remainder. The results in Table IV indicate that a larger % of the dose exists in the combined metabolite fraction than can be assayed for as hydroxytolbutamide equivalents as seen in Table VI. This difference may be due to the presence of sulfonamides as mentioned before or even dealkylated sulfonylureas. A word of caution should be given though, since different animals were used (c.f. Tables IV and VI). In addition, an unextractable portion of the dose exists in the aqueous phase and this also remains unidentified.

Conclusion:

About 75% of an I.V. administered dose of tolbutamide in the sheep can be accounted for in terms of parent compound (13%) and metabolites

(62%). Hydroxytolbutamide represents about 90% of the accountable metabolites.

B. Kinetics of Tolbutamide in the Sheep:

Administration of I.V. boluses of tolbutamide to the sheep resulted in plasma concentration-time curves as seen in Figure XIII. It should be noted that Experiment I represents a bolus in sheep #368 while Experiments II - V are from sheep #1494. All curves were fitted by eye. The data may be described by either a 1 or 2 compartment model as illustrated in Figure XIV. Using conventional methods as described by Portmann[85] the data were analysed for kinetic parameters, noting that residuals were obtained after eye fitting. The parameters for each set of data appear in Table VII. Plasma analysis for the major metabolite, hydroxytolbutamide was also carried out in Experiment II and the results may be seen in Figure XV. The curves were again obtained by eye fitting of the data. In Figure XVI is found the rate of urinary excretion of parent tolbutamide again corresponding to Experiment II. Rates of excretion are plotted against the mid-point of the urinary collection interval and the lines are drawn visually.

Discussion:

As can be seen from Figure XIII, I.V. administration of tolbutamide results in a very rapid distribution of the compound into its apparent volume of distribution as evidenced by the short or even insignificant α phase (fast distribution phase). The apparent volume of distribution in the sheep represents a value which is close to the volume of the extracellular space (25% of 50 Kg sheep is 12500 ml). This is not to indicate though that the apparent volume of distribution is in fact the extracellular space. Most of the β phases (slow elimination

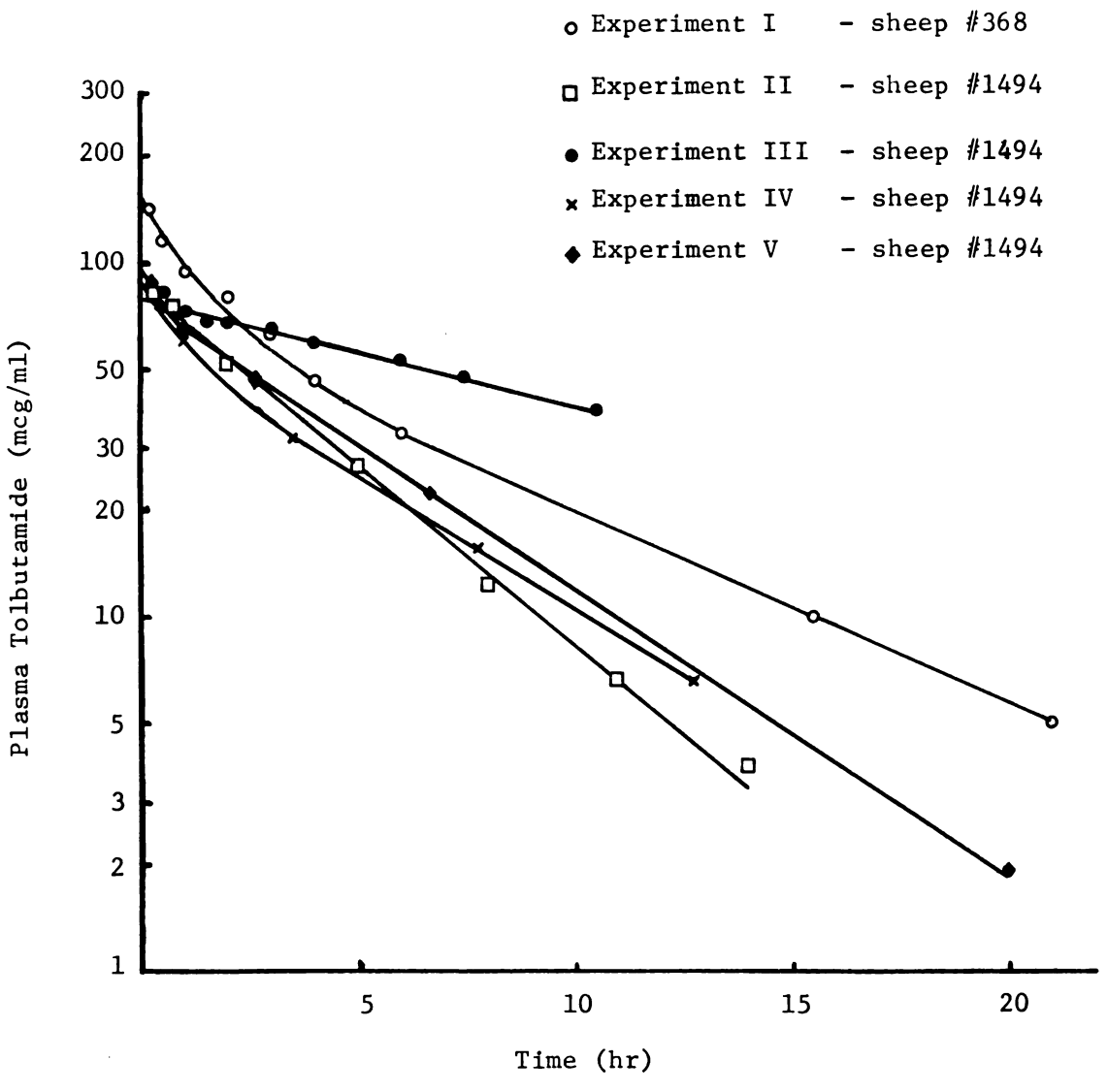
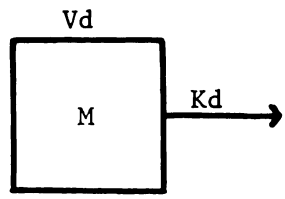


Fig. XIII: Semi-logarithmic plot of tolbutamide plasma concentrations with time following an I.V. bolus of tolbutamide.

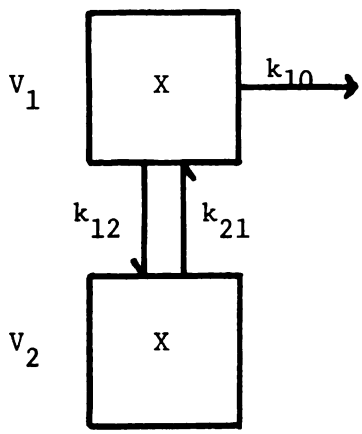
1 Compartment



Vd = volume of compartment

Kd = first order rate constant describing the loss of mass (M) from the compartment.

2 Compartment



V_1 = volume of central compartment

V_2 = volume of periferal compartment

$Vd_{ss} = V_1 + V_2$

k_{12}, k_{21} = first order rate constants describing the transfer of mass (X) between the two compartments.

k_{10} = first order rate constant describing the loss of mass (X) from the central compartment.

Fig. XIV: Compartmental models

Table VII: Kinetic data of I.V. tolbutamide bolus

Experiment	Sheep	Date	Dose (mg)	Model	A (mcg/ml)	α (hr^{-1})	B (mcg/ml)*1	β (hr^{-1})	k_{12} (hr^{-1})	k_{21} (hr^{-1})
I	368	2/18/72	900	2	70	0.693	72	0.126	0.194	0.413
II	1494	2/21/73	865	1	--	--	85	--	--	--
III	1494	4/26/73	900	1*2	--	--	77	--	--	--
IV	1494	6/6 /73	860	2	41.5	1.5	58.5	0.173	0.313	0.745
V	1494	6/10/73	900	2	24	1.54	74	0.183	0.283	1.21

$t_{\frac{1}{2}}$(hr)*3	k_{10} (hr^{-1})	V_1 (ml)	V_2 (ml)	Vd (ml)*5	CL (ml/min)*4
5.5	0.211	6338	2977	9315	22.5
3.0	0.231	--	--	10200	39.1
10.6	0.065	--	--	11700	12.7
4.0	0.267	8600	3612	12212	38.3
3.8	0.230	9184	2153	11337	35.2

*1 represents Cp^o in 1 compartment model

*2 had a very small α phase which was neglected

*3 half-life of terminal log-linear phase

*4 plasma clearance

*5 $Vd = Vd_{ss}$ i.e. $V_1 + V_2$

Note: see Appendix for definition of symbols

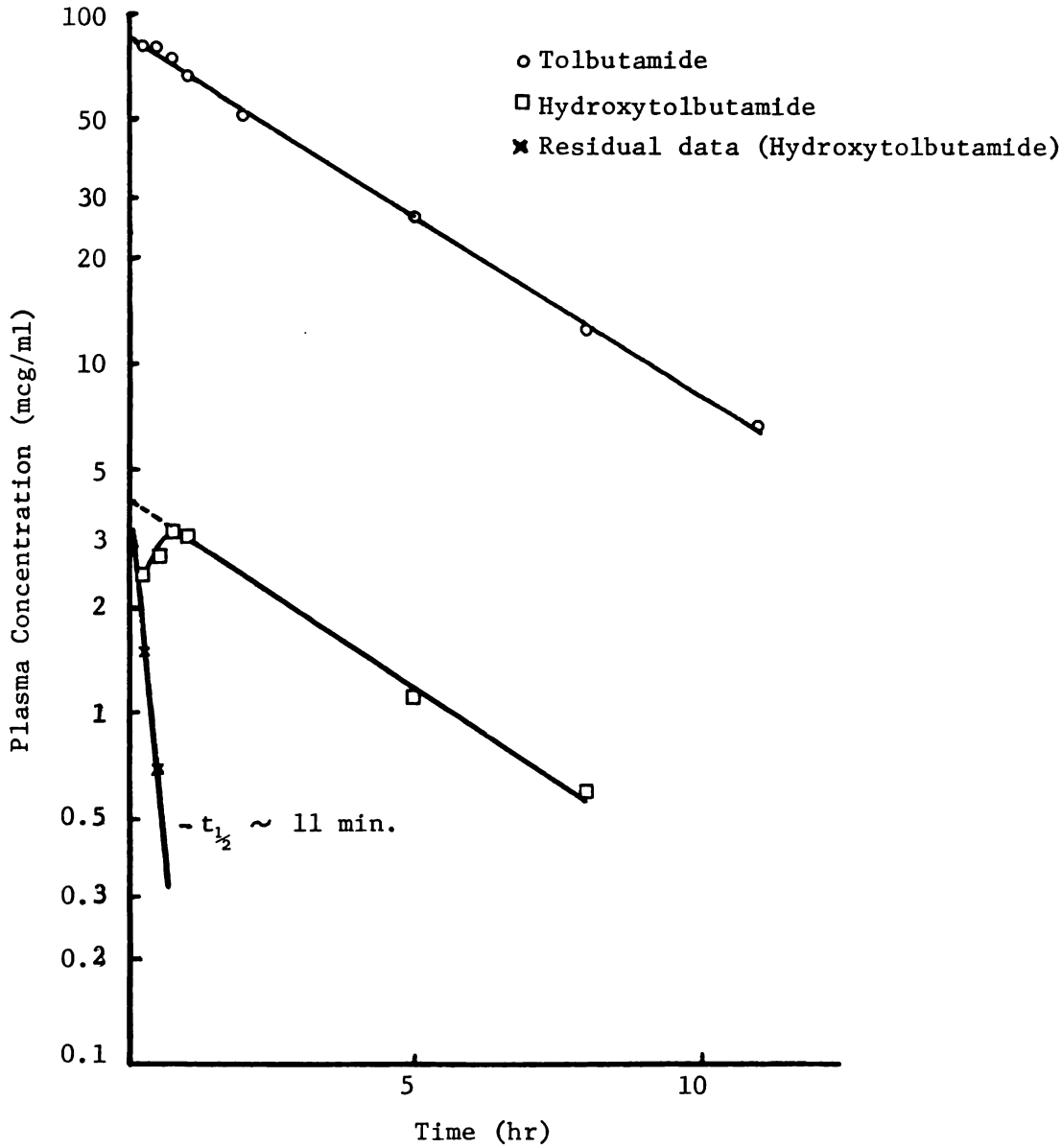


Fig. XV: Plasma hydroxytolbutamide after 865 mg. I.V. tolbutamide bolus (Experiment II - Sheep #1494)

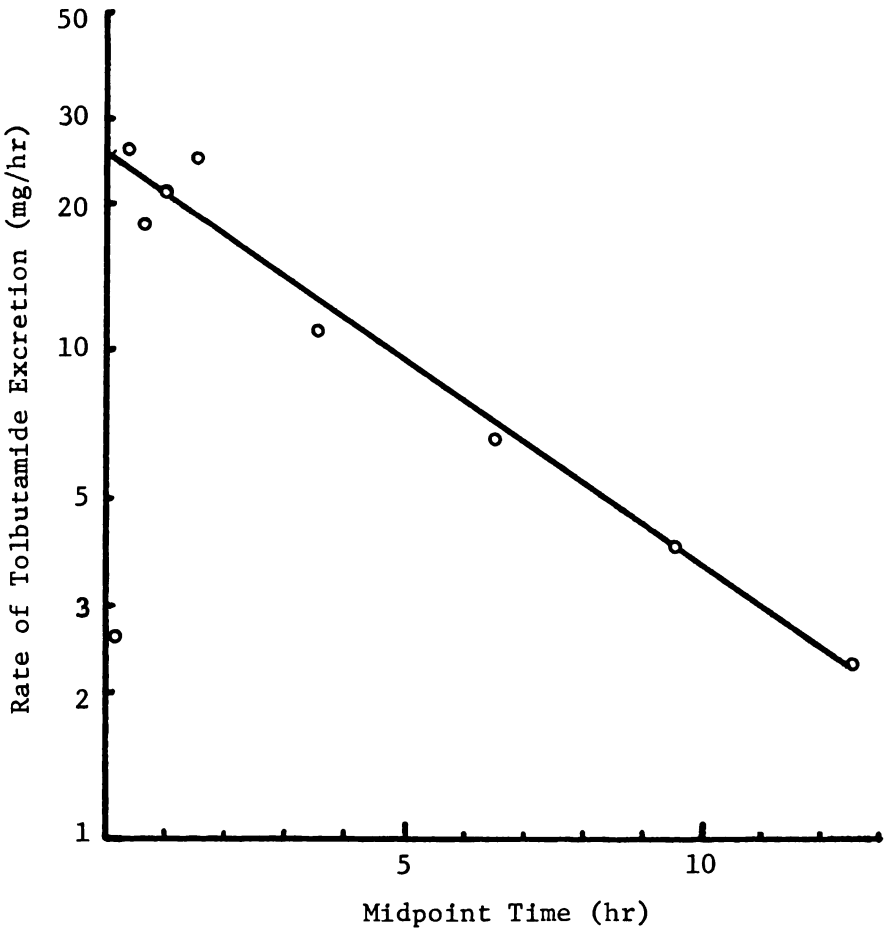


Fig. XVI: Rate of urinary tolbutamide excretion after 865 mg. I.V. tolbutamide bolus (Experiment II - Sheep #1494)

phase) in Figure XIII have about the same half-life. The exception is Experiment III which is considerably different although it should be noted that this particular experiment was followed only for about 11 hrs. while the other experiments were followed for 13 to 21 hrs. It was noted at the time of Experiment III that the sheep had developed an infection in the hind limb in which the vascular catheters were inserted. The infection was visible due to the extensive swelling and wool loss. It is suspected that the infection may have been responsible for the change in kinetics. Although a culture was not taken of the infected leg, plasma electrophoresis was performed subsequently on plasma obtained during (Experiment III) and after (Experiment V) the infection. These values along with standard values appear in Table VIII. As can be seen, no differences exist to which the change in half-life might be attributable. As the Vd is comparable before and during the infection (Table VII), the data suggests that the clearance is diminished during the infection. Since most of the tolbutamide is excreted in a degraded form, one might speculate that the infection caused a change in activity of the metabolic site resulting in a decreased metabolic rate of tolbutamide. After removal of the catheters and treatment with I.V. ampicillin (Polycillin: Bristol) and Topical Neosporin (Burroughs and Wellcome) the infection cleared up. Subsequent tolbutamide administration resulted in a return to preinfection kinetics (Experiments IV and V). This points out a potential problem when using a chronic animal preparation to investigate the kinetics of compounds. Not only may there be considerable inter-animal variations, but intra-animal variation may be due to a state of health in the animal.

The plasma hydroxytolbutamide concentration data in Figure XV suggest that its disappearance rate from plasma is rate limited by its rate of formation from tolbutamide. The half-lives of plasma tolbutamide

Table VIII: Plasma electrophoresis; Sheep - #1494

Plasma	Tolbutamide $t_{\frac{1}{2}}$ *1	Component	Conc. (Gm %)
Experiment III (4/26/73)	10.6	Total Protein	9.2
		Albumin	2.3
		Alpha 1	0.4
		Alpha 2	1.1
		Beta	3.7
		Gamma	1.7
Experiment V (6/10/73)	3.8	Total Protein	9.2
		Albumin	2.7
		Alpha 1	0.5
		Alpha 2	0.9
		Beta	2.6
		Gamma	2.5
Normal	-	Total Protein	7.1-8.1
		Albumin	3.1-3.7
		Alpha 1	0.8-0.9
		Alpha 2	-
		Beta	1.0-1.1
		Gamma	1.9-2.4

*1 half-life of terminal log-linear phase

*2 Kaneko, J.J.[86]

and hydroxytolbutamide disappearance appeared to be identical ($t_{\frac{1}{2}}$ = 3 hr). Back extrapolation of the terminal linear phase of hydroxytolbutamide concentration to time zero and calculation of residuals resulted in a phase with half-life of about 11 min. This suggested that the half-life for elimination of hydroxytolbutamide was about 11 min. Evidence to support this will be shown later to indicate that the rate of

elimination of hydroxytolbutamide is very rapid after an I.V. bolus of the metabolite. A question may be raised as to the low concentration of hydroxytolbutamide in plasma relative to that of tolbutamide. Explanation for this might lie in 1) a large volume of distribution of hydroxytolbutamide, 2) only small amounts of it are formed, or 3) it is very rapidly eliminated. Further reference to this question will be made in the section on an I.V. bolus of hydroxytolbutamide.

Regression analysis of plasma tolbutamide concentration (Fig. XV) on the rate of urinary tolbutamide excretion (Fig. XVI) can be seen in Figure XVII. The plasma concentrations represent those at the midpoint of the excretion interval. The correlation coefficient, regression coefficient and intercept with standard errors of estimate are 0.94 (0.14), 0.29 (0.05) and 1.28 (2.40) respectively. It is evident that the rate of unchanged tolbutamide excreted in the urine is proportional to the tolbutamide plasma concentration (proportionality constant is renal clearance) and that the 95% confidence interval for the intercept would contain Y = 0 and X = 0.

A further note might be made about the plasma clearance of tolbutamide as seen in Table VII. The range is seen to be 12.7 to 39.1 ml/min. Assuming a negative extrapolation from man (70 Kg) to sheep (50 Kg) then the cardiac output (8.6% of body weight per min[94]) in the sheep would be 4300 ml/min. The corresponding plasma output would be 2700 ml/min for a hematocrit of 0.38[86]. As can be seen then, plasma clearance of tolbutamide is only a fraction of the total plasma circulated per minute. Even if one were to consider the liver as the sole clearing organ (700 ml/min) the observed clearance still represents only a fraction of the flow. Once again, there is room for speculation. The plasma clearance of tolbutamide might be related to its unbound concentration.

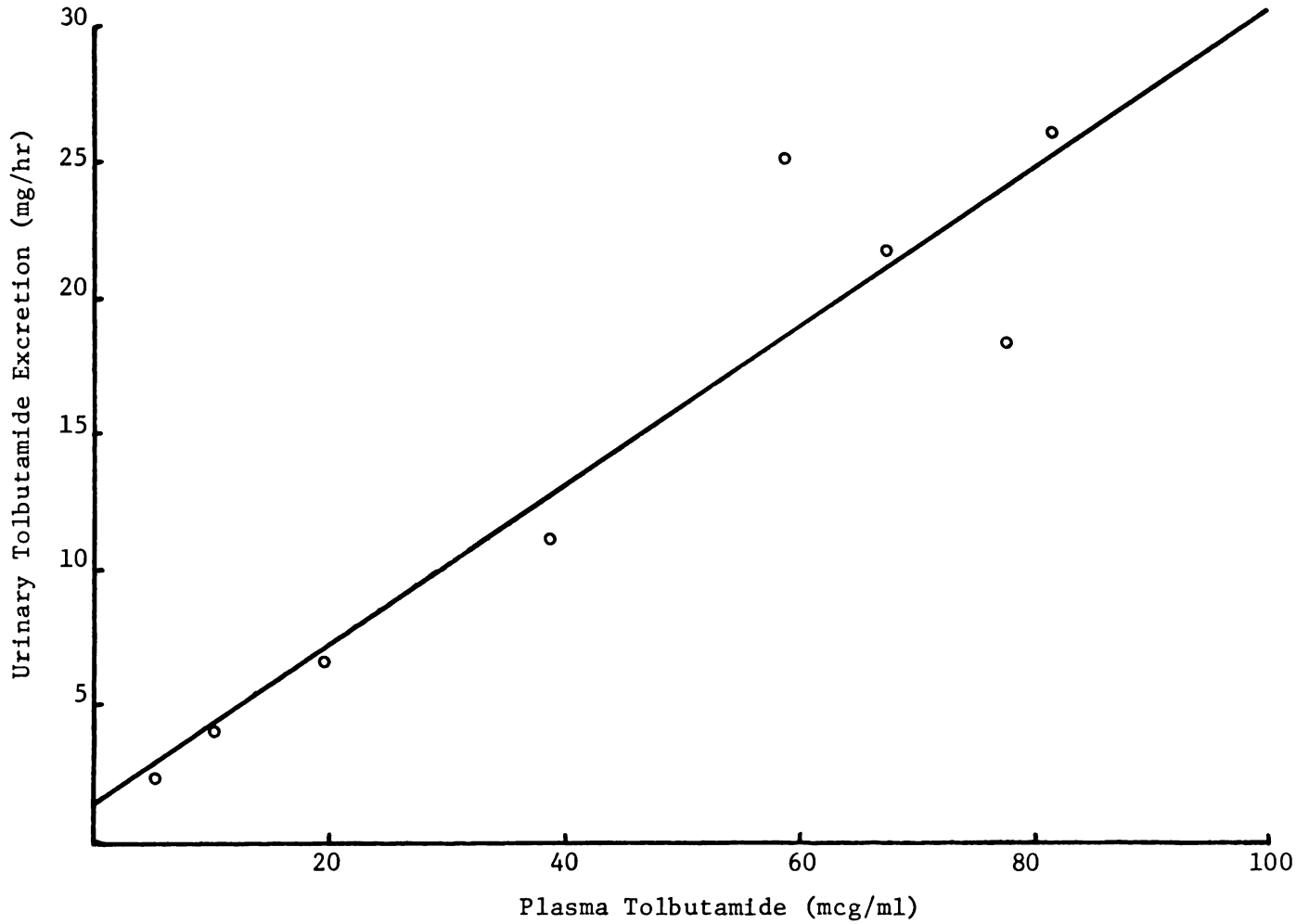


Fig. XVII: Regression of rate of urinary excretion of unchanged tolbutamide on the plasma tolbutamide concentration (Experiment II - Sheep #1494 - plasma values by linear interpolation to the midpoint of the excretion interval - standard error of slope and intercept is 0.045 and 2.40 respectively)

Conclusion:

The kinetics of an I.V. bolus of tolbutamide in the sheep may be described by a one compartment model, or a two compartment model with a rapid (α= 0.693 to 1.54 hr^{-1}) distribution phase. The normal terminal log-linear half-life in plasma was found to have a range of 3 to 5.5 hr. The apparent volume of distribution (liters) had a range of 18.5 to 24.5% of body weight given a mean body weight of 50 Kg. The total body plasma clearance of tolbutamide had a range of 12.7 to 39.1 ml/min.

C Pharmacologic Response to Tolbutamide:

In order to assess the potential hypoglycemic effect of tolbutamide, blood was collected in appropriate vacutainer tubes and the plasma analyzed for glucose. Figure XVIII, Experiment III, represents the results obtained after a 900 mg bolus of tolbutamide I.V. in sheep #1494.

Discussion:

It is quite evident that tolbutamide produces a hypoglycemic state in sheep. It cannot be stated by what means this effect is produced, although it is probably attributable to an increase in insulin release from the β - cells of the pancreas as discussed in Chapter I. A more appropriate pharmacologic response index might then have been circulating insulin levels. A question exists why the plasma glucose returns to a normal value about 2 hrs after the administration of the tolbutamide while the plasma level of tolbutamide has only changed fractionally during this interval. One explanation commonly given is that compensatory forces in the body mediated by the adrenal glands attempt to nullify the state of hypoglycemia[95]. This is possibly the case. Alternately, tolbutamide may only release a quickly depleted labile pool of insulin thus causing the transitory effect. Yet

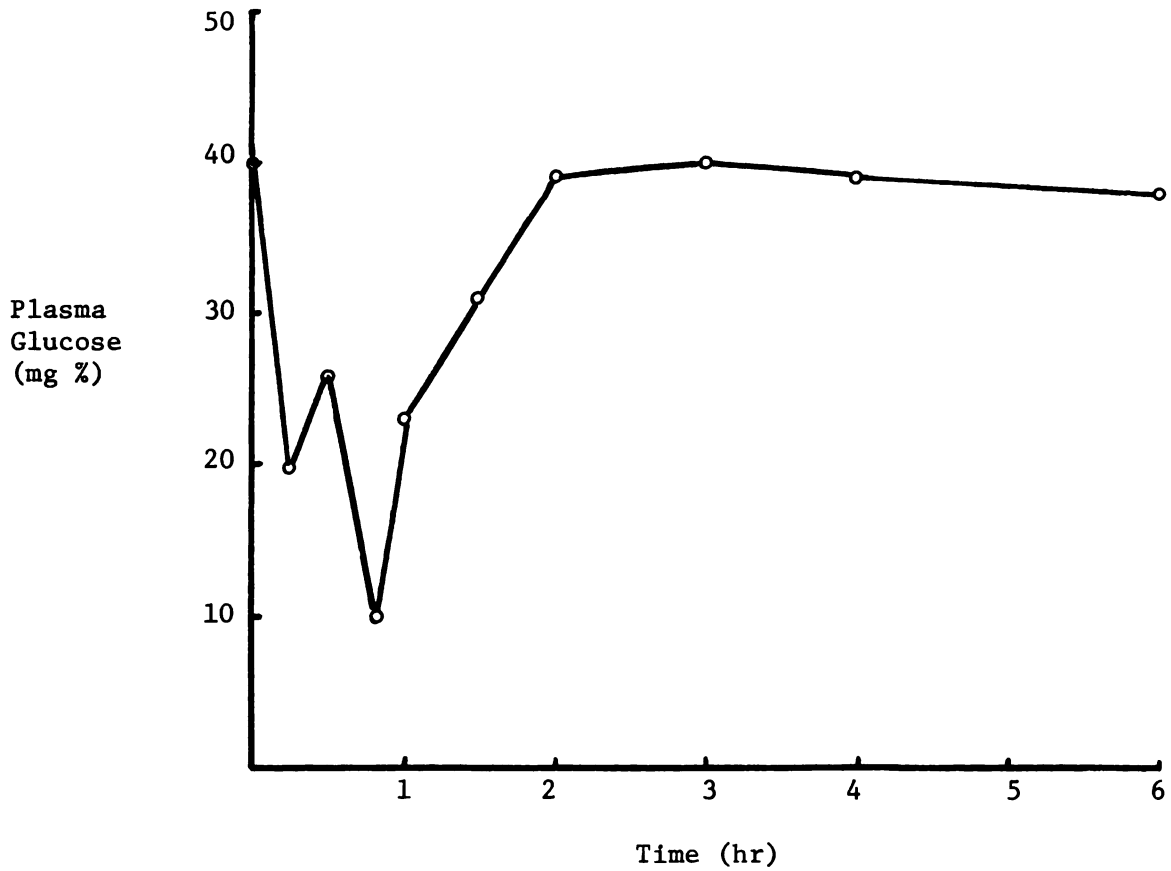


Fig. XVIII: Plasma glucose after 900 mg I.V. tolbutamide bolus given over 30 seconds to sheep #1494 (Experiment III)

another possibility exists. If the release of insulin is dependent upon the concentration of unbound tolbutamide reaching the pancreas then the release would be greatest in the first minute or two after administration when most of the dose is present in the vascular system. Suppose tolbutamide's initial apparent volume of distribution is 6 to 12L (V_1 or Vd, Table VII) and the plasma cardiac output is 2700 ml/min. If the drug is administered in 30 seconds, then the initial concentration in the plasma could be in the order of 5 times that found after distribution which might result in a higher unbound fraction and a higher unbound concentration reaching the pancreas. Yet, greater initial total tolbutamide plasma concentrations which are also seen according to the hypothesis could explain the greater initial hypoglycemia resulting from increased circulating insulin. This thus remains mere speculation. Preliminary work suggests that the release of insulin in an isolated perfused rat pancreas depends upon the unbound tolbutamide concentration[66].

Conclusion:

An I.V. bolus of tolbutamide in the sheep causes a pronounced hypoglycemia whereafter recovery to baseline values takes place in 2 hrs.

II INTRAVENOUS HYDROXYTOLBUTAMIDE BOLUS

Hydroxytolbutamide was administered I.V. to determine its disposition kinetics and to evaluate its hypoglycemic activity.

A. Kinetics of Hydroxytolbutamide:

Administration of I.V. boluses of hydroxytolbutamide resulted in plasma concentration-time curves as shown in Figure XIX. The two curves represent a dose of 250 mg. in different sheep. Again, all curves were fitted by eye and their biexponential character suggested that they could be described by a 2 compartment model (Figure XIV). Using the conventional methods as was done with tolbutamide, the data was analyzed for its kinetic parameters with the results tabulated in Table IX. The urine for each experiment was collected and analyzed. Urine from Experiment VI, collected for 20 hrs was analyzed for both parent hydroxytolbutamide and carboxytolbutamide. Urine from Experiment VII, collected for 3 hrs was analyzed merely for total metabolite. The results appear in Table X.

Discussion:

As can be seen in the two experiments, hydroxytolbutamide is very rapidly eliminated from the body having a terminal log-linear half-life about 1/12 of that for tolbutamide. This confirms the preliminary estimate (Figure XV), that the overall elimination kinetics of hydroxytolbutamide, given a dose of tolbutamide, is rate-limited by its formation. Virtually all of the dose could be accounted for in the two experiments and the results further support the suggestion that carboxytolbutamide represents a minor metabolic product. The fate of hydroxytolbutamide after its I.V. administration is not necessarily the same as that after formation at a metabolic site. I.V. administered

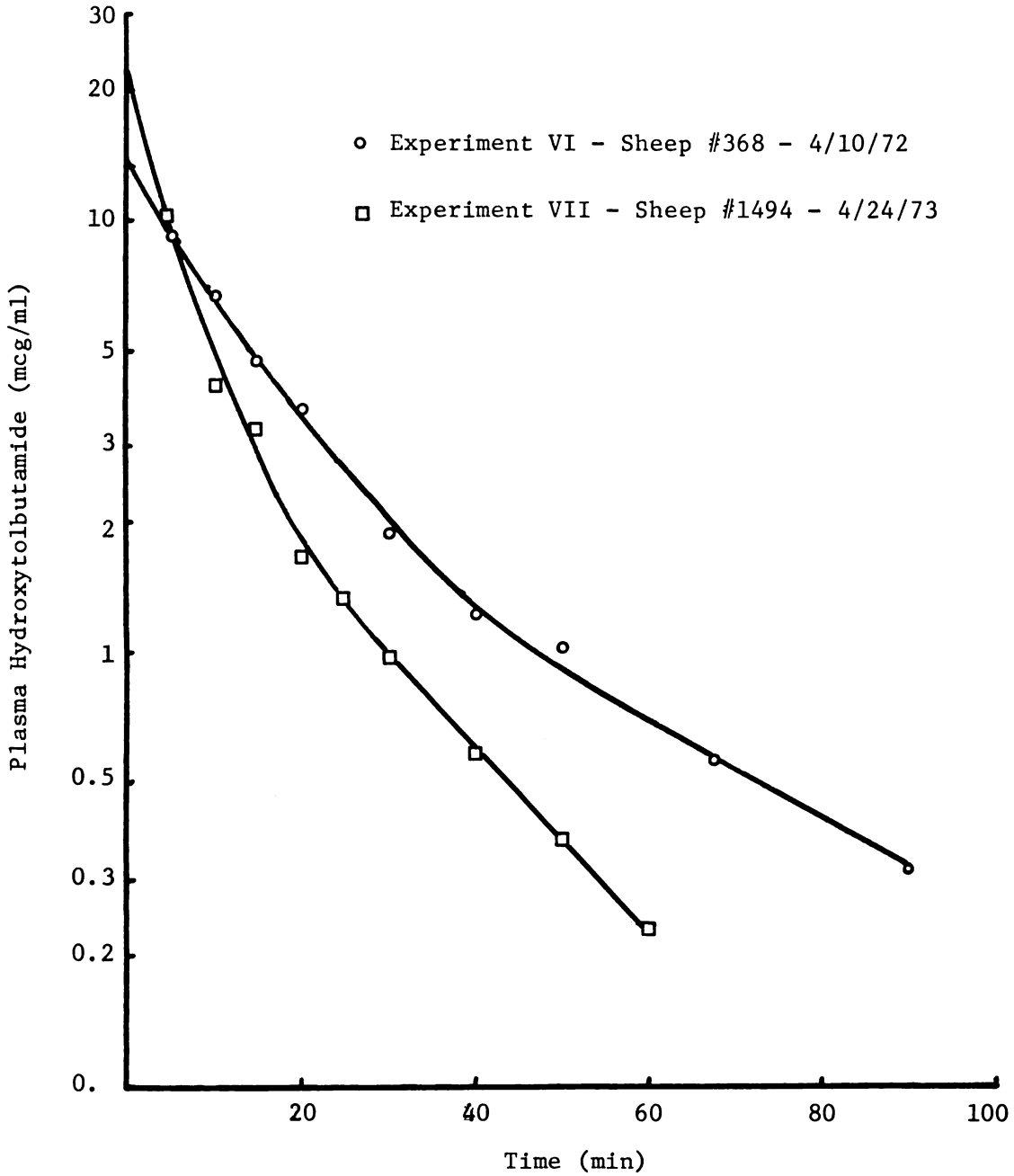


Fig. XIX: Plasma hydroxytolbutamide - time plots following an I.V. bolus of 250 mg hydroxytolbutamide.

Table IX: Kinetic data of I.V. hydroxytolbutamide bolus

Experiment	Sheep	Date	Dose (mg)	Model	A (mcg/ml)	α (min^{-1})	B (mcg/ml)	β (min^{-1})	$t_{\frac{1}{2}\beta}$ (min)	k_{12} (min^{-1})	k_{21} (min^{-1})
VI	#368	4/10/72	250	2	10	0.043	3.5	0.027	26	0.019	0.044
VII	#1494	4/24/73	250	2	22	0.231	4.6	0.051	13.5	0.056	0.082

k_{10} (min^{-1})	V_1 (ml)	V_2 (ml)	Vd (ml)*1	Cl (ml/min)*2
0.056	18520	8000	26520	1490
0.144	9400	6420	15820	1350

*1 $Vd = Vd_{ss}$, i.e. $V_1 + V_2$

*2 Plasma clearance

Note: See Appendix for definition of symbols

Table X: Urinary analysis after 250 mg. I.V. hydroxytolbutamide bolus

Experiment	Sheep	Urine Collection (hr)	Dose (mg)	Carboxy-tolbutamide (mg)	Hydroxytolbutamide (mg)	Total Metabolite (mg)	% Recovery*2
VI	#368	20	250	20.7	229	249.7	99.9
VII	#1494	3	250	--	--	229.5*1	91.8

*1 hydroxytolbutamide equivalents (see p. 51)

*2 $\frac{\text{mg total metabolite}}{\text{dose}} \times 100$

Table XI: Hydroxytolbutamide area analysis in sheep #1494

Experiment	Date	Compound	Dose (mg)	Area (mcg/ml x hr)	%*3,4 Dose
VII	4/24/73	Hydroxytolbutamide	250	3.09*1	-
II	2/21/73	Tolbutamide	865	15.60*2	138
III	4/26/73	Tolbutamide	900	11.02*2	93.5

*1 $A/\alpha + B/\beta$

*2 trapezoid rule

*3 % resulting in hydroxytolbutamide formation

*4 calculated: $\frac{\frac{(\text{Area T}) \times 250 \times 270}{(\text{Area H}) \quad 286}}{\text{Dose T}} \times 100$

Area T = area hydroxytolbutamide generated by tolbutamide

Area H = area hydroxytolbutamide after its administration

Dose T = dose tolbutamide

*5 mol. wt. tolbutamide/mol. wt. hydroxytolbutamide

hydroxytolbutamide may never reach the metabolic site required for conversion to carboxytolbutamide due to its own rapid elimination and partition characteristics.

Use can be made of the area under the plasma concentration time curve after tolbutamide and hydroxytolbutamide administration to suggest the fraction of a tolbutamide dose being converted to hydroxytolbutamide. The results in Table XI do not appear to be meaningful when looking at the % Dose column. However, it should be pointed out that Experiment VII was generated two days before Experiment III while Experiment II was obtained two months before either of these. Experiments III and VII were also obtained at a time when the sheep had the infected hind limb. Thus Experiment III and VII may be very meaningful together, suggesting that most of the dose is metabolized to hydroxytolbutamide. Together with Table VI, the data suggest that a small fraction of a dose of tolbutamide is excreted unchanged while the balance is metabolized to hydroxytolbutamide. An unaccounted for discrepancy exists here since only about 62% of a tolbutamide dose (Table VI) can be accounted for in terms of hydroxytolbutamide equivalents. Possibly an error exists in the area estimate after an I.V. hydroxytolbutamide bolus or unexpected properties are exhibited after its I.V. administration. The possible discrepancy in the area estimate reflects itself in exceedingly high plasma clearance value found in Table IX. This value (1400 ml/min) appears to exceed the plasma flow to the kidney (about 90% eliminated unchanged). The question of the high plasma clearance was further investigated. Freshly drawn blood (Sheep #1887) was incubated with hydroxytolbutamide at 37° C. The results could adequately be explained by Equation 9 in which λ

represents the

$$\lambda = 1 - H \qquad \text{Eq. 9}$$

concentration ratio between blood and plasma and H is the hemotocrit. The experimentally determined value of λ was 0.66 while the hematocrit was 0.39. Equation 9 is then a simplification of Equation 10

$$\lambda = \frac{H}{Kp} + (1 - H) \qquad \text{Eq. 10}$$

used by Rowland[96] where Kp is the apparent partition coefficient of the drug between plasma and erythrocytes. When Kp is very high, Equation 10 simplifies to Equation 9.

Blood clearance (CLb) can be calculated from plasma clearance (CLp).

$$CLb = \frac{CLp}{\lambda} \qquad \text{Eq. 11}$$

This would indicate that the blood clearance still exceeds kidney blood flow. The question remains unresolved.

Conclusion:

A 2 compartment model characterized the plasma concentration-time curve of hydroxytolbutamide after an I.V. bolus. The terminal log-linear half-life in two sheep was 13.5 and 26 min. While most of the dose (91.8 - 99.9%), was recovered, the unchanged compound represented about 90%. The plasma clearance appeared to exceed renal plasma flow.

B. Pharmacologic Response to Hydroxytolbutamide:

Along with the measurement of hydroxytolbutamide in plasma, Experiment VII served to provide information on the plasma glucose levels. The results are depicted in Figure XX.

An unexpected response observed was that of a great change in normal urinary output. The urine samples were then analyzed for total

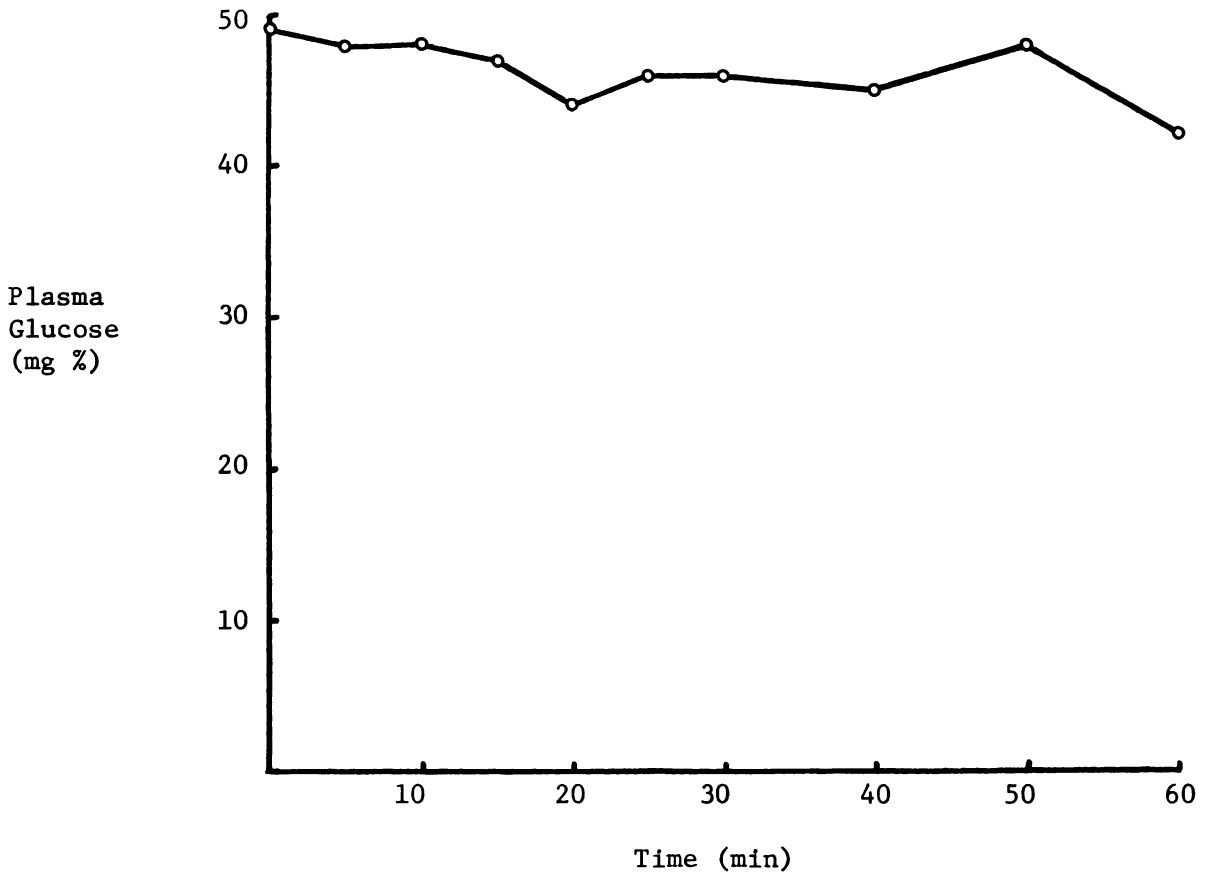


Fig. XX: Plasma glucose after 250 mg I.V. hydroxytolbutamide bolus in sheep #1494 (Experiment VII)

osmolality and electrolyte content. The results can be seen in Table XII. Blank urine from two other experiments where hydroxytolbutamide was not given was also analyzed for osmolality and electrolyte content.

Discussion:

Although Figure XX seems to indicate that hydroxytolbutamide does not possess hypoglycemic activity, yet, it should be pointed out that the dose used was about 1/4 that of tolbutamide which had pronounced plasma glucose depression activity (Figure XVIII). Were an equal dose used, an effect might be seen.

Surprisingly hydroxytolbutamide appeared to elicit a decided diuretic effect. Using the terminology of Goodman and Gilman[25c], hydroxytolbutamide could be classified as a compound resulting in a positive free-water production. In this case, there is a reduction in concentration of osmotically active solute in the urine. The cited authors indicate that there is no evidence that the available diuretic drugs directly alter the action of the antidiuretic hormone itself. Classically, the nephron of the kidney is functionally organized into 3 zones: proximal convolution, ascending loop of Henle and distal nephron. Compounds which cause a positive free-water production are thought to act either by an inhibition of sodium reabsorption in the proximal tube or an augmented sodium reabsorption in the most distal nephron where the antidiuretic hormone exerts its effect. Although the diuretic effect was very visible in Experiment VII, Experiment VI (Table XII) did not exhibit the same magnitude of diuresis. Further studies are needed to confirm this action and then compare equivalent doses of other sulfonylureas. Especially of interest is the compound chlorpropamide which is reported to possess antidiuretic activity in man[87].

Table XII: Urinary data after 250 mg I.V. hydroxytolbutamide bolus in sheep #1494 (Experiment VII)

Experiment VI					Experiment VII							
Sample	t (min)	Δt (min)	Vol (ml)	ΔV/Δt (ml/hr)	Sample	t (min)	Δt (min)	Vol (ml)	ΔV/Δt (ml/hr)	Na (meq/L)	K (meq/L)	Cl (meq/L)	Osm (mosm/Kg)
Blank	-	120	-	-	Blank A*1	-	92	103	67	124	324	261	1249
1	30	30	85	170	Blank B*2	-	60	85	85	222	372	294	1605
2	70	40	77.5	116	Blank C*3	-	60	77	77	63	492	142	1540
3	90	20	55	165	1	10	10	27	162	81	165	170	718
4	120	30	43	86	2	20	10	49	294	29	135	106	463
5	150	30	50	100	3	30	10	66	396	12	78	64	277
6	180	30	54	108	4	40	10	92	552	10	44	57	213
7	1200	1020	1250	74	5	50	10	32	192	11	85	63	294
					6	60	10	25	150	20	153	106	551
					7	90	30	45	90	37	196	126	816
					8	120	30	36	72	62	192	148	1019
					9	180	60	42.5	42.5	58	227	153	1165

*1 corresponds to blank of Experiment VII

*2 blank urine from Experiment IV

*3 blank urine from Experiment VIII

Conclusion:

A dose of 250 mg hydroxytolbutamide I.V. bolus had no apparent hypoglycemic activity, but a decided diuretic activity. This diuretic activity described by a positive free-water production manifested itself in the formation of osmotically dilute urine.

III INTRAVENOUS SULFADIMETHOXINE BOLUS

The sulfadimethoxine given to the sheep served to assess the kinetics in this particular species and to determine whether this sufonamide possessed any hypoglycemic activity. Linkenheimer and Stolzenberg[88] have investigated a number of sulfonamides in sheep including sulfadimethoxine. They have reported a half-life of 8.4 hr. with a volume of distribution of 310 ml/Kg at a dose of 110 mg/Kg given I.V. Their volume of distribution was calculated by dividing the dose by the maximum observed plasma concentration. No compartmental modeling was done and all data were plotted using linear coordinates. Although the majority of data will be recorded and discussed in Chapter IV, selected information will be given at this time.

A. Kinetics of Sulfadimethoxine:

A 4 Gm I.V. bolus of sulfadimethoxine (Experiment VIII) given I.V. over a period of 4 minutes resulted in the plasma concentration-time curve as demonstrated in Figure XXI. The Bratton-Marshall assay used to make the measurements failed to detect the presence of any N^4 conjugated derivative of sulfadimethoxine in the plasma. The data of Figure XXI could be explained by means of a 2 compartment model (Figure XIV) and thus the resultant pharmacokinetic parameters have been tabulated in Table XIII after fitting the curve by eye. An attempt was also made to

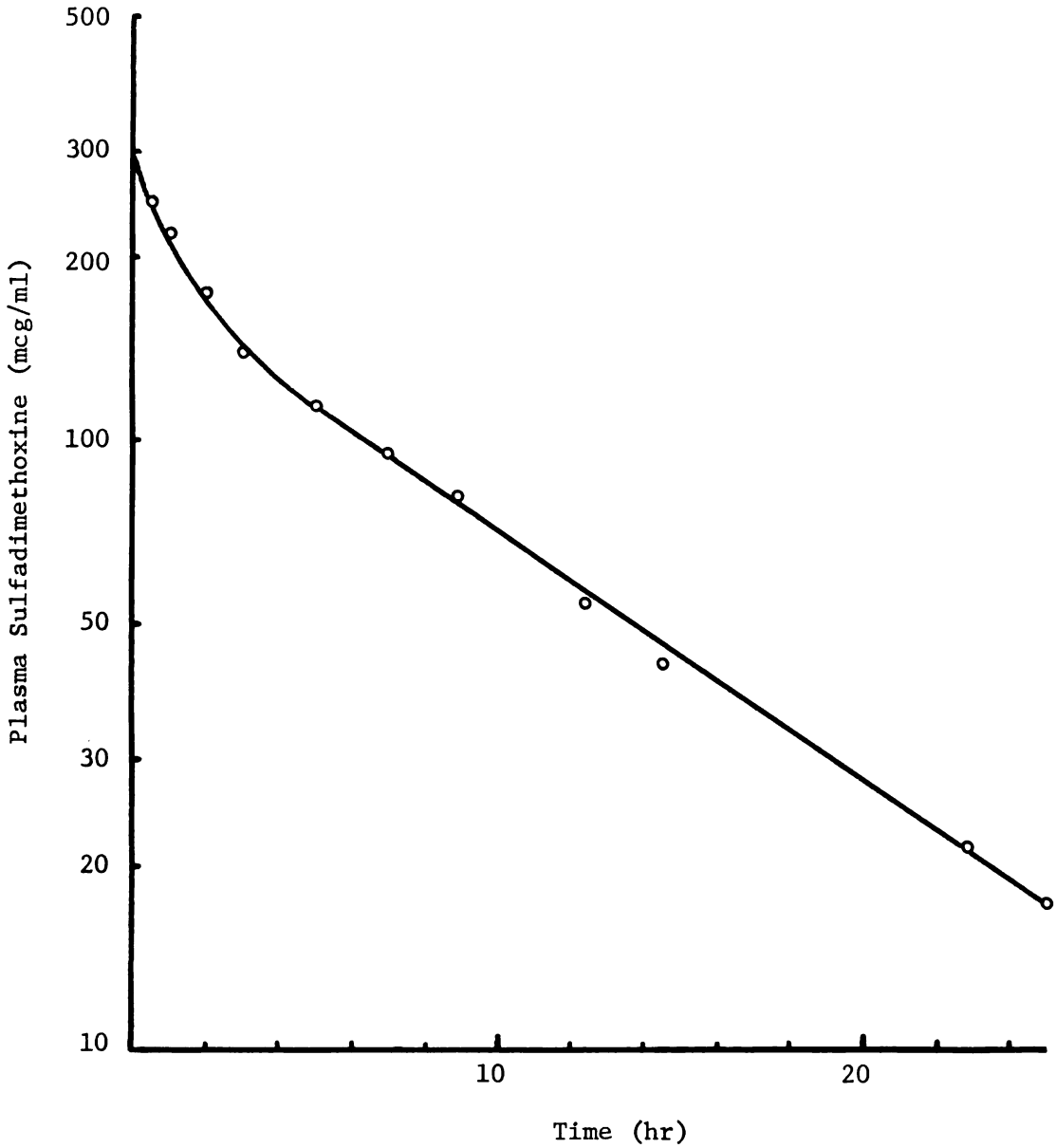


Fig. XXI: Plasma sulfadimethoxine after a 4 Gm I.V. bolus of sulfadimethoxine in sheep #1494 (Experiment VIII)

recover sulfadimethoxine or its N^4 conjugated metabolites in urine. However, it was found that blank urine contained a large component which gave a sulfonamide reaction. A pH profile extraction was performed with ether using blank urine and then blank urine containing 50 mcg/ml sulfadimethoxine. The results given in Figure XXII indicate the absorbance obtained after Bratton-Marshall assay for blank urine, blank urine plus 50 mcg/ml sulfadimethoxine, and by subtraction the absorbance for sulfadimethoxine.

Discussion:

The kinetics of sulfadimethoxine after an I.V. bolus indicate a distribution half-life of 1 hr and an apparent body elimination half-life of about 7.4 hrs. Although only one bolus is mentioned with more to follow in Chapter IV, the results mentioned here were used as the basis for Interaction Study III. It is of interest that the observed plasma clearance of 31.8 ml/min corresponds to the values obtained for tolbutamide (Table VII). The low magnitude of the value along with the knowledge that sulfadimethoxine is plasma protein bound to a considerable degree (see next section) lend consideration to the hypothesis that the clearance may depend upon the concentration of the unbound component.

The sulfonamide blank urine reading obtained suggests the presence of a compound with a diazotizable amino group. Th pH extraction profile also suggests the presence of an acidic moeity on the compound. A compound fitting these characteristics would be p-aminobenzoic acid. However, chemical ionization mass spectral analysis failed to detect a compound with m/e = 138 corresponding to protonated p-aminobenzoic acid. The question thus remains unresolved and further work on urinary excretion of sulfadimethoxine was abandoned.

Table XIII: Kinetic parameters after 4 Gm sulfadimethoxine I.V. bolus (Experiment VIII, sheep #1494 4/19/73)

Parameter	Value
Dose (gm)	4
Model	2
A (mcg/ml)	105
α (hr^{-1})	0.693
B (mcg/ml)	183
β (hr^{-1})	0.094
$t_{\frac{1}{2}}$ β (hr)	7.4
k_{12} (hr^{-1})	0.175
k_{21} (hr^{-1})	0.475
k_{10} (hr^{-1})	0.137
V_1 (ml)	13890
V_2 (ml)	5110
Vd (ml)	19000
CL (ml/min)*	31.8

* plasma clearance

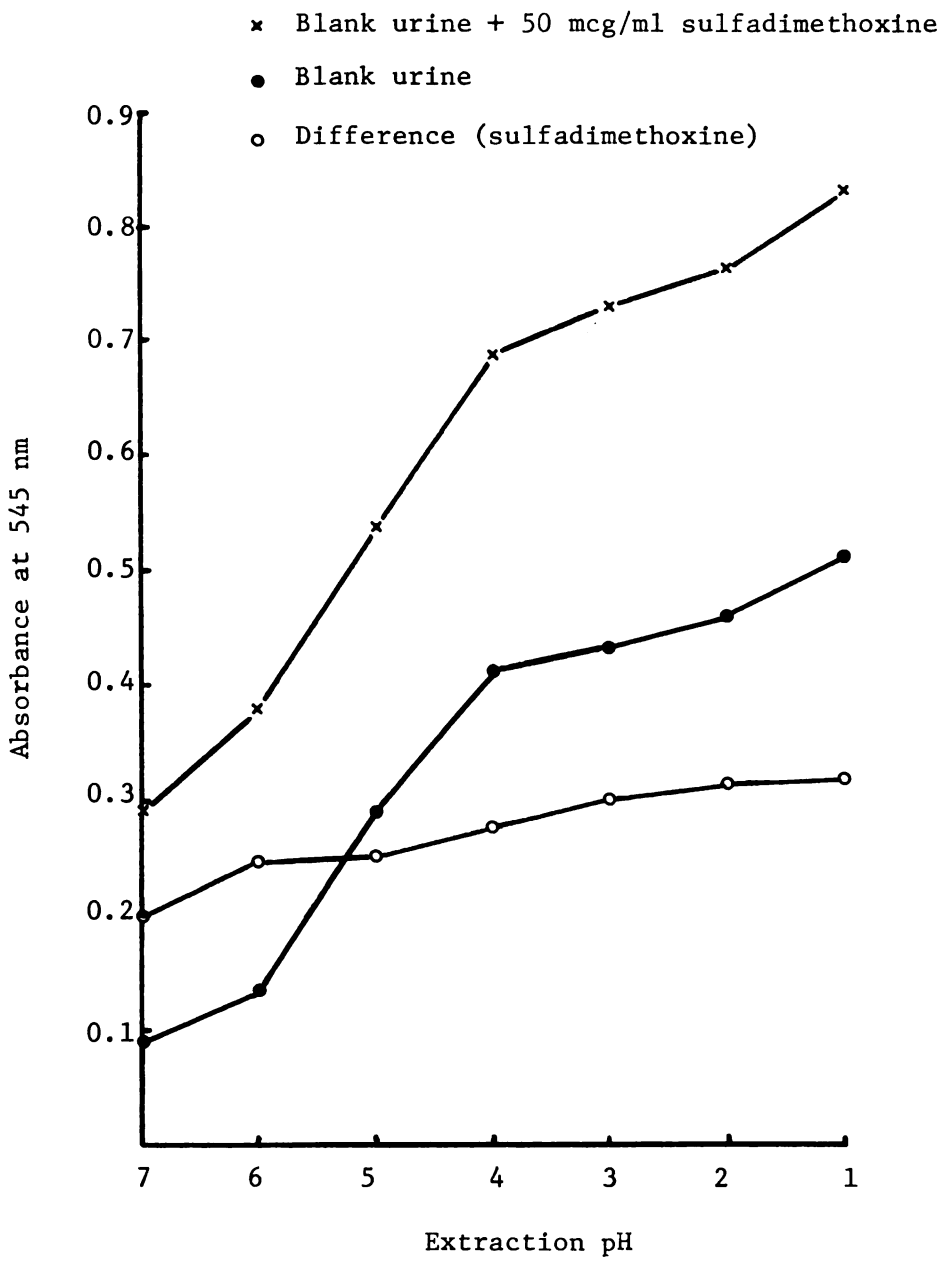


Fig. XXII: Urinary extraction profile for sulfonamide analyzed by Bratton-Marshall assay (sheep urine).

Conclusion:

A 4 Gm I.V. bolus of sulfadimethoxine resulted in a plasma concentration-time curve described by a 2 compartment model. Kinetic computation provided a distribution half-life of 1 hr., terminal log-linear half-life of 7.4 hr. and a plasma clearance of 31.8 ml/min.

B. Pharmacologic Response to Sulfadimethoxine:

The results obtained on the possible hypoglycemic effect of sulfadimethoxine are seen in Figure XXIII. There appears to be a slight plasma glucose depression. Yet when it is considered that the dose used was over 4 times that of tolbutamide, then the relative effect appears to be very small.

Conclusion:

An insignificant hypoglycemic effect was seen in the sheep after a 4 Gm I.V. bolus of sulfadimethoxine.

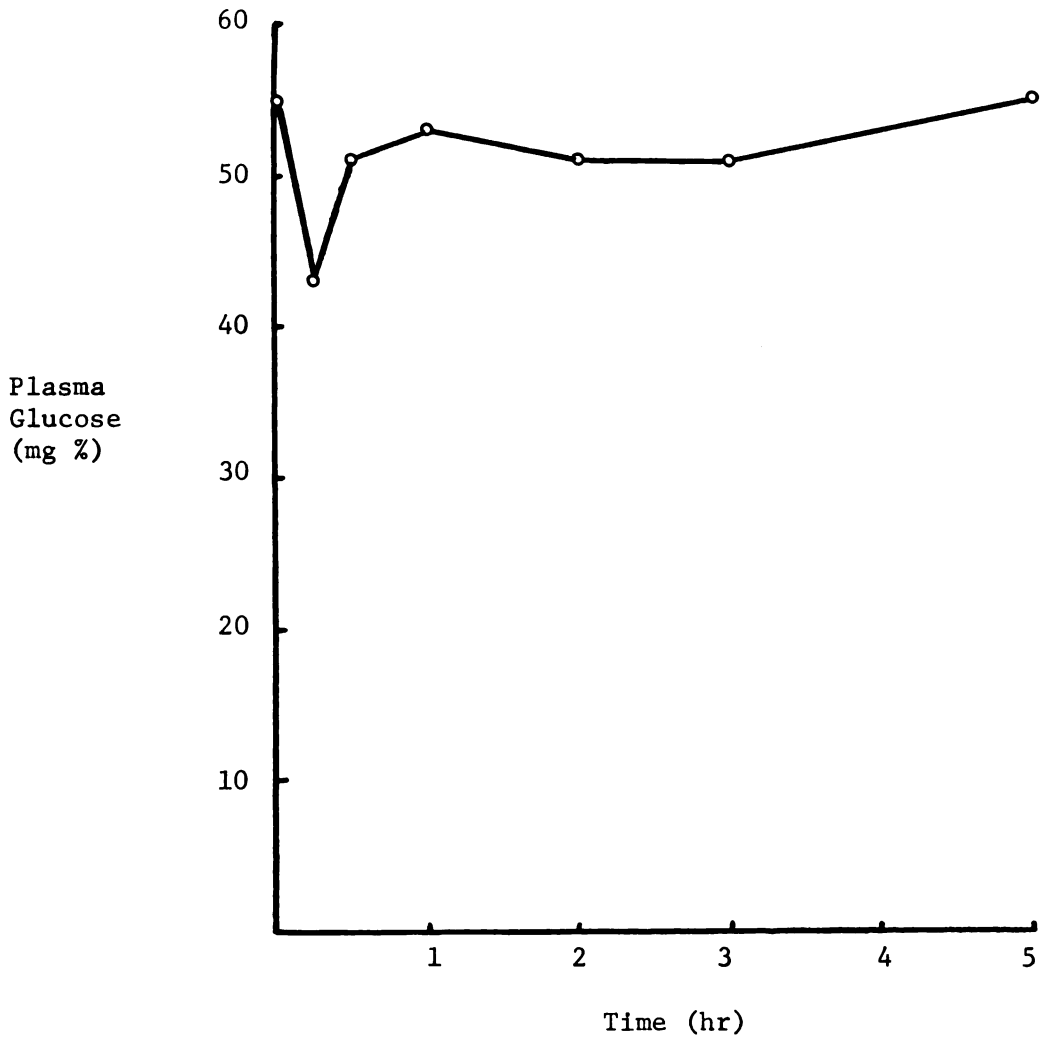


Fig. XXIII: Plasma glucose after 4 Gm I.V. sulfadimethoxine bolus in sheep #1494 (Experiment VIII)

IV IN VITRO PROTEIN BINDING

An assessment of the degree of protein binding of compounds has been performed in vitro for a number of reasons such as attempting to relate the concentration of the unbound moeity to a pharmacologic response, attempting to explain a drug interaction or possibly to explain the compound's pharmacokinetic behavior. In this study, the purpose of the in vitro binding experiments was to establish baseline values obtained in vivo and then also to find a compound which would displace tolbutamide from plasma proteins. No information was found for the binding of tolbutamide to sheep plasma although it is reported to be bound 97% to bovine serum albumin at a concentration of 100 mcg/ml[61]. Of the compounds used, sulfadimethoxine binding has been investigated in the sheep using whole plasma[88]. For a concentration less than 50 mcg/ml a little more than 90% was bound. Between 140 and 200mcg/ml the values were 90-78% bound, respectively.

In the listing of the results, or during the discussion a few abbreviations will be used. With regards to assay methods, R = radioactivity, B.M. = Bratton-Marshall sulfonamide and GLC = gas liquid chromatography. When dealing with methods used to determine protein binding, E.D. = equilibrium dialysis, U.C. = ultracentrifugation and U.F. = ultrafiltration. Throughout this section different whole plasma samples were used. Table XIV indicates the designations for the various samples of plasma.

Table XIV: Plasma sources and designations

Plasma Source	Designation
Sample #1, Sheep #368	368A
Sample #2, Sheep #368	368B
Sample #1, Sheep #1494	1494A
Sample #2, Sheep #1494	1494B
Hemoline*, Sample #1	HA
Hemoline*, Sample #2	HB

* Hemoline Pacific, Belmont Ca. 94002

The in vitro protein binding studies have been divided into three sections - work relating to the binding studies with tolbutamide or hydroxytolbutamide, binding studies with sulfadimethoxine and finally displacement studies involving tolbutamide and other agents. In all cases, % unbound was calculated by dividing the radioactivity or concentration in the plasma water obtained by E.D., U.C. or U.F., by the radioactivity or concentration in the initial whole plasma, multiplied by 100.

A. Tolbutamide and Hydroxytolbutamide Binding:

Much of the early work in the binding of tolbutamide served not only to determine the % unbound at various concentrations but also served to investigate the protein binding determination methods. No including time required for the preparation of the samples nor their subsequent analysis, E.D., U.C., and U.F. were performed for 6 hr., 24 hr., and 4 min. respectively during the binding experiments. As can be seen in Table XV, the results obtained by the three methods at a tolbutamide plasma concentration of 100 mcg/ml are virtually identical. Of interest as well as was the affect of temperature (25 and 37^{o}C) on the binding of tolbutamide.

Table XV: Tolbutamide binding* using 3 methods at 37°C.

Method	Plasma	Assay	% Unbound
E.D.	HA	GLC	14.4
U.C.	368A	R	14.9
U.F.	368A	R	14.6

* Initial plasma concentration of 100 mcg/ml

The results are tabulated in Table XVI.

Table XVI: The effect of temperature on the sheep plasma protein binding of tolbutamide.

Method	Plasma	Temp(°C)	Assay	Tolbutamide conc(mcg/ml)*	% Unbound
U.C.	368A	25	R	10	10.1
"	"	37	"	10	10.7
"	"	25	"	50	12.4
"	"	37	"	50	12.7
"	"	25	"	100	15.2
"	"	37	"	100	14.9
U.F.	"	25	"	10	8.9
"	"	37	"	10	9.5
"	"	25	"	50	11.9
"	"	37	"	50	12.0
"	"	25	"	100	14.2
"	"	37	"	100	14.6

* Initial whole plasma concentration

The methods were also investigated to determine the protein content of the generated plasma water. As stated in Chapter II, Experimental

Methods, the protein value obtained represented a fraction of the original whole plasma. Table XVII summarizes the results.

Table XVII: Protein content of generated plasma water*1

Method	Temp(°C)	Fraction*2	n*6	% Protein*3
E.D.	37	complete	-	N.D.*4
U.C.	25	A	3	0.073
"	"	B	3	0.15
"	"	C	3	0.23
"	37	A	3	0.12
"	"	B	3	0.18
"	"	C	3	0.20
U.F.	25	complete	5	0.43*5
"	37	"	6	0.87

*1 plasma 368A

*2 complete = bulked plasma water; A = top 1 ml; B and C are subsequent 1 ml layers

*3 average of n samples expressed as % of plasma proteins in sheep plasma 368A

*4 not detectable

*5 one sample greater than 1% not included

*6 number of samples

It should be noted that various layers obtained after ultracentrifugation were analyzed. These layers were 1 ml in volume beginning at the top of the ultracentrifuge tube. Table XVIII includes all the <u>in vitro</u> tolbutamide plasma binding performed.

Table XVIII: Tolbutamide sheep plasma protein binding at 37°C.

Plasma	Method	Assay	Tolbutamide (mcg/ml)	% Unbound
368A	U.F.	R	10	9.5
"	U.C.	"	"	10.7
HA	E.D.	GLC	40	9.2
368A	U.F.	R	50	12.0
"	U.C.	"	"	12.7
HB	U.F.	GLC	"	14.1
368B	U.C.	R	"	14.7
"	U.F.	"	"	16.3
1494B	E.D.	"	"	18.0
HA	U.F.	GLC	65	10.8
1494A	"	"	75	17.4
HA	E.D.	"	100	14.4
368A	U.F.	R	"	14.6
"	U.C.	"	"	14.9
HA	E.D.	GLC	200	17.9

In addition, the Table provides one sample (1494A) which was obtained after an I.V. bolus of tolbutamide. The data obtained with plasma sample 368A has been utilized to prepare a Rosenthal plot[89] of the binding of tolbutamide as seen in Figure XXIV. Equilibrium dialysis was used to assess the binding of the major metabolite, hydroxytolbutamide, and the results can be seen in Table XIX.

Table XIX: Hydroxytolbutamide sheep plasma protein binding at 37° C.

Plasma	Method	Assay	Hydroxytolbutamide (mcg/ml)	% Unbound
H.B.	E.D.	R	2	19.7
"	"	"	10	21.4
"	"	"	20	21.0

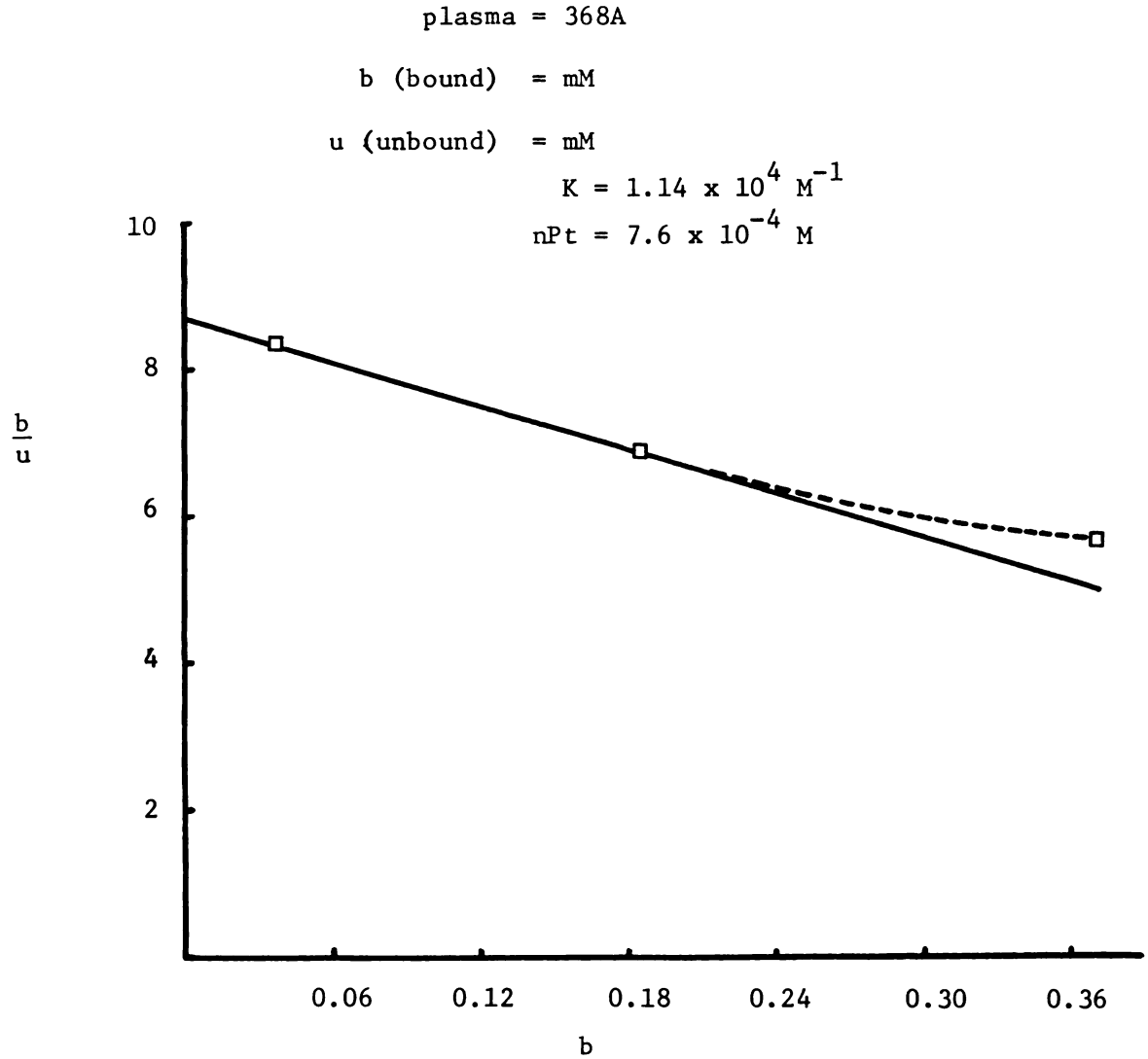


Fig. XXIV: Rosenthal plot of tolbutamide sheep plasma protein binding after ultracentrifugation at 37°C.

Discussion:

As can be seen, all 3 methods (E.D., U.C., U.F.) gave essentially the same result for the binding of tolbutamide. Differences that did occur as seen in Table XVIII may be attributable to a difference in the binding seen in a particular plasma sample, which may in turn be a function of the concentration or inherent binding character of the binding protein. Electrophoresis was performed on a few plasma samples with the results of the fractional separation recorded in Table XX.

Table XX: Fractional separation of plasma proteins

		Gm/100 ml				
Sample	Total Protein	Albumin	Alpha 1	Alpha 2	Beta	Gamma
HB	6.9	3.5	0.3	0.7	1.7	0.8
1494B	9.2	2.7	0.5	0.9	2.6	2.5
1494C*1	9.2	2.3	0.4	1.1	3.7	1.7
1494D*2	9.2	2.7	0.4	1.0	2.4	2.7

*1 plasma from Experiment III

*2 plasma from Interaction Study #3

The differences found with 50 mcg/ml tolbutamide in plasma HB and 1494B might be due to their difference in albumin content, the protein reported to be the chief binder of tolbutamide in man[61]. Other differences which occur in the same plasma sample with the same concentration of tolbutamide but different methods must be attributable to the technique employed. Of the methods used, U.F. permits the greatest entry of protein into the plasma water. Since the binding of tolbutamide in sheep plasma is less than 90%, this protein is of minor consequence. Thus, due to the short time required for the method, U.F. was selected for

use in the interaction studies. The Rosenthal[89] plot of tolbutamide while limited in data points, yet gives a few indications about the binding characteristics. The gentle curvature is an indication that the binding is not limited to one site. Although the line drawn through the 2 lower concentration points is not the actual line describing the binding to the predominant site, yet it is an approximation. The association constant indicates that the binding is moderately strong with the binding site concentration (nPt = 7.6×10^{-4} M) approximately twice the albumin concentration (2.7 Gm%). It was interesting to note that hydroxytolbutamide was also bound to plasma proteins but apparently to a lesser degree than tolbutamide.

Conclusion:

The binding of 100 mcg/ml tolbutamide to sheep plasma as determined by E.D., U.C. and U.F. was found to be 85.6, 85.1 and 85.4% respectively. Since all methods gave comparable results, U.F. was chosen for subsequent work due to the short time (4 min) required by the method. Tolbutamide bound to a principle plasma site with an apparent association constant (K) of 1.14×10^{4} M^{-1} with the binding site concentration (nPt = 7.6×10^{-4} M) approximating twice the albumin concentration. Hydroxytolbutamide (2 - 20 mcg/ml) was found to be bound to sheep plasma 78.5 to 80%.

B. Sulfadimethoxine Binding:

The binding results of sulfadimethoxine are summarized in Table XXI.

Table XXI: Sulfadimethoxine sheep plasma protein binding at 37°C

Plasma	Method	Assay	Sulfadimethoxine (mcg/ml)	% Unbound
1494B	E.D.	R	12.5	4.9
"	"	"	25	5.4
"	"	"	50	7.3
"	"	"	100	15.2
"	"	"	200	30.4
"	"	"	300	40.0
"	"	"	400	46.2
"	"	"	600	53.8
"	"	"	800	59.4
HB	U.F.	"	100	11.2
"	"	"	200	18.8
"	"	"	300	26.6
"	"	"	400	33.2
"	E.D.	"	100	11.5
"	"	"	200	20.0
"	"	"	400	32.5

The results obtained for plasma HB after ultrafiltration have been illustrated in a Rosenthal plot as seen in Figure XXV.

Discussion:

The same plasma sample, analyzed by U.F. or E.D., gives about the same binding value. This is surprising because sulfadimethoxine levels of greater than 100 mcg/ml, using E.D., should give a lower % unbound value due to the dilution aspect. This problem encountered with E.D. will become more evident in Chapter IV, when the problem will also be discussed further. The association constant (K) determined is an apparent value because the Rosenthal plot[89] exhibits curvature indicative of more

plasma = HB

b (bound) = mM

u (unbound) = mM

$K = 1.54 \times 10^{4}\ M^{-1}$

$nPt = 8 \times 10^{-4}\ M$

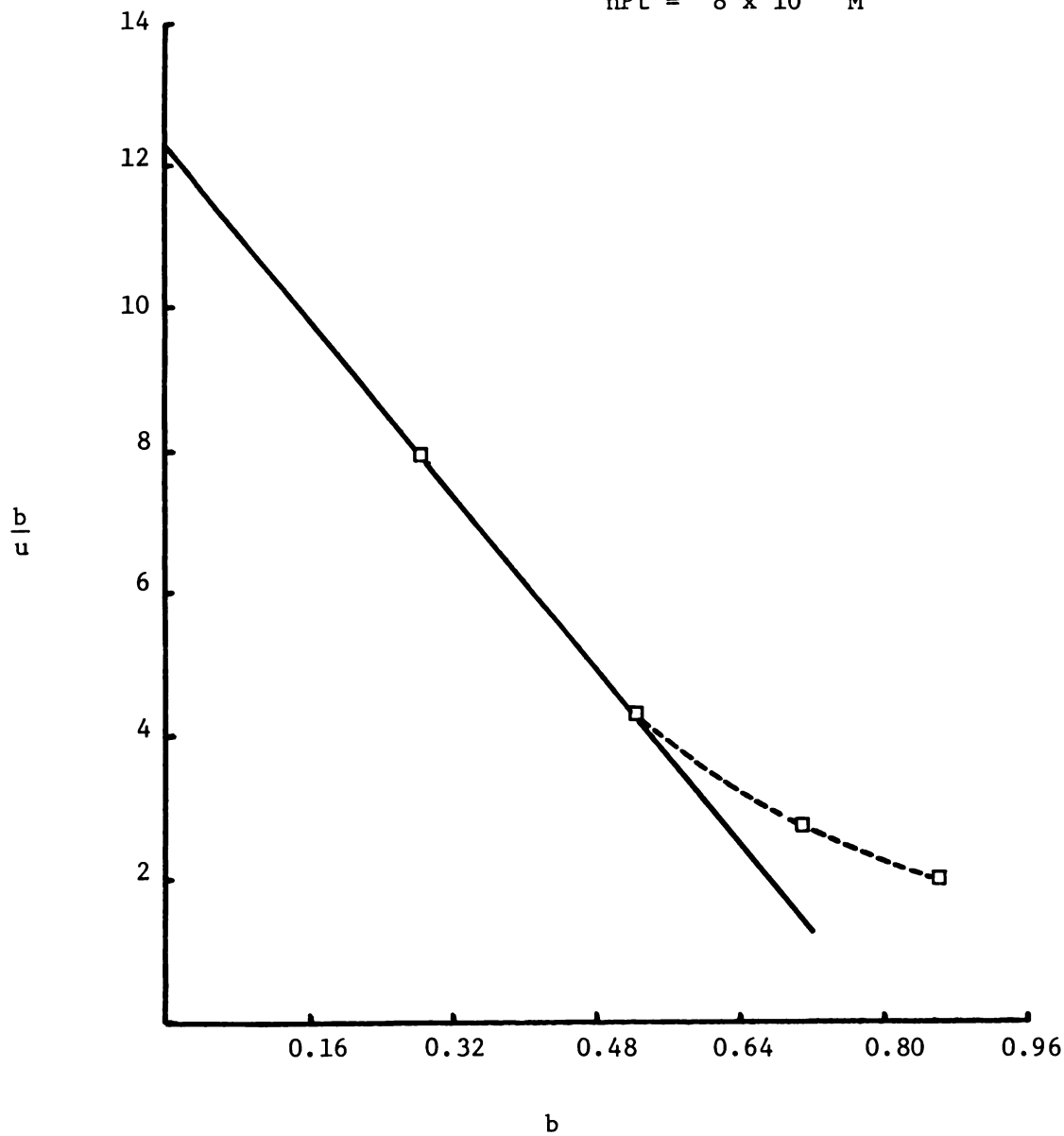


Fig. XXV: Rosenthal plot of sulfadimethoxine binding after ultrafiltration at 37^{o} C.

than one binding site. The value obtained (1.54×10^4 M^{-1}) is about 35% higher than that for tolbutamide (1.14×10^4 M^{-1}) but it is interesting again that the nPt value (8×10^{-4} M) approximates twice the albumin concentration. Thus it appears that sulfadimethoxine represents a logical choice to investigate the interaction hypothesis. This will be more evident subsequently when it will be seen that sulfadimethoxine displaces tolbutamide from plasma proteins *in vitro*.

Conclusion:

Sulfadimethoxine binds to a principal plasma site with an apparent association constant (K) of 1.54×10^4 M^{-1} with the binding site concentration (nPt = 8×10^{-4} M) approximating twice the albumin concentration.

C. Tolbutamide Displacement:

The initial experiments were aimed at finding a compound which would displace tolbutamide from plasma proteins. Dicloxacillin (Beecham), indocyanine green (Cardiogreen: Hynson, Westcott & Dunning), sulfadimethoxine (Roche) and rose bengal (Mattheson, Coleman & Bell) were investigated in sheep plasma (HA) with a tolbutamide concentration of 60 mcg/ml. The results are seen in Table XXII.

Table XXII: Alteration of tolbutamide binding to sheep plasma (HA) by various agents, determined by U.F. at 37°C

Tolbutamide (mcg/ml)	Compound	Mol. Wt.	Displacer Conc. (mcg/ml)	Conc. (mcM/ml)	% Unbound Tolbutamide
65	--	--	--	--	10.8
60	Dicloxacillin	492	212	0.43	9.5
"	Indocyanine Gr.	775	500	0.65	14.6
"	Sulfadimethoxine	310	216	0.70	22.3
"	Rose Bengal	974	710	0.73	22.6

The peculiar result for dicloxacillin was repeated in plasma 368B in which 50 mcg/ml tolbutamide alone had 14.7% unbound while in the presence of 50 mcg/ml dicloxacillin this value fell to 9.9%. Thereafter sulfadimethoxine was investigated further and the summary of results can be seen in Table XXIII.

Table XXIII: Alteration in tolbutamide (50 mcg/ml) binding to sheep plasma by sulfadimethoxine (Methods at 37°C)

			% Unbound Tolbutamide Sulfadimethoxine (mcg/ml)									
Plasma	Method	Assay	0	10	20	50	100	200	300	400	600	800
368B *1	U.C.	R	14.7	14.2	15.6	16.5	20.9	-	-	-	-	-
" *2	U.F.	"	16.3	16.8	18.3	19.4	22.9	-	-	-	-	-
HB	U.F.	GLC	14.1	-	-	18.3	21.4	28.5	32.1	35.2	-	-
1494B	E.D.	R	18.0	-	-	25.8	32.7	39.3	43.0	45.4	49.7	52.8

Note: *2 collected from sheep #368 two days after *1

Of the results appearing in the Table, plasma HB and 1494B have been recorded graphically in Figure XXVI.

Discussion:

Of the compounds investigated, three cause a measure of displacement while the exception, dicloxacillin, appears to increase the binding of tolbutamide at the concentration studied. A similar phenomenon to that seen with dicloxacillin has been observed with phenylbutazone which has been reported to increase the binding of thiopental to bovine plasma[90]. Possibly dicloxacillin,an acidic compound, has an affect upon the structure of the binding protein(s) causing a liberation of additional binding sites (increasing nPt) thus causing the increase in binding of tolbutamide. It is interesting to note that the plasma which had the highest % unbound (368B) tolbutamide before the addition of dicloxacillin exhibited the greatest increase in % bound. Of the compounds investigated, rose bengal and sulfadimethoxine in the same molar concentration cause the same degree of displacement. Sulfadimethoxine was chosen for further study.

Sulfadimethoxine appears to be a fairly strong displacer, with a concentration of 100 mcg/ml causing a 50% increase in % unbound tolbutamide (50 mcg/ml) while 200 mcg/ml appears to double the % unbound. Although both the interactions determined by E.D. and U.F. have been plotted in Figure XXVI, the results for E.D. are misleading. The E.D. method allows the unbound moeity of either drug to pass through the semi-permeable membrane thus in effect causing a dilution of both drugs in the half-cell which contains the protein. In actual fact, the results reflect a lower tolbutamide and sulfadimethoxine concentration at equilibrium. This will be evident in Chapter IV. The E.D. curve in Figure XXVI would be expected to be higher after about 50 mcg/ml sulfadimethoxine. However, how much higher the curve should be is complicated

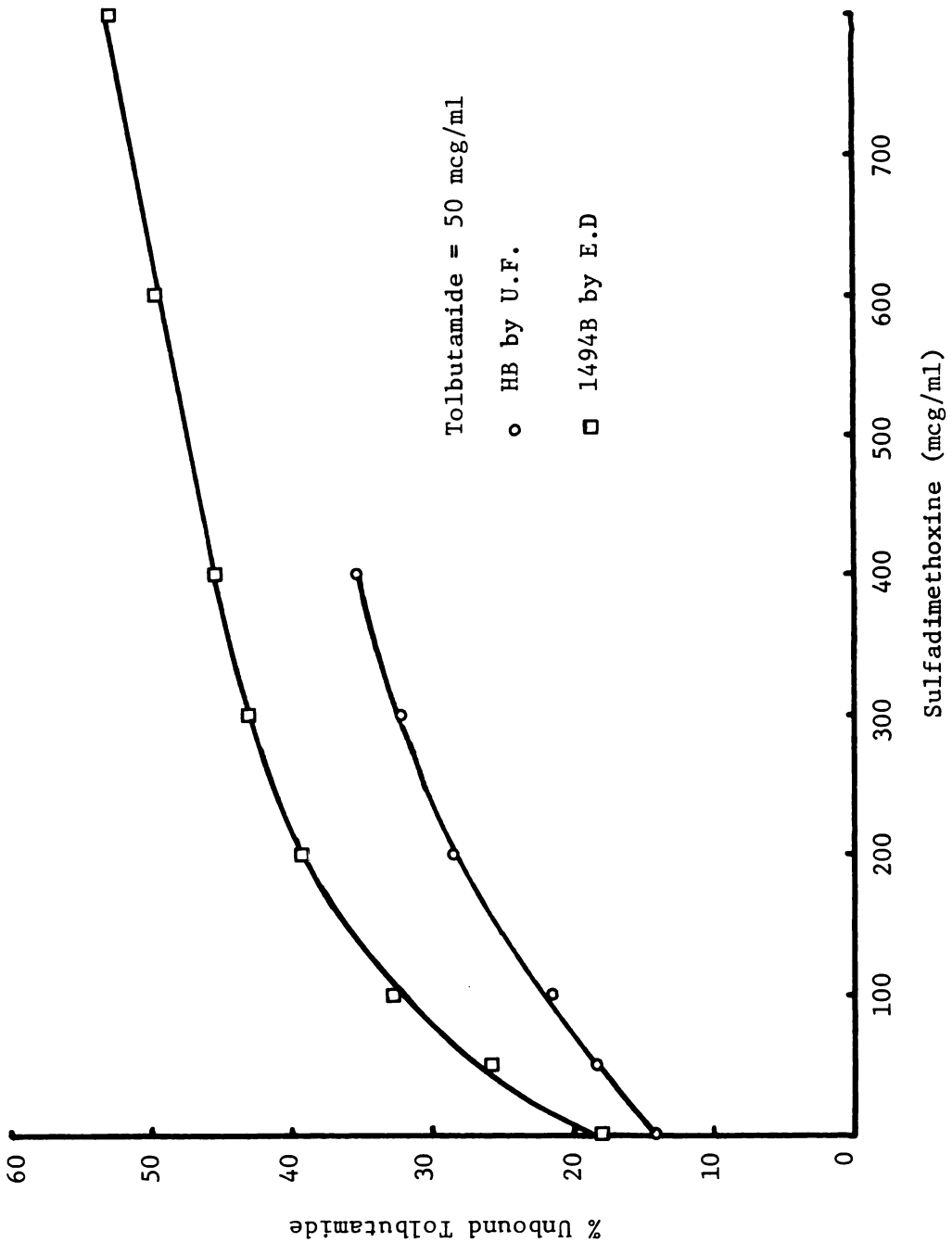


Fig. XXVI: Alteration in tolbutamide binding to sheep plasma by sulfadimethoxine

by the fact that the effective tolbutamide concentration (concentration of total tolbutamide in the plasma half-cell) has also gone down.

Conclusion:

Dicloxacillin has been shown to decrease while indocyanine green, sulfadimethoxine and rose bengal have been shown to increase the % unbound tolbutamide in sheep plasma. The increase with sulfadimethoxine has been about 50% at 100 mcg/ml and 100% at 200 mcg/ml, when using a tolbutamide concentration of 50 mcg/ml.

V INTERACTION STUDY #1 (Tolbutamide-Sulfadimethoxine)

After the discovery that sulfadimethoxine was capable of significantly displacing tolbutamide from sheep plasma proteins, interaction studies were begun *in vivo*. It was decided that the most sensitive method of examining the interaction consisted of evaluating the influence of sulfadimethoxine on steady-state levels of tolbutamide. Calculation based on previous kinetic data (Table VII, Experiment II) indicated that a bolus of 510 mg tolbutamide followed immediately with a constant infusion of 95 mg/hr. should immediately attain and maintain a steady-state plasma concentration of 60 mcg/ml tolbutamide. After 3 hours, a simultaneous zero-order infusion of sulfadimethoxine (2 Gm/hr) was initiated and allowed to proceed for 3 hours. Throughout the experiment blood and urine samples were taken. The animal was allowed food and water ad-lib. The results obtained for total plasma tolbutamide, unbound tolbutamide and plasma glucose appear in Figure XXVII. The corresponding plasma levels of sulfadimethoxine during and after its infusion can be seen in Figure XXVIII. When the sulfadimethoxine data is plotted semi-logarithmically the post infusion curve exhibits bi-exponential character with the apparent distribution phase having a half-life of 1.6 hr. and the terminal log-linear phase a half-life of 7.5 hr. Plasma analysis was also carried out for hydroxytolbutamide and the results are depicted in Figure XXIX. In addition, urinary analysis was performed for parent tolbutamide and metabolite measured as hydroxytolbutamide. The results given as excretion rates per hour appear in Figure XXX. The total tolbutamide equivalents excretion data points have been corrected for molecular weight differences between hydroxytolbutamide and tolbutamide. Some of the urine samples have been pooled and this

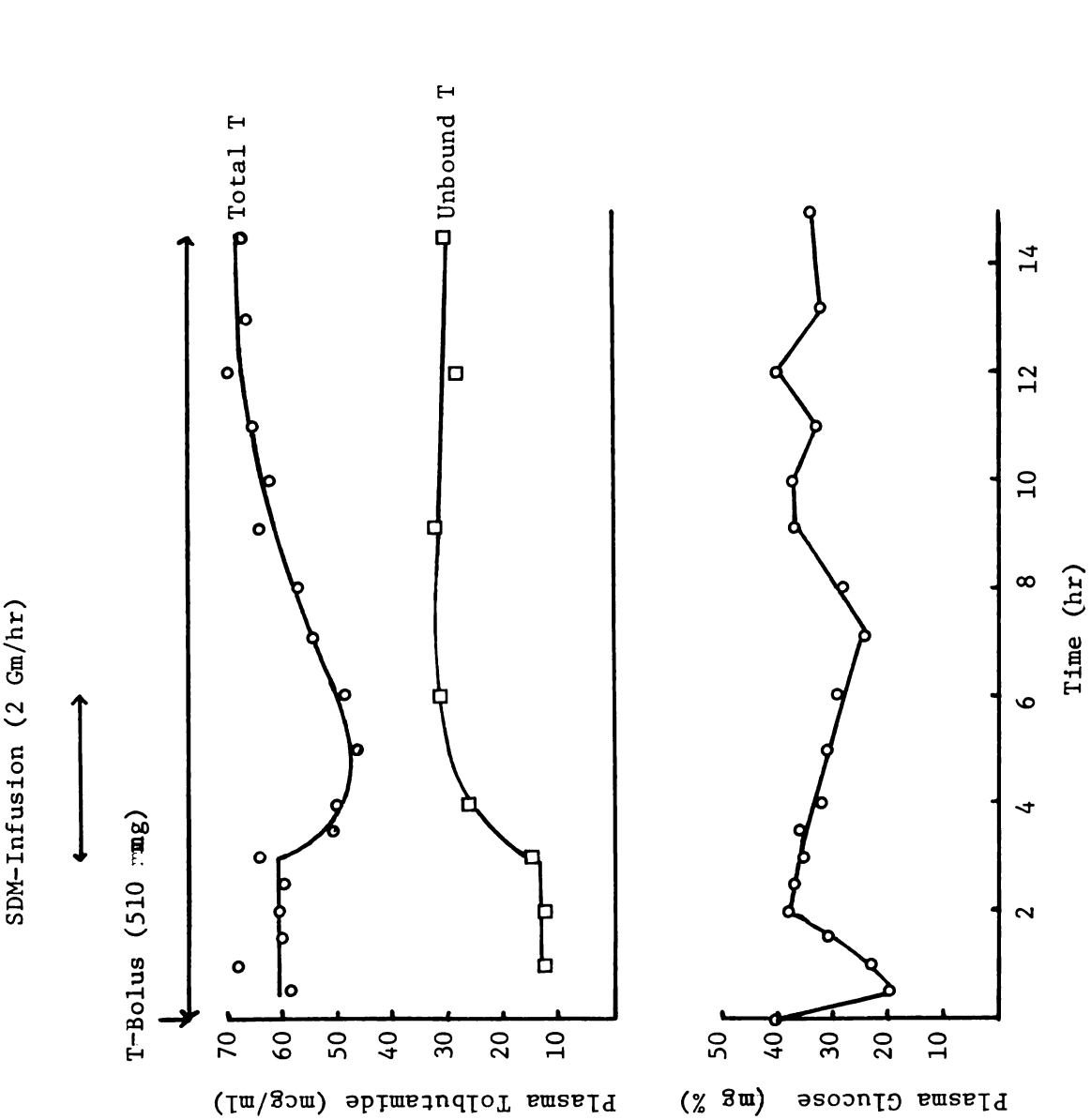


Fig. XXVII: Total and unbound plasma tolbutamide concentrations and plasma glucose as seen in interaction study #1 (Experiment IX; T = tolbutamide; SDM = sulfadimethoxine)

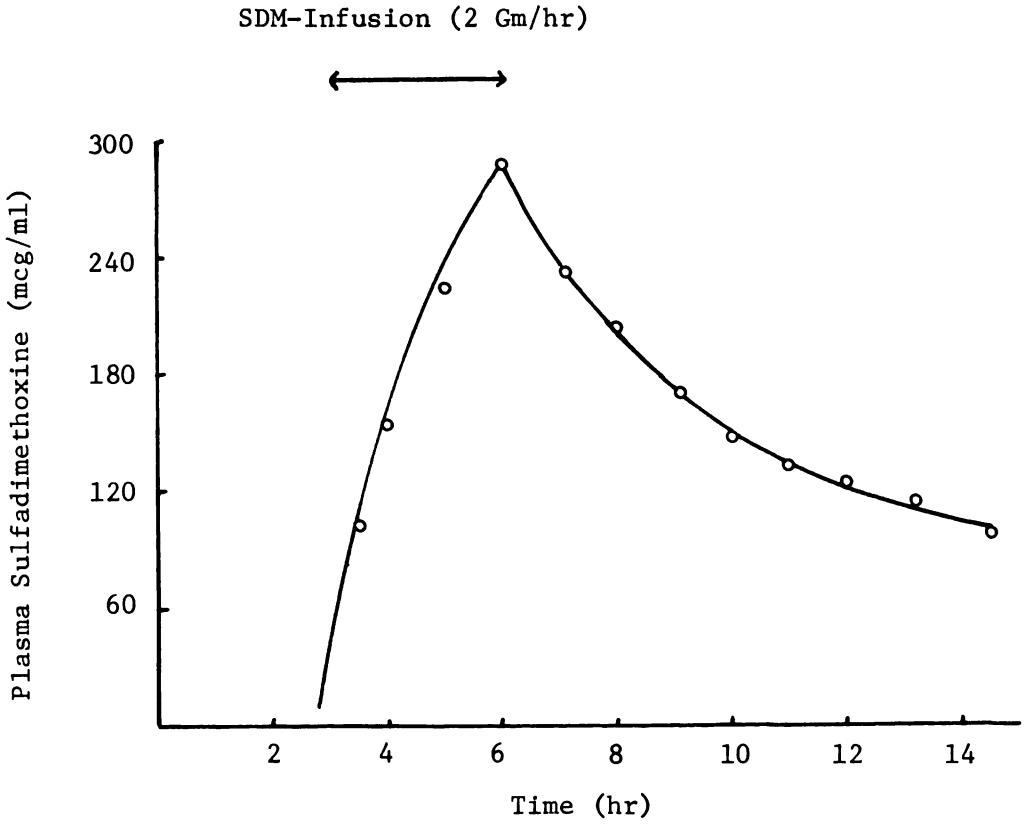


Fig. XXVIII: Plasma sulfadimethoxine of interaction study #1. (Experiment IX; SDM = sulfadimethoxine)

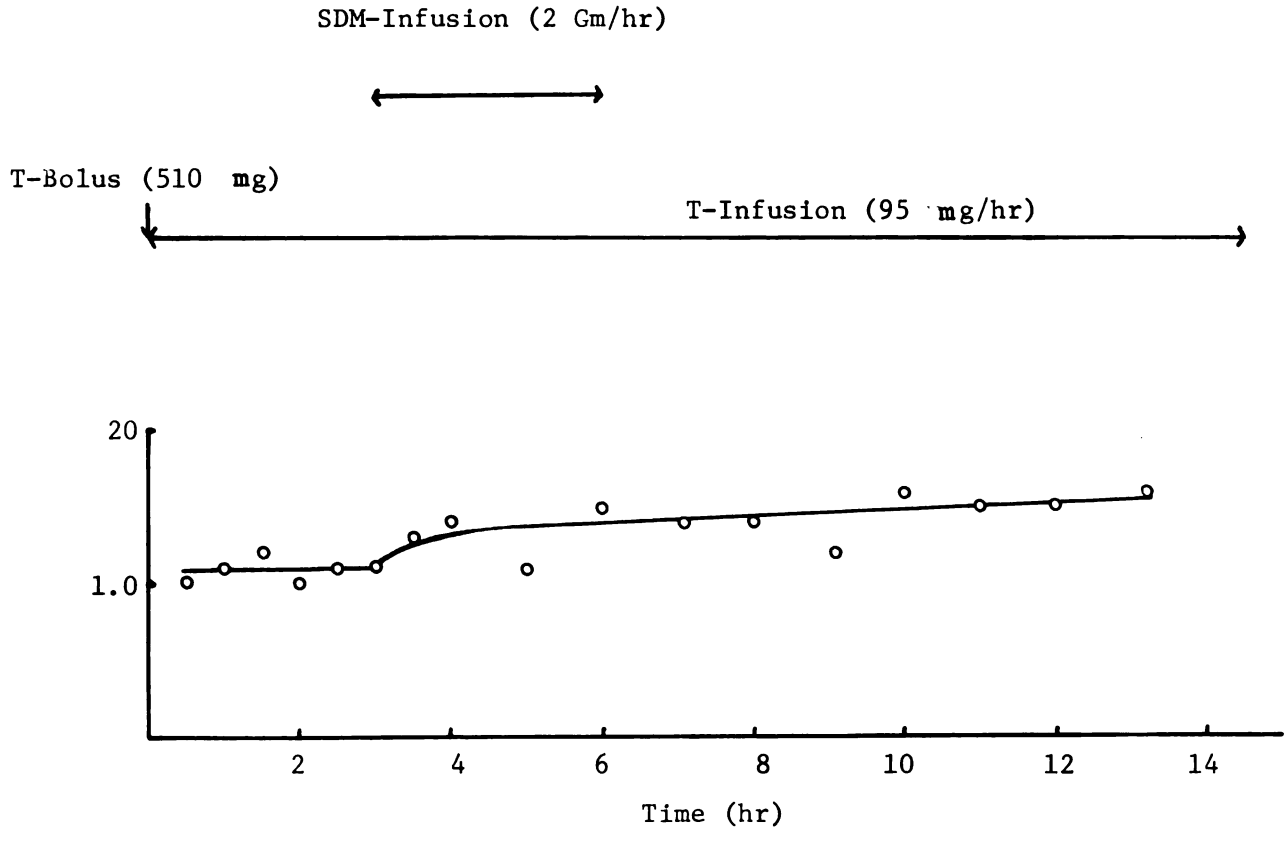


Fig. XXIX: Plasma hydroxytolbutamide observed during interaction study #1. (Experiment IX; T = tolbutamide; SDM = sulfadimethoxine)

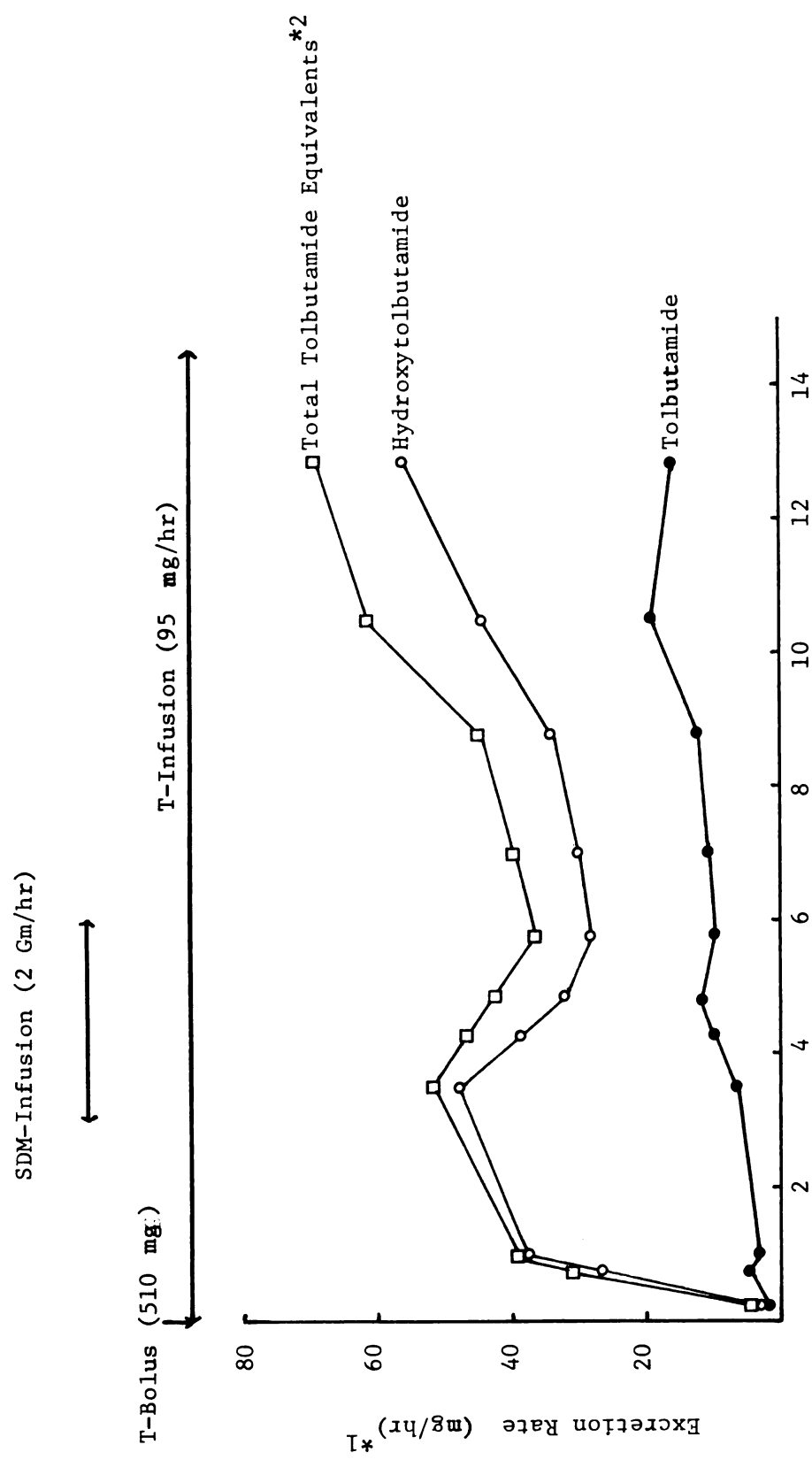


Fig. XXX: Urinary excretion rates of interaction study #1 (Experiment IX; T = tolbutamide; SDM = sulfadimethoxine)

*1 midpoint plots

*2 data corrected for molecular weight

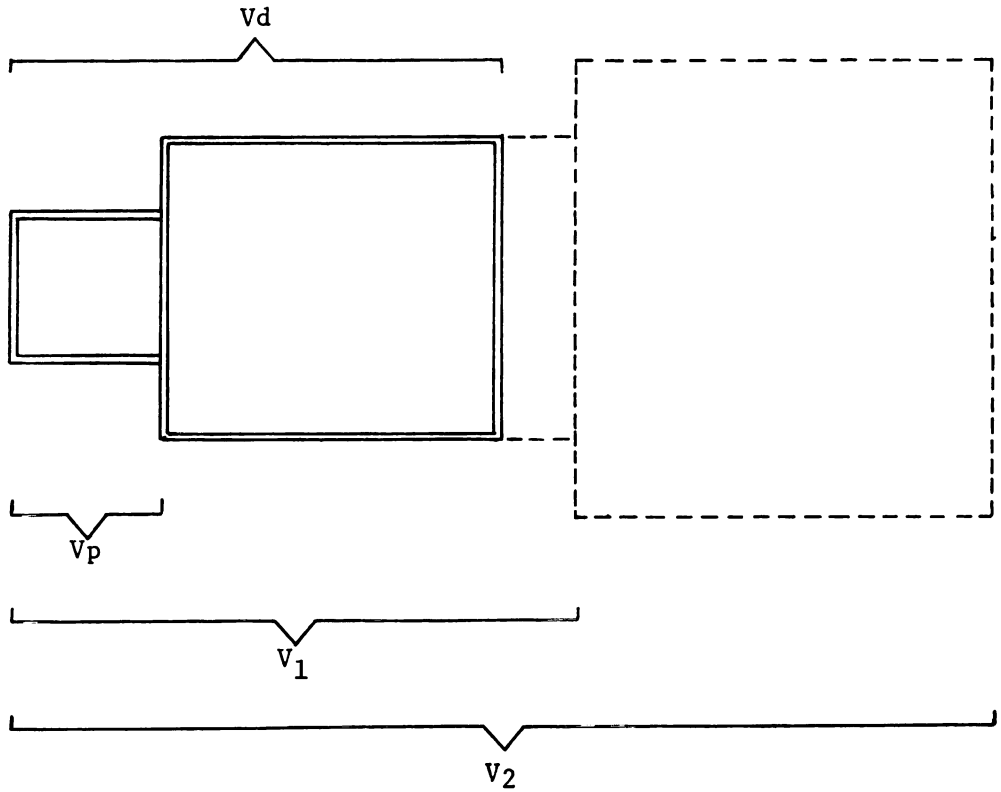
information is provided in the data of Experiment IX. During the first 2 to 3 hours the animal displayed a nervousness which resulted in some loss of unanalyzed urine via the urethra and the normal collection via the bladder catheter was impeded.

Discussion:

As can be seen in Figure XXVII sulfadimethoxine has a profound effect upon the total and unbound plasma concentration of tolbutamide.

The total tolbutamide levels fell indicative of plasma protein displacement. This fall was accompanied by an expected rise in the unbound tolbutamide concentration. However, both the magnitude of the initial changes and the subsequent levels were greater than anticipated. Table XXIV indicates the % unbound tolbutamide in plasma as well as the % increase in the unbound fraction in the presence of sulfadimethoxine. The"% unbound "values obtained may provide some information about the actual distribution of tolbutamide. To explain the large increase in "% unbound" in plasma (98 to 221%) several models of drug distribution were considered. One may compartmentalize the body of the sheep into 4 volumes, 3 of which are real - the plasma, extracellular fluid and total body water (Figure XXXI). The fourth, the apparent volume of distribution, as described in Chapter I, was graphically estimated from Experiment II (Table VII) which represented the results from the last tolbutamide bolus just before the interaction study. Now, 3 cases might be envisaged.

Case 1 - drug bound only in Vp: Since the plasma volume (Vp) represents 18.3% of the apparent volume of distribution (1860/10200 x 100), and assuming about 20% of the drug in Vp is unbound (Table XXIV), then only 14.6% of a dose would be in a bound form. If all of this



Vd = apparent volume of distribution of tolbutamide (10200 ml - Experiment II)
Vp = volume of plasma = 1860 ml (3.72% of 50 Kg sheep)
V_1 = extracellular fluid = 12500 ml (25% of 50 Kg sheep)
V_2 = total body water = 30,000 ml (60% of 50 Kg sheep)

Fig. XXXI: Diagrammatic representation of the various sheep body fluid compartments.

drug were displaced, then the maximum increase in the unbound fraction in the body would be 17.1%. However, as will be evident in Case #2, Case #1 cannot exist for tolbutamide in the sheep.

Table XXIV: Percent unbound tolbutamide in plasma interaction study #1 (Sheep #1494)

Time (hr)	% Unbound*1	% Increase*2	Sulfadimethoxine (mcg/ml)
1.0	18.2	-	-
2.0	20.0	-	-
3.0	22.5	-	-
4.0	51.7	156	155
6.0	64.8	221	288
9.1	50.4	150	170
12.0	40.0	98	125
14.5	45.2	124	98

*1 obtained from actual data values

*2 obtained from the mean (20.2%) of the first 3 data points in absence of sulfadimethoxine

Case 2 - drug bound and the unbound moeity allowed to diffuse into total body water: To assess the possible changes in % unbound with displacement one needs to determine the fraction of the dose bound in the body. Using Experiment II, Equation 12 may be written

$$\text{Dose} = Vd\ Cp \qquad \text{Eq. 12}$$

where Cp represents the total tolbutamide plasma concentration. Since the unbound concentration (C_f) in plasma represents 20% of the total concentration

$$Cp = 5\ C_f \qquad \text{Eq. 13}$$

Equation 13 applies. Therefore by substitution of Eq. 13 into Eq. 12 and using a Vd of 10.2L,

$$\text{Dose} = 51\ C_f \qquad \text{Eq. 14}$$

The same dose may also be accounted for by Fig. XXXI:

$$\text{Dose} = \text{Amount in } V_2 \qquad \text{Eq. 15}$$

$$= 1.86\ (5\ C_f) + 28.14\ C_f + x$$

where x represents the amount of the dose bound in $(V_2 - Vp)$ in Fig. XXXI. Therefore Eq. 16

$$x = \text{Dose} - 37.44\ C_f \qquad \text{Eq. 16}$$

may be obtained. Now dividing both sides of Eq. 16 by Dose and substituting Eq. 14 for Dose, results in Eq. 17 describing the

$$\frac{x}{\text{Dose}} = 1 - \frac{37.44\ C_f}{51\ C_f} = 0.265 \qquad \text{Eq. 17}$$

fraction of the dose bound in $(V_2 - Vp)$. Together with the knowledge that the fraction of the dose bound in the plasma is 0.146, the total fraction bound in this case would be about 41% of the dose. Total displacement of the bound drug could maximally produce a 70% increase in the unbound fraction in the body. Since the results obtained were higher than 70%, this case may also not apply. A cautionary word might, however, be added. Possibly the redistribution of the unbound moeity into the cell is slow relative to displacement thus causing a higher % unbound in the plasma than calculated in Case #2. Also, since the rate of excretion of total tolbutamide equivalents does not equal the rate of tolbutamide infusion after the administration of sulfadimethoxine (Fig. XXX), the amount of undistributed tolbutamide in the body (V_2-Vp) could be rising causing the exaggerated % unbound.

Case 3 - drug bound but the unbound moeity limited to the extracellular fluid: Using similar calculations as in Case 2, one arrives at Eq. 18 which describes the fraction of the dose bound in

$$\frac{x}{\text{Dose}} = 1 - \frac{19.94\ C_f}{51\ C_f} = 0.609 \qquad \text{Eq. 18}$$

(V_1 - Vp). Therefore the total fraction of the drug bound would be about 75% and displacement could maximally produce a 300% increase in the unbound fraction in the body. This value exceeds those obtained in the interaction study and suggests that the actual distribution of tolbutamide may be limited to the extracellular fluid.

Continuing, if the % unbound tolbutamide in any isolated sample is purely due to displacement by sulfadimethoxine then the value observed should be obtainable in vitro by the addition of a similar tolbutamide and sulfadimethoxine concentration. However, the % unbound determined at the resultant sulfadimethoxine levels (Table XXIV) are much higher than would be approximately expected from the in vitro displacement data (Fig. XXVI). Either a chemical species is generated in the animal which has potent displacing properties or another change must have taken place. This matter will be further discussed in Interaction Study #3.

The secondary fall in plasma glucose levels, generated (Fig. XXVII) after the initial depression (tolbutamide bolus), may be accounted for by the substantial and rapid increase in unbound tolbutamide during the sulfadimethoxine infusion since the unbound tolbutamide concentration is maximal at the point of greatest glucose depression. Alternately, this secondary depression of glucose may be due to the presence of sulfadimethoxine (Fig. XXIII, p.114). However the effect seen in the interaction study appears to be greater than what would be expected from Fig. XXIII.

The explanation for the entire shapes of the total and unbound tolbutamide plasma curves may be twofold. In either case, however, an assumption is made about the factor controlling the rate of removal of tolbutamide. The rate of removal may be dependent upon the unbound concentration (Chapter I). Alternately, the rate of removal could be dependent upon the total tolbutamide concentration. This latter method of removal will be dealt with first.

Case 1 - clearance dependent upon total plasma tolbutamide: In this case the shape of the curve would reflect only redistributional changes. Initially sulfadimethoxine would predominantly displace tolbutamide from plasma proteins with the unbound moeity largely diffusing out of plasma into tissues. Subsequently after sufficient distribution of sulfadimethoxine into tissues, displacement would occur there and cause a reversal of distribution of some unbound tolbutamide back into the plasma. Two aspects of the curves, however, cloud this case. First, the level of total tolbutamide in plasma begins to rise before the point of maximum sulfadimethoxine concentration. This will occur if sulfadimethoxine displaces tolbutamide from the tissues to a greater extent than from plasma proteins. Using the tolbutamide distribution, Case 3, it can be seen that 80% of tolbutamide is bound in the plasma while calculations show that about 75% is bound in the tissues. Possibly more drug displaced off the tissue binding sites enters than leaves the plasma volume. Although difficult to defend or refute, it seems a remote possibility. Another contradicting aspect is that the total tolbutamide levels appear to be higher after 11 hrs. than before the sulfadimethoxine infusions. According to this case, since the rate of tolbutamide infusion remains the same, the total tolbutamide concentration should return to

the presulfadimethoxine level. Yet the presiding problem is that sulfadimethoxine levels are non-steady-state, hindering the explanation.

Case 2 - clearance dependent upon unbound plasma tolbutamide: In this case, the unbound tolbutamide concentration would initially rise due to displacement and then eventually return to the presulfadimethoxine level as seen in Chapter I. The total level should fall and then due to the disappearance of sulfadimethoxine, begin to rise and return to the presulfadime thoxine level when all the sulfonamide has been eliminated. This case is clouded by the fact that again the total level begins to rise before the maximum sulfadimethoxine plasma concentration has been reached and by the fact that the unbound levels remain at a high value even when sulfadimethoxine plasma levels are dropping. The data could be explained by this case if in fact sulfadimethoxine was also blocking the metabolism of tolbutamide. It is felt that this case with the latter hypothesis might account for the results. Since the sulfadimethoxine levels are non-steady-state, it is difficult to evaluate at this time. Subsequent discussion will refer to the hypotheses as Case 1, Case 2 and Case 2-metabolic block.

The urinary data unfortunately does not provide good base-line information in the first 3 hours due to urine loss. Total tolbutamide equivalents excreted per hour should have reached about 75% of the tolbutamide infusion rate (Table VI, Experiment II). The regression of urinary rate of tolbutamide excretion on the total tolbutamide plasma concentration is seen in Figure XXXII.

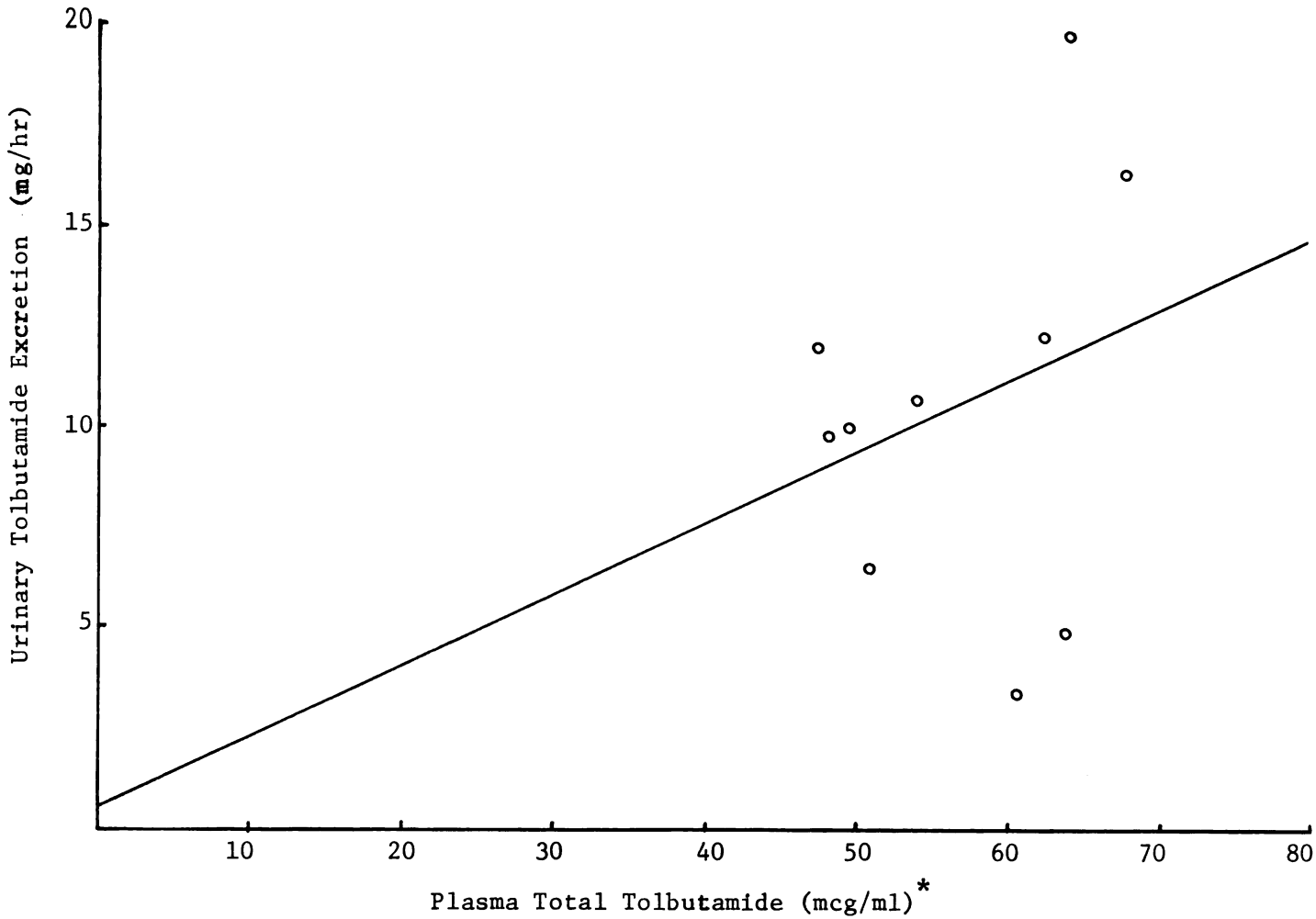


Fig. XXXII: Regression of urinary rate of tolbutamide excretion on the total plasma tolbutamide concentration (Interaction Study #1 - Experiment IX - standard error of slope and intercept is 0.22 and 12.7 respectively)

* at midpoint of excretion interval

Although the scatter is quite local, the correlation coefficient (r) of 0.27 indicates a lack of correlation between the two experimental measurements. However, the correlation coefficient (r) of 0.72 suggests the rate of urinary tolbutamide excretion is possibly related to the unbound tolbutamide plasma concentration as seen in Figure XXXIII. Both of these regression plots were obtained by linear interpolation of plasma concentrations to coincide with the midpoints of urinary excretion rates. The reduction in excretion rate of hydroxytolbutamide could be explained by Case 1 since the urinary curve is a delayed mirror image of the total plasma tolbutamide up to about 9 hrs., but the reduction in rate is greater than reduction in total plasma concentration. Thereafter the excretion rate is still rising while the plasma tolbutamide has levelled off, contradicting Case 1. The date could also be described by Case 2 - metabolic block, for the minimum in excretion rate takes place at the point of maximum plasma sulfadimethoxine. In this case the rise in plasma unbound tolbutamide should result in greater hydroxytolbutamide excretion but due to the metabolic block the excretion is in fact reduced. This case would also explain why the excretion rate continues to rise after 9 hours. The sulfadimethoxine blocking effect becomes less predominant with the unbound concentration (still high) causing an ever increasing excretion rate.

While considerable scatter is present in the plasma hydroxytolbutamide levels, coupled with the reduction in its rate of urinary excretion, the data suggest that sulfadimethoxine might impede the urinary excretion of this metabolite, because the metabolic rate of conversion of tolbutamide to hydroxytolbutamide has been decreased by sulfadimethoxine.

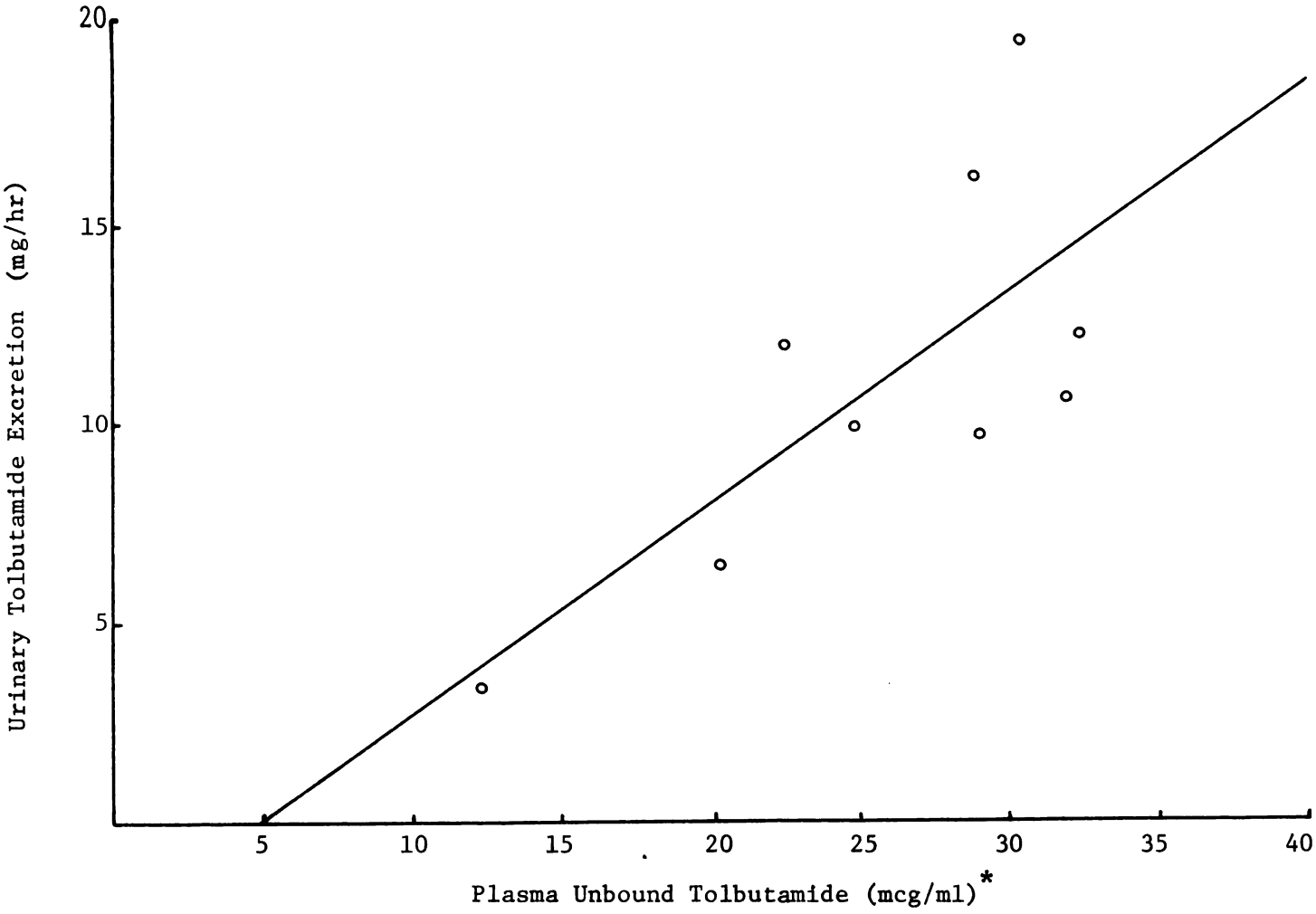


Fig. XXXIII: Regression of urinary rate of tolbutamide excretion on the unbound plasma tolbutamide concentration (Interaction study #1 - Experiment IX - standard error of slope and intercept is 0.19 and 5.1, respectively)

* at midpoint of excretion interval

Conclusion:

A bolus and constant infusion established a steady state tolbutamide plasma concentration of 61 mcg/ml. Sulfadimethoxine zero-order infusion (2 Gm/hr for 3 hrs) superimposed on an existing and continuing tolbutamide infusion caused a prolonged increase in the unbound tolbutamide concentration ranging from 100-220% above the previous steady state unbound concentration unaccountable by the mere presence of the determined sulfadimethoxine plasma levels. This large increase in unbound concentrations suggests that the major fraction occurring extravascularly is protein bound. Although inconclusive, the data suggest that the renal and metabolic clearance of tolbutamide are dependent upon the unbound concentration and that sulfadimethoxine is a metabolic inhibitor of tolbutamide. The results also suggest that sulfadimethoxine decreases the renal clearance of formed hydroxytolbutamide. Pharmacologically the data indicates that plasma glucose depression after the initiation of sulfadimethoxine infusion may be related to the resultant increase in the unbound tolbutamide concentration.

VI INTERACTION STUDY #2 (Hydroxytolbutamide-Sulfadimethoxine)

The observation that sulfadimethoxine administration caused an apparent decrease in renal clearance of hydroxytolbutamide in interaction study #1, prompted further investigation. Using kinetic data of Experiment VII, Table IX, as baseline information, a zero-order intravenous infusion of hydroxytolbutamide (155 mg/hr) was initiated in sheep #1494 permitted food and water ad-lib. After 1.75 hr. a simultaneous zero-order sulfadimethoxine infusion (2 Gm/hr) was begun and the combined infusions continued for another 3 hours after which time they both were discontinued. Blood and urine samples were collected with the results recorded as Experiment X. The plasma hydroxytolbutamide levels as well as the urinary hydroxytolbutamide excretion rates from the experiment can be seen in Figure XXXIV. The corresponding plasma sulfadimethoxine levels obtained after initiation of its infusion can be seen in Figure XXXV.

Discussion:

The steady state hydroxytolbutamide plasma concentration reached appears to be 3.5 mcg/ml. However, sulfadimethoxine appears to temporarily lower this value a little. This may be due to plasma protein displacement of hydroxytolbutamide (bound about 80% - Table XIX) by sulfadimethoxine. The rate of urinary excretion of hydroxytolbutamide approximates the rate of infusion of hydroxytolbutamide thus indicating that essentially all the hydroxytolbutamide is excreted unchanged. The apparent plasma clearance of hydroxytolbutamide based upon the infusion rate and a plasma concentration of 3.5 mcg/ml is 738 ml/min, a value of about 33% of cardiac plasma output. Using this clearance value instead of that (1350 ml/min) found in Experiment VII (Table IX p.89) would result in an area of

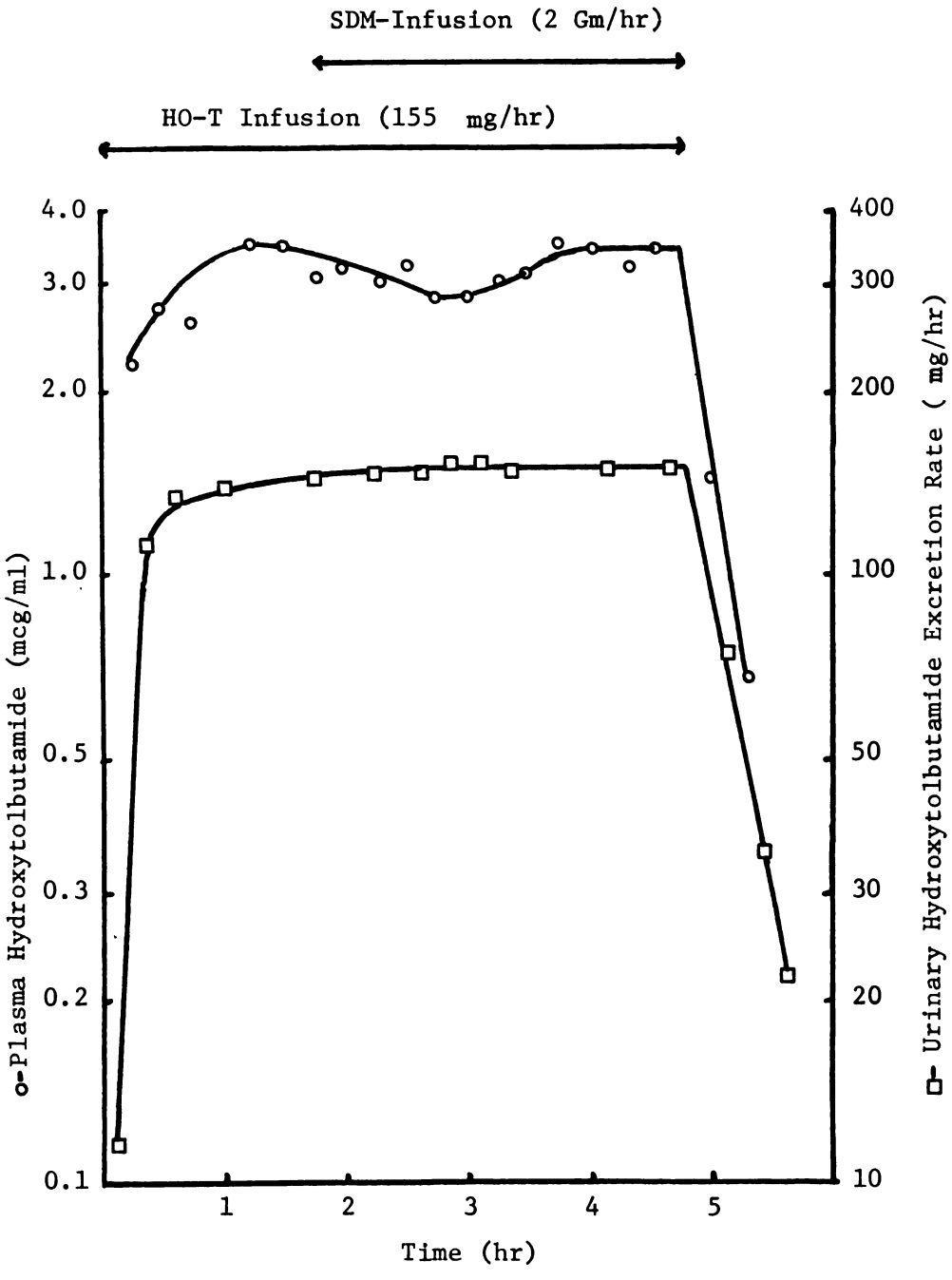


Fig. XXXIV: Plasma hydroxytolbutamide and urinary excretion rate of interaction study #2 (Experiment X - sheep #1494) SDM = sulfadimethoxine; HO-T = hydroxytolbutamide.

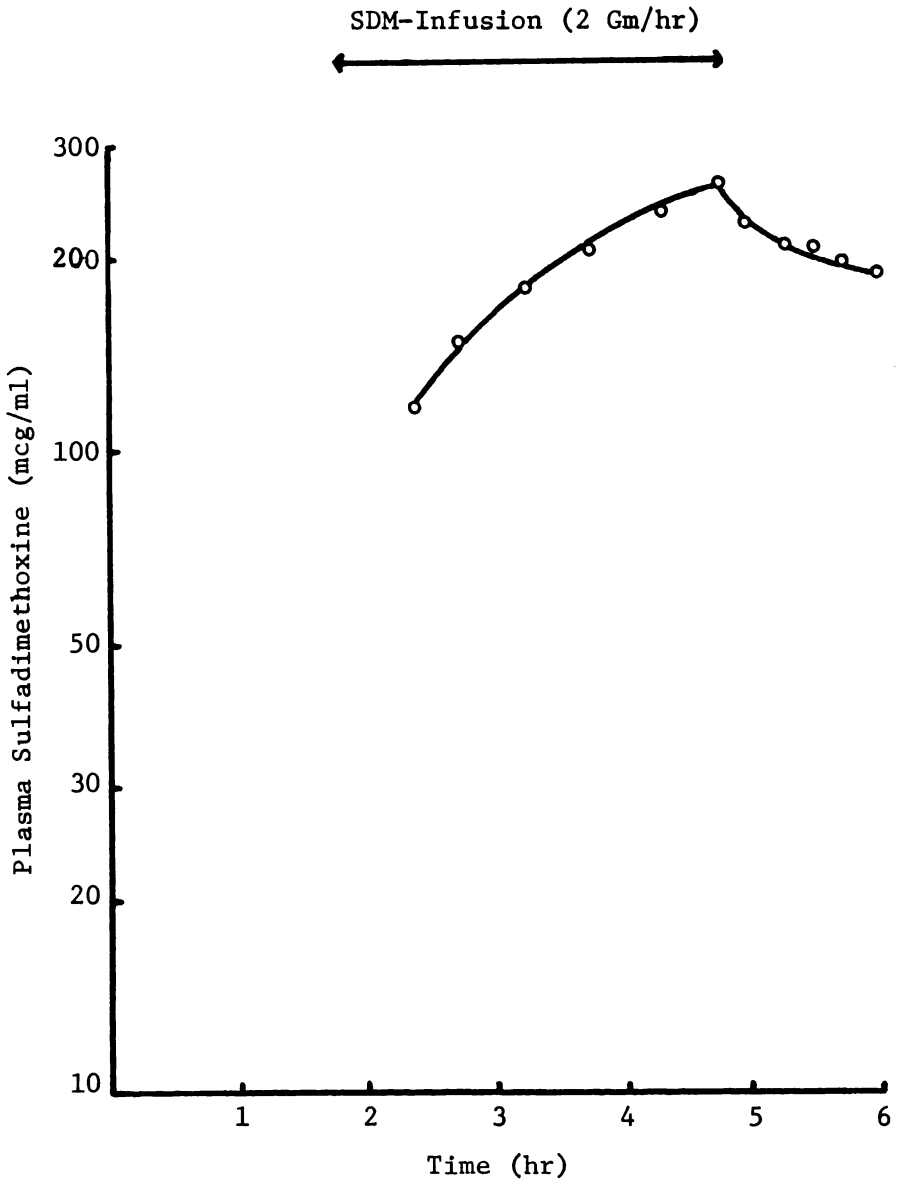


Fig. XXXV: Plasma sulfadimethoxine of interaction study #2 (Experiment X).
SDM = sulfadimethoxine

5.65 mcg/ml x hr based upon a hydroxytolbutamide dose of 250 mg (Dose/clearance = area). If this area value were used in Table XI p.90 , the percent of the tolbutamide dose converted to hydroxytolbutamide for Experiments II and III would be 75.3 and 51.1% respectively. These values are fairly close to those (54 to 66%) actually found in Table VI p.71 . The clearance (738 ml/min) must indicate that the clearance is dependent upon the total plasma concentration. Since the urinary excretion rate has not changed during the sulfadimethoxine co-administration, the apparent slight drop in plasma hydroxytolbutamide does suggest an increase in the renal clearance of hydroxytolbutamide. However, considerable weight is placed on the plasma values at 1.25 and 1.5 hr in order to make this suggestion for which there is no established explanation. The results in Interaction Study #1 which suggested a decrease in renal clearance of hydroxytolbutamide due to the presence of sulfadimethoxine cannot be due to an interaction by the chemical entities but may be an artifact of that particular study. It was noted in this connection that the urinary output after initiation of sulfadimethoxine infusion in Interaction Study #1 was down (about 25%). A decrease in urine flow might simply reflect a greater reabsorption of water in the renal tubules, but this would not be expected to decrease the renal clearance of hydroxytolbutamide. Alternately, a decrease in urine flow could be indicative of a decrease in renal plasma flow. Since the clearance of hydroxytolbutamide must be near renal plasma flow, a reduction in renal plasma flow would result in a decreased clearance of hydroxytolbutamide.

A sample of blood in this study was analyzed for hydroxytolbutamide and found to have a lower value than that found in its plasma. The blood concentration was found to equal the plasma concentration if corrected for hematocrit indicating as shown earlier that hydroxytolbutamide does

not appear to partition significantly into red blood cells. It is interesting to note that the plasma clearance value obtained in this study (738 ml/min) is much lower than that obtained by an I.V. bolus of hydroxytolbutamide (1350 ml/min, Experiment VII, Table IX). Evidently, the I.V. bolus doesn't provide a good estimate of the area under the curve although the half-life (13.5 min) obtained from the I.V. bolus study (Experiment VII, Table IX) is the same obtained from the log-linear plasma hydroxytolbutamide concentrations after the steady-state infusion is stopped (Fig.XXXIV). The discrepancy in clearances remains unaccounted for.

Conclusion:

Sulfadimethoxine (2 Gm/hr for 3 hrs) given during steady-state plasma hydroxytolbutamide (infusion 155 mg/hr) does not decrease the renal clearance of hydroxytolbutamide which in this study was found to be 738 ml plasma per minute.

VII INTERACTION STUDY #3 (TOLBUTAMIDE-SULFADIMETHOXINE)

After confirmation in Interaction Study #2 that the clearance of hydroxytolbutamide was not reduced by the co-administration of sufadimethoxine, it was decided to repeat Interaction Study #1 except that sulfadimethoxine was also to be at a steady-state. This would then permit a more thorough assessment of the interaction between tolbutamide and sulfadimethoxine.

In order to facilitate urine flow, the lack of which had presented a problem in Study #1, the sheep was administered about 2 ml/min (I.V. drip) normal saline throughout the experiment. The animal was placed in the experimentation cage the night before the study to alleviate nervousness encountered in other studies. The study was initiated with an I.V. bolus of 565 mg tolbutamide followed immediately with a zero-order infusion of 95 mg/hr; a regimen estimated, from previous I.V. bolus data, to immediately attain and maintain a plasma concentration of 50 mcg/ml. At 4 hrs, a bolus of 2.44×10^7 dpm ^3H - hydroxytolbutamide was given. Then at 6 hrs the co-administration of sulfadimethoxine was started using a bolus of 3.2 Gm and a zero-order infusion of 285 mg/hr. At 12 hrs a repeat dose of 2.44×10^7 dpm ^3H - hydroxytolbutamide was administered followed at 14 hrs with a dose of 2.47×10^8 dpm ^{14}C - tolbutamide. Then at 19 hrs the sulfadimethoxine administration was changed - an additional 1.5 Gm bolus given followed by a new infusion rate of 400 mg/hr. Tolbutamide infusion was halted at 20 hrs while the infusion of sulfadimethoxine was stopped at 28.5 hrs. Throughout the study, blood and urine samples were taken.

Many results have been obtained from this study. Figures XXXVI a & b indicate what changes took place in total and unbound tolbutamide

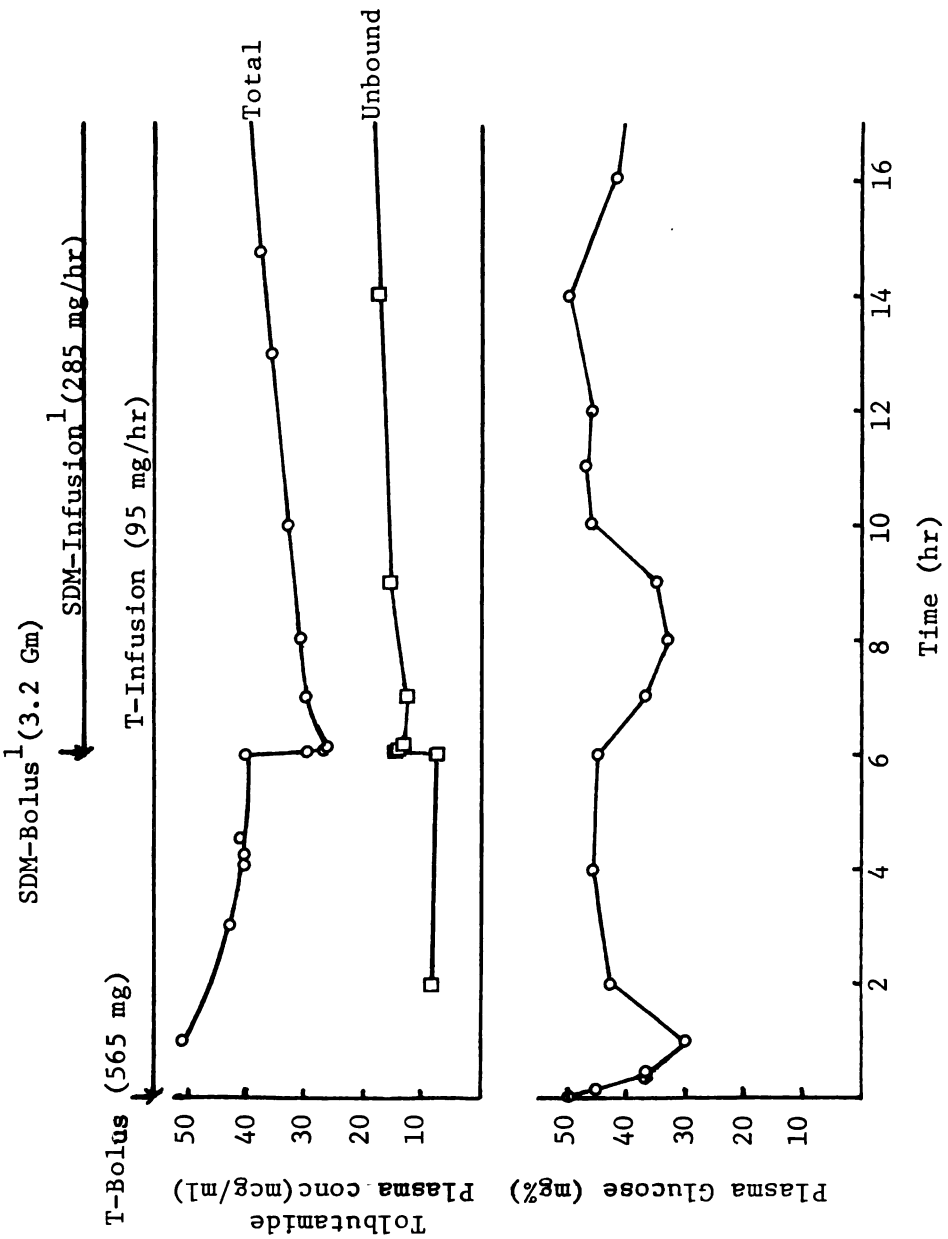


Fig. XXXVIa: Plasma total tolbutamide, unbound tolbutamide and glucose observed in Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73
SDM = sulfadimethoxine; T = tolbutamide

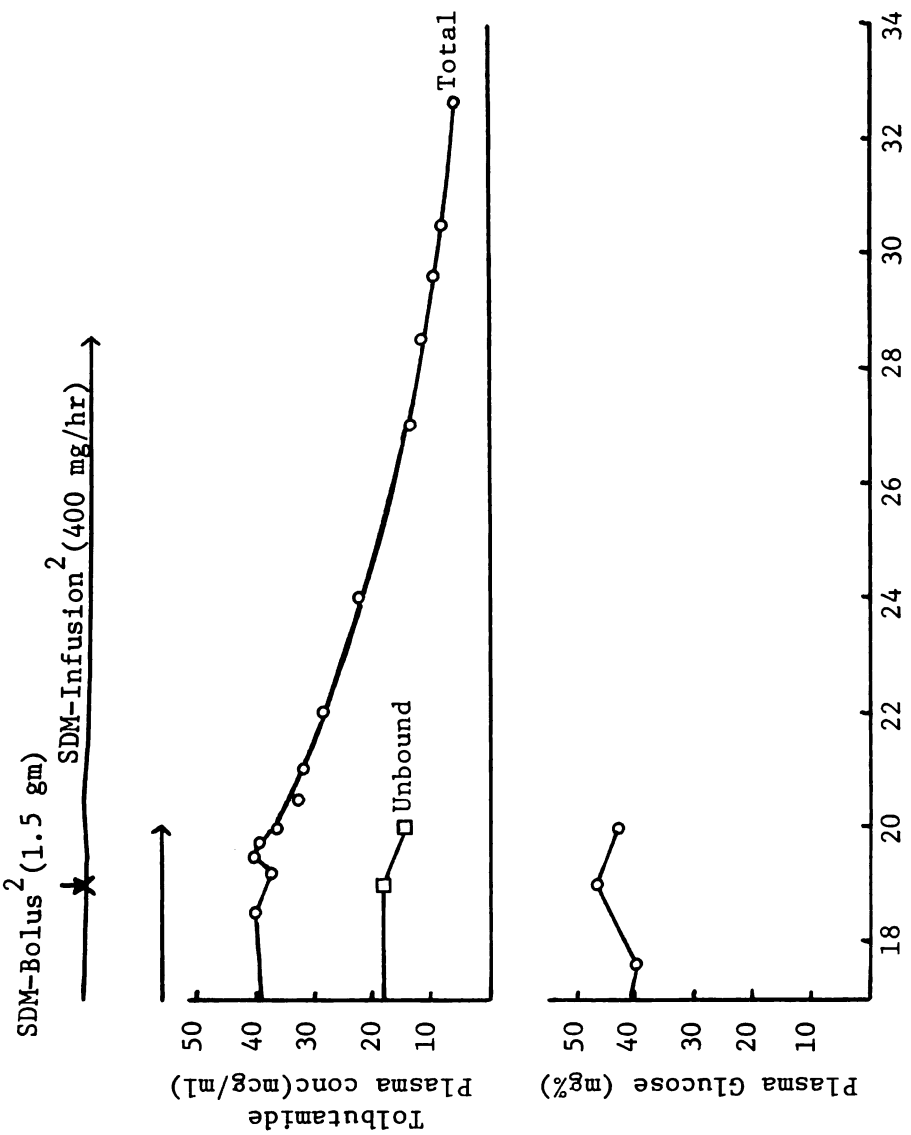


Fig. XXXVI b: Plasma total tolbutamide, unbound tolbutamide and glucose observed in Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73) SDM = sulfadimethoxine

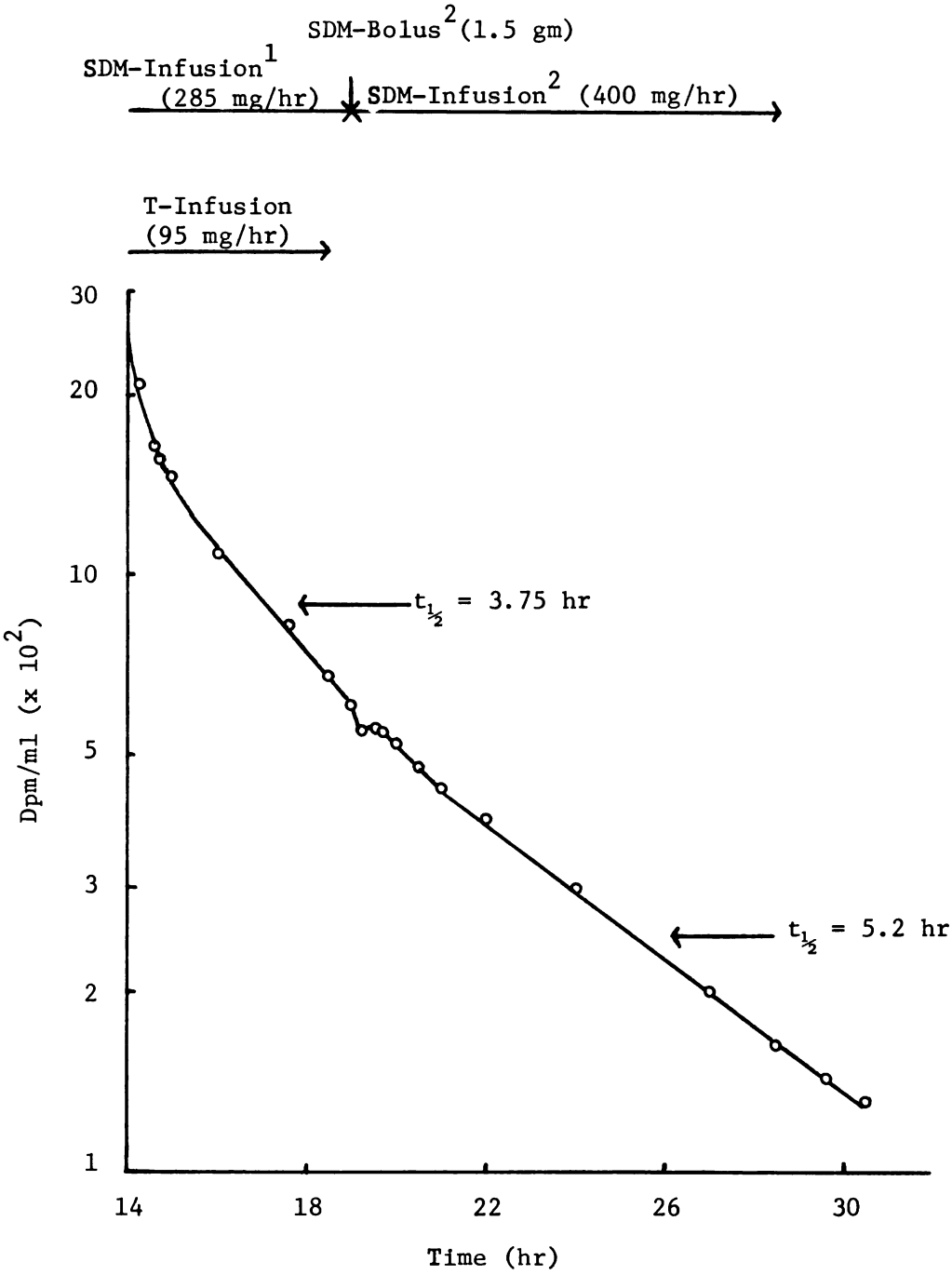


Fig. XXXVII: Plasma ^{14}C activity found after the I.V. administration of 2.47 x 10^8 dpm ^{14}C - tolbutamide in Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73)
SDM = sulfadimethoxine; T = tolbutamide

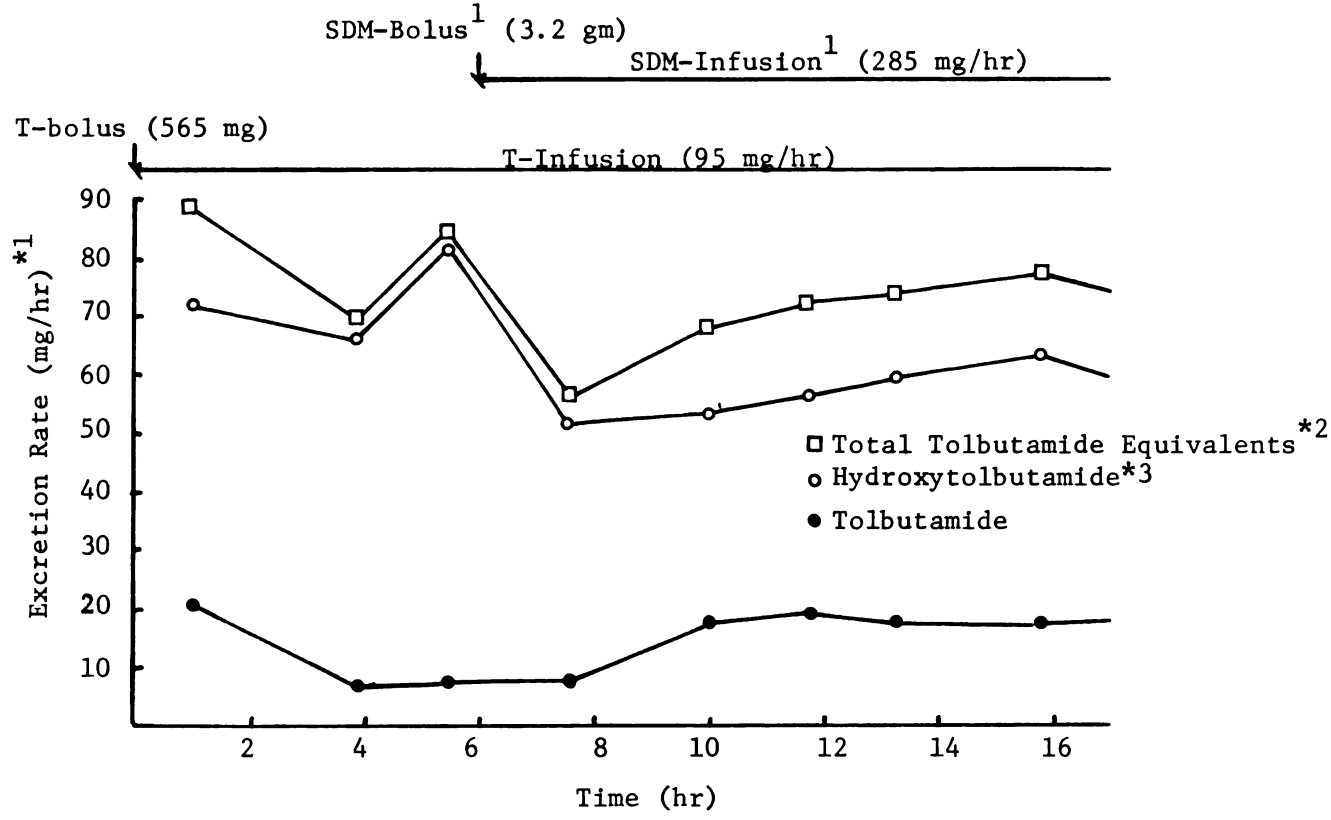


Fig. XXXVIIIa: Urinary excretion rates of tolbutamide, hydroxytolbutamide and total tolbutamide equivalents observed in Interaction Study #3 (Sheep #1494 Experiment XI - 5/22/73)

*1 midpoint plots SDM = sulfadimethoxine; T = tolbutamide
*2 see p. 51
*3 see p. 51

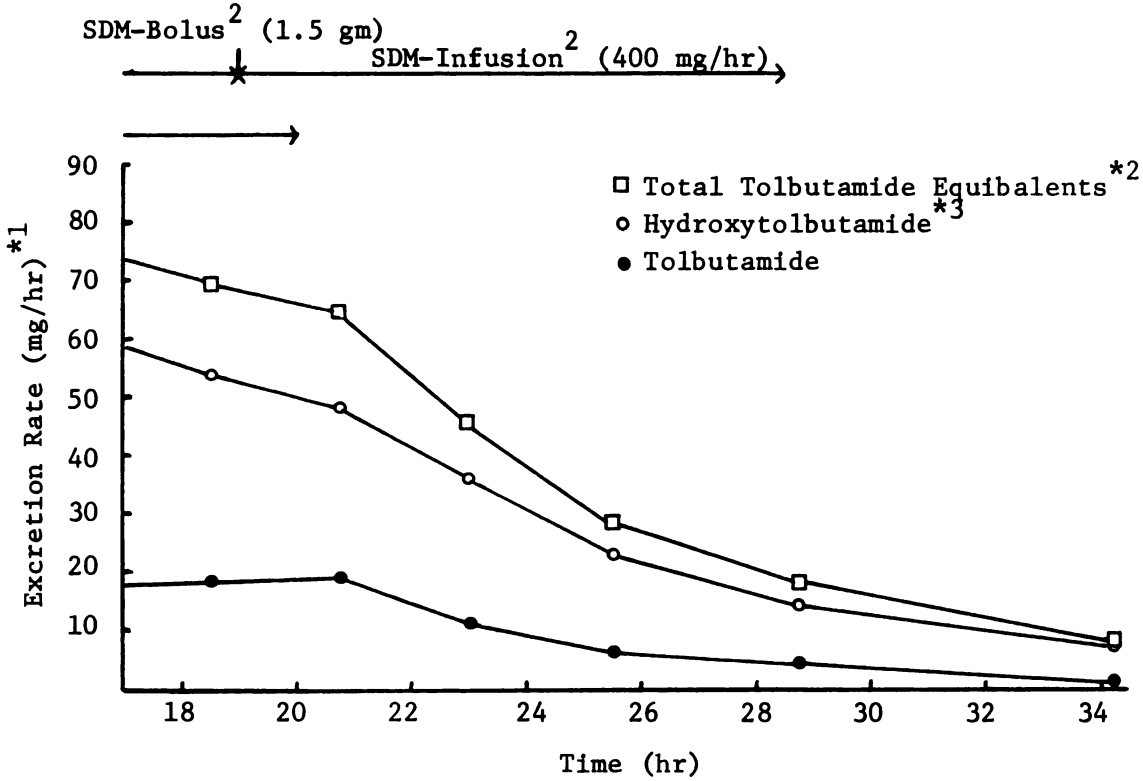


Fig. XXXVIIIb: Urinary excretion rates of tolbutamide, hydroxytolbutamide and total tolbutamide equivalents observed in Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73)

*1 - midpoint plots
*2 - see p.51
*3 - see p.51

SDM = sulfadimethoxine

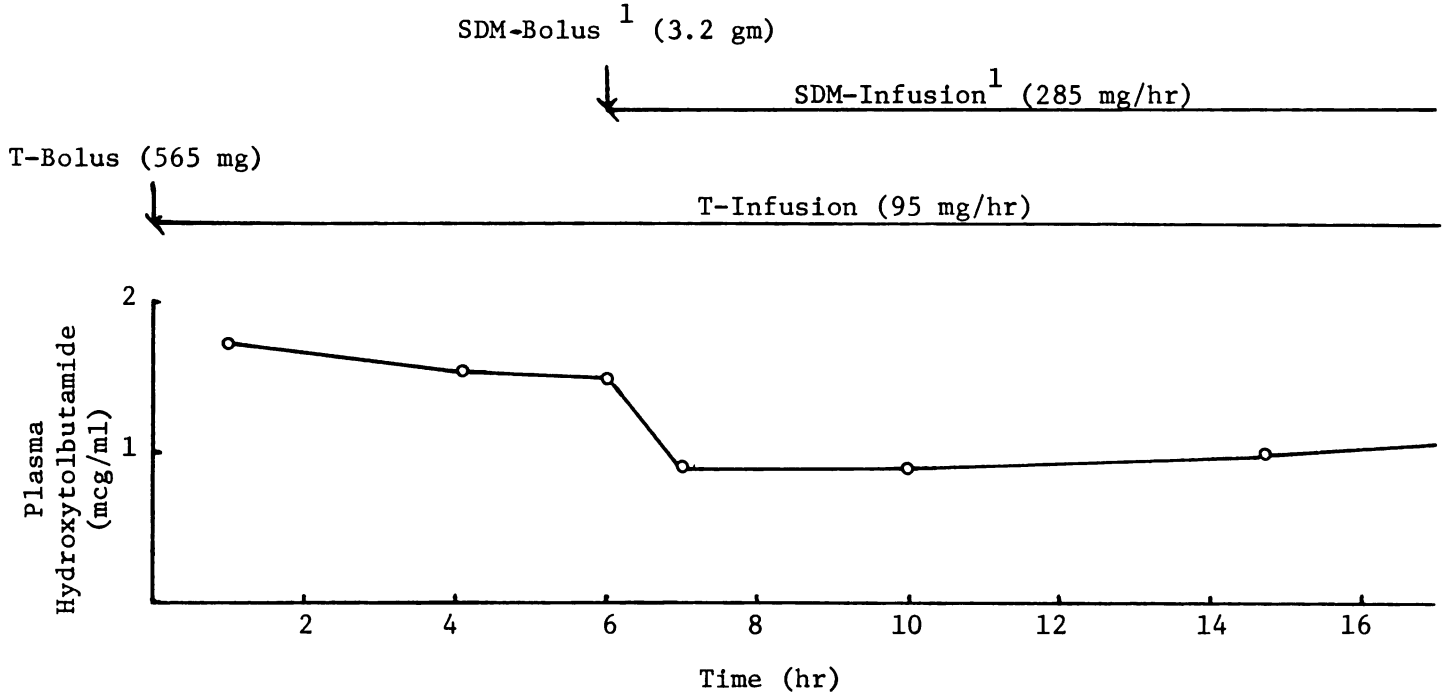


Fig. XXXIXa: Plasma hydroxytolbutamide found in Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73)

SDM = sulfadimethoxine; T = tolbutamide

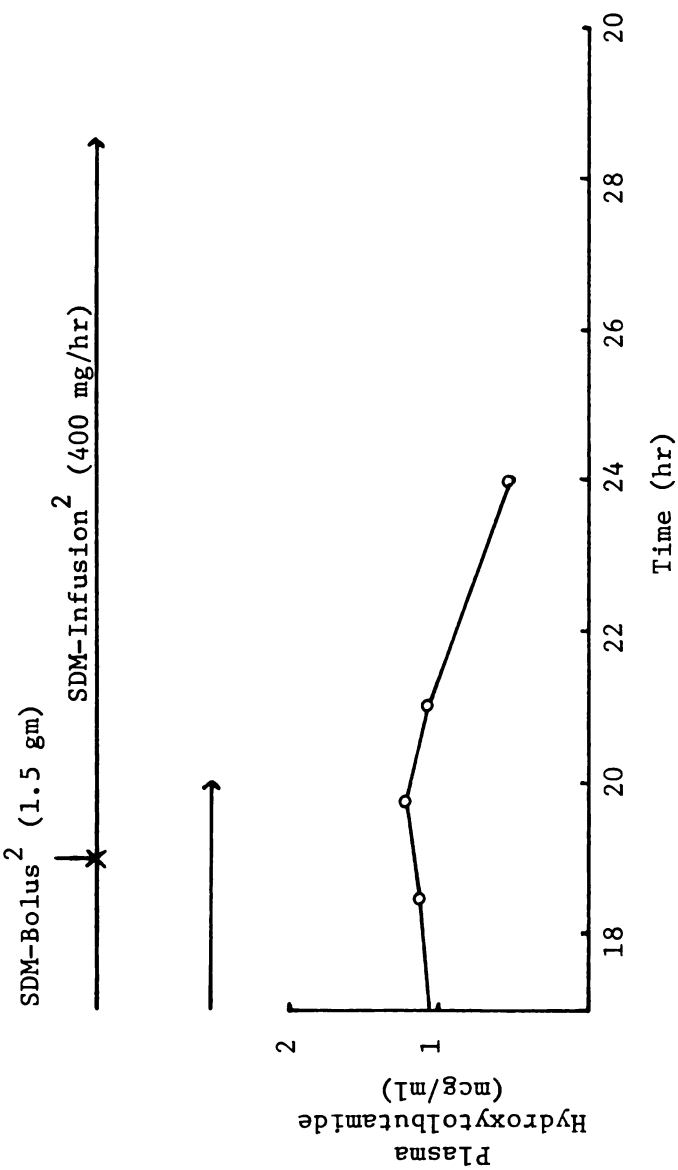


Fig. XXXIXb: Plasma hydroxytolbutamide found in Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73)

SDM = sulfadimethoxine

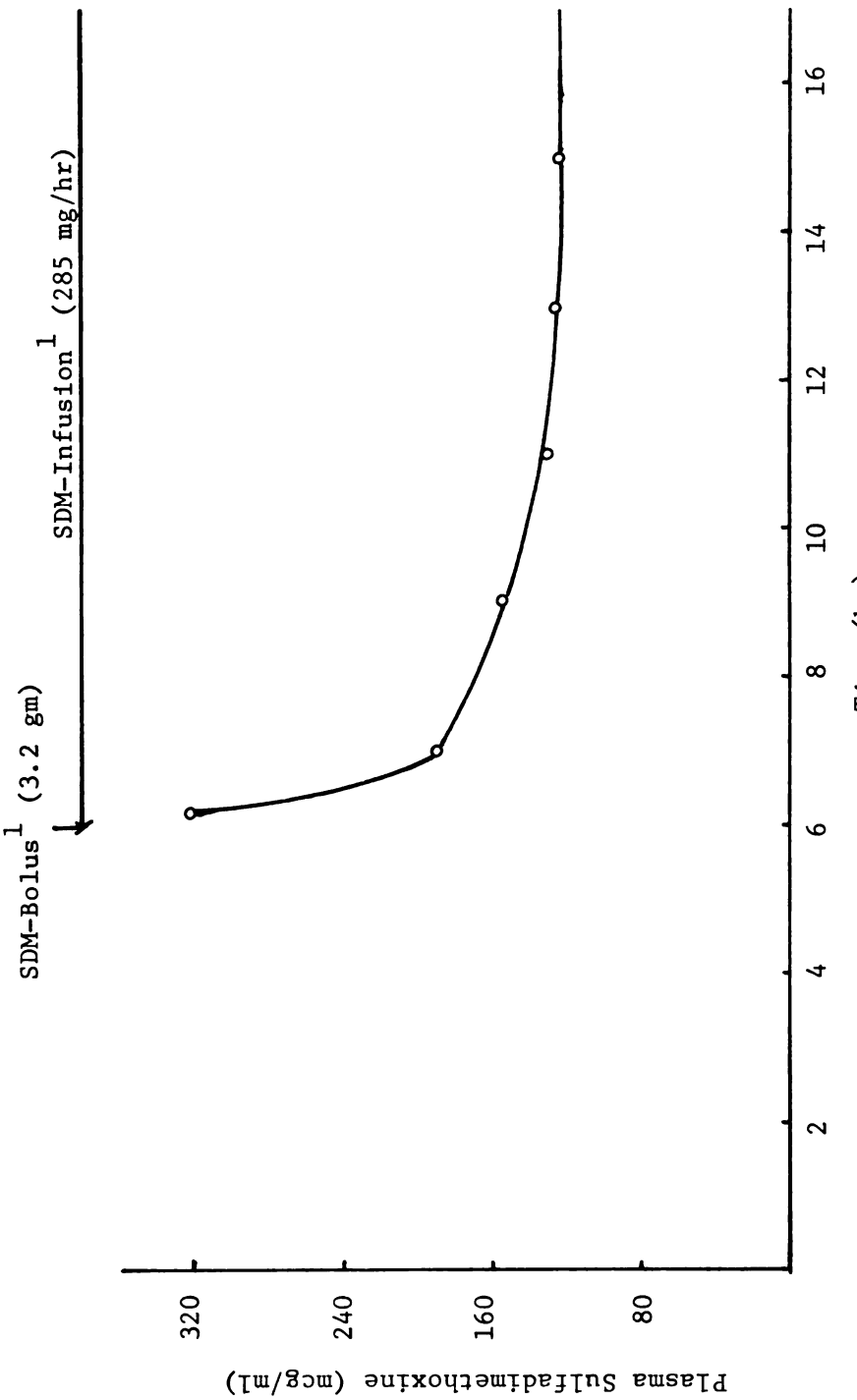


Fig. XLa: Plasma sulfadimethoxine observed during Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73)

SDM = sulfadimethoxine

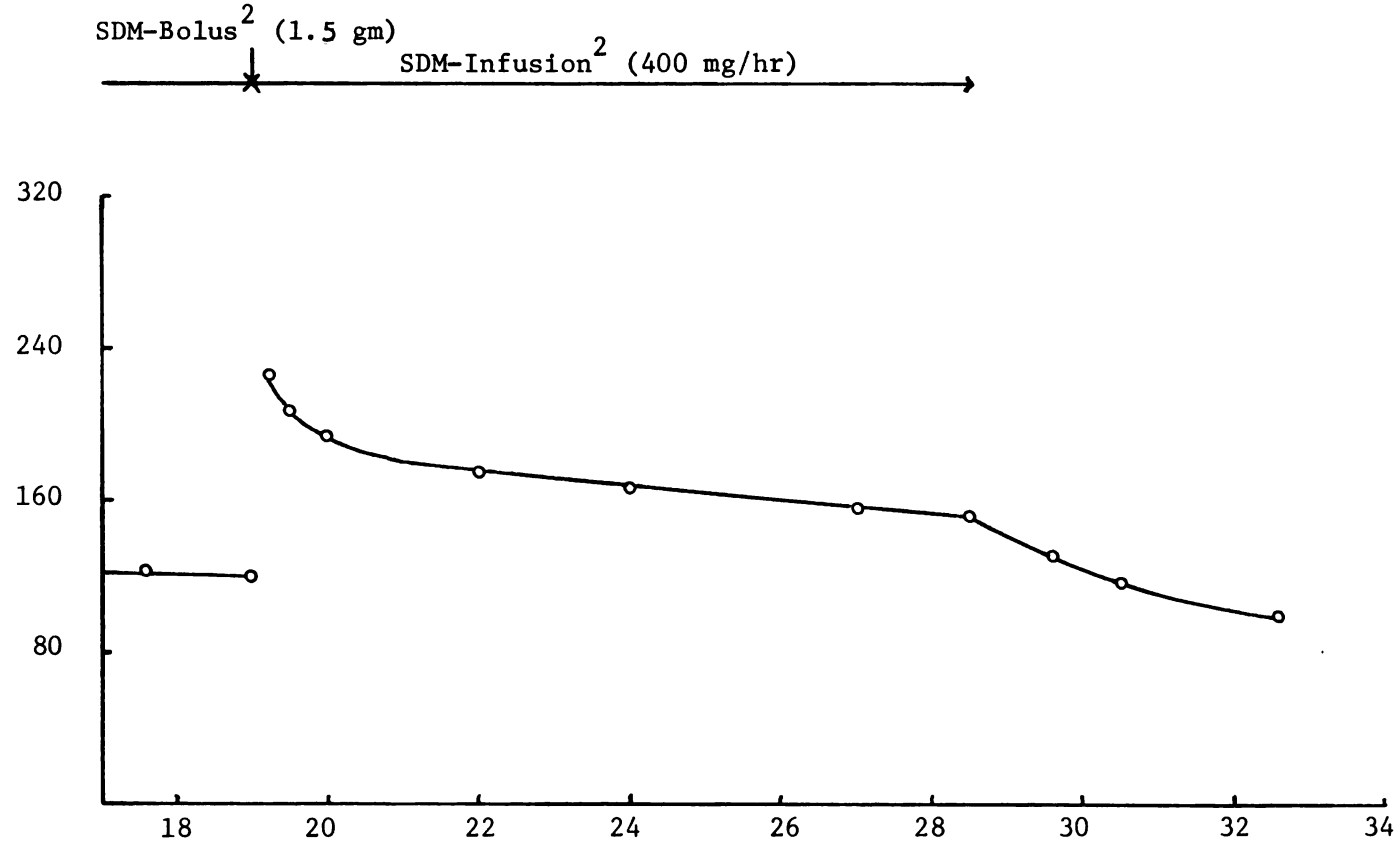


Fig. XLb: Plasma sulfadimethoxine observed during Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73)

SDM = sulfadimethoxine

as well as in plasma glucose. The results of the bolus of ^{14}C - tolbutamide are seen in Figure XXXVII. The urinary rates of excretion data of parent tolbutamide, hydroxytolbutamide and total tolbutamide equivalents are seen in Figures XXXVIII a & b. The corresponding plasma hydroxytolbutamide levels are seen in Figures XXXIX a & b while the plasma levels of sulfadimethoxine are seen in Figures XL a & b. All of the data is recorded as Experiment XI in the Appendix.

Discussion:

During the discussion, each set of figures will be dealt with separately. Throughout the discussion., Phase 1 refers to times 0.6 hrs when tolbutamide is at steady-state, Phase 2 refers to times 6-19 hrs when sulfadimethoxine infusion is maintained at 285 mg/hr while Phase 3 encompasses events 19 hrs onwards, i.e. after the second bolus of sulfadimethoxine followed by the new infusion rate (400 mg/hr). The tolbutamide picture seen in Figures XXXVI a & b again is rather interesting. The initial changes after the first bolus of sulfadimethoxine (6 hr) again reflect plasma protein displacement of tolbutamide by sulfadimethoxine. The sharpness in the drop in total plasma, due to extravascular redistribution of displaced unbound tolbutamide, indicates the rapidity of the distribution phenomenon. Such results in the time period 6.00 to 6.13 hrs are very similar to those obtained by McQueen and Wardell[68] who followed the displacement of sulfadoxine by phenylbutazone for 30 minutes also in the sheep. The results in this study (#3) are again very much like those in study #1 in which the initial changes are followed by a rise in both total and unbound tolbutamide to a new apparent steady-state at 19 hrs. Using the data in Table XXIII, p.114 (Plasma 1494B), certain values for the fraction of tolbutamide unbound (α) could be

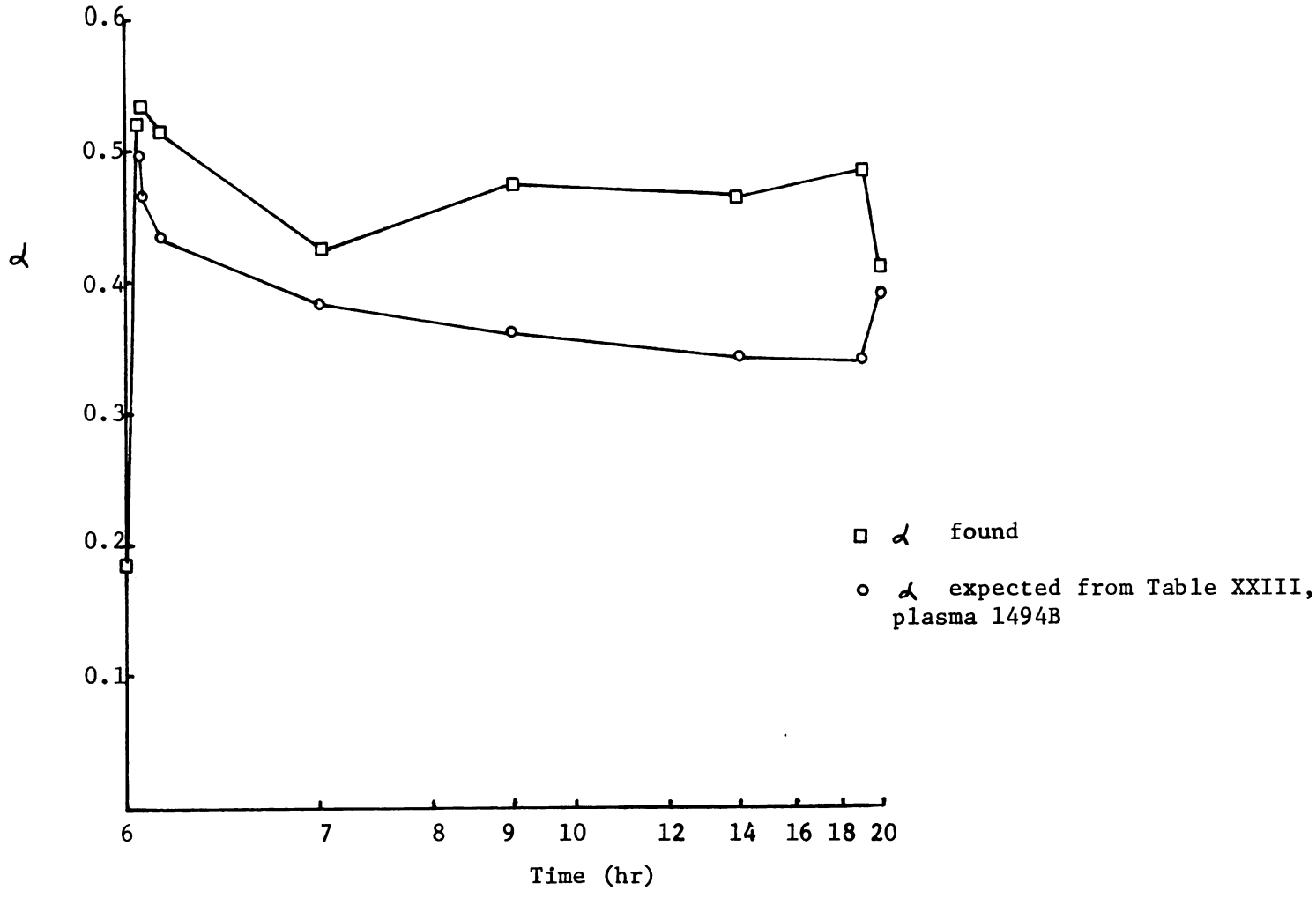


Fig. XLI: The fraction of tolbutamide unbound (α) found in Interaction Study #3, and expected based upon the plasma sulfadimethoxine concentration (Table XXV) found during the study and the in vitro alteration of tolbutamide binding by sulfadimethoxine (Table XXIII, Plasma 1494B)

anticipated based upon the sulfadimethoxine concentrations found during Interaction Study #3. The expected, and found values of α agree reasonably well at the 6.85 and 19.97 hr Time point (Figure XLI). However, thereafter the unbound tolbutamide levels are unaccountably high in the light of the concurrent plasma sulfadimethoxine levels (Table XXV). The question arose whether an ^{4}N-conjugated sulfonamide metabolite was generated in sufficiently high concentrations to account for the high unbound tolbutamide levels. The Bratton-Marshall assay prior to heating (p.51) will measure all sulfonamides which have an unconjugated ^{4}N position. In the light of evidence that ^{4}N-conjugated sulfonamides are in general more strongly bound to plasma proteins than the parent sulfonamide[97] and could displace tolbutamide, raised the question whether an accumulation of ^{4}N-conjugated metabolite(s) might have occurred to account for the high values of α found.

Table XXV: Fraction unbound plasma tolbutamide (α) found and expected in Interaction Study #3 (5/22/73)

Time (hr)	SDM (mcg/ml)	α^1 Found	α^2 Expected*1	α^1 / α^2	FFA*2 (mcM/ml)
0	-	-	-	-	0.66
2.00	-	0.183	-	-	0.51
6.00	-	0.185	-	-	0.42
6.05	620	0.520	0.499	1.04	-
6.06	525	0.533	0.468	1.14	-
6.13	324	0.515	0.436	1.18	-
7.01	190	0.426	0.386	1.10	0.33
9.04	154	0.475	0.363	1.31	0.44
13.97	124	0.465	0.343	1.36	-
18.51	122	-	-	-	0.52
19.00	120	0.485	0.340	1.43	-
19.97	196	0.410	0.390	1.05	-

*1 using the presiding sulfadimethoxine concentrations and by linear interpolation of the data of Table XXIII, p.114 (Plasma #1494B; 8/17/73)
*2 non-esterified fatty acids SDM = sulfadimethoxine

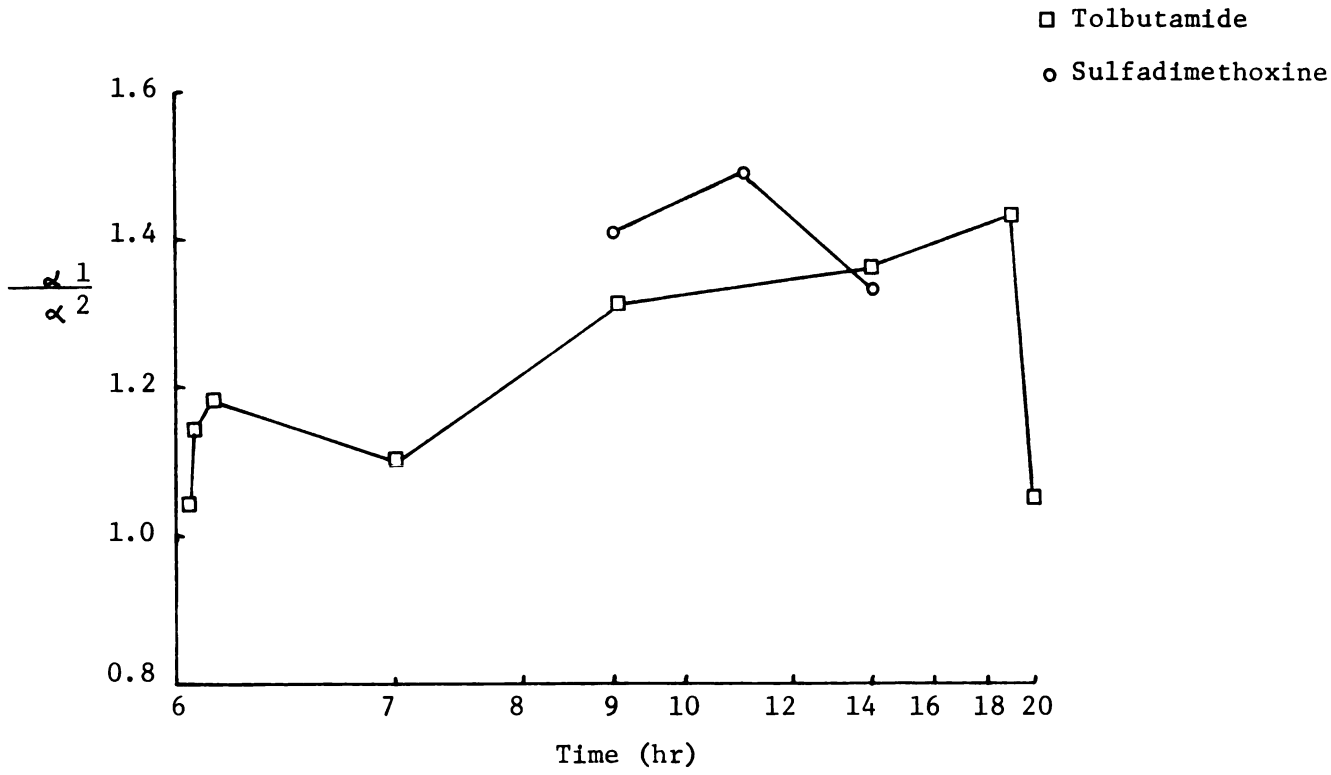


Fig. XLII: Ratios of fractions of unbound tolbutamide or sulfadimethoxine found (α^1) to fractions of unbound tolbutamide or sulfadimethoxine expected (α^2) during Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73) α^2 tolbutamide - from presiding sulfadimethoxine levels and Table XXIII, p.114 (Plasma #1494B, 8/17/73)
α^2 sulfadimethoxine - from Table XXXI

Samples at 7.01, 9.04, 11.00 and 19.00 hrs when analyzed did not contain detectable concentrations of ^{4}N-conjugated sulfadimethoxine metabolite(s) so this possibility was ruled out. It was decided to analyze some of the sulfadimethoxine samples to see whether in fact its unbound levels were also higher than expected. Using the method of equilibrium dialysis, the fraction unbound sulfadimethoxine (α) at 9.04, 11.00 and 13.97 hrs was found to be 0.33, 0.294 and 0.25 respectively. These values are higher than would be expected from the equilibrium dialysis data on the plasma binding of sulfadimethoxine (Table XXI, p. 111 , linear interpolation). The ratios of α (found)/ α (expected) for various times have been plotted for both tolbutamide and sulfadimethoxine in Figure XLII. Plasma protein analysis was performed on the sample taken at 13.03 hr with the results seen in Table XX, p. 109 (Sample - 1494 D), almost identical to other plasma samples analyzed from sheep #1494. Evidently, the composition of the plasma proteins could not account for the differences seen. It was then speculated that an endogenous species might have been generated which would displace both sulfadimethoxine and tolbutamide thus giving the unaccountably high results. Such a species might be non-esterified fatty acids (FFA). However some discrepancies exist about the effect of FFA as displacers of drugs from plasma proteins. Rudman *et al* [91] have reported that *in vitro* palmitate at a molar ratio of 7 times that of bovine serum albumin inhibits the binding of 8 ligands (including salicylic acid and phenylbutazone) to the protein. However they indicated the displacement effect was minor or absent *in vivo* in rabbits when the FFA concentrations were below 2 mcEq/ml. Mukherjee[29] has shown the reverse in that the addition of 0.61 to 1.41 mcEq/ml FFA (palmitate) to human serum causes an increase of 5.6 to 26.5% in the

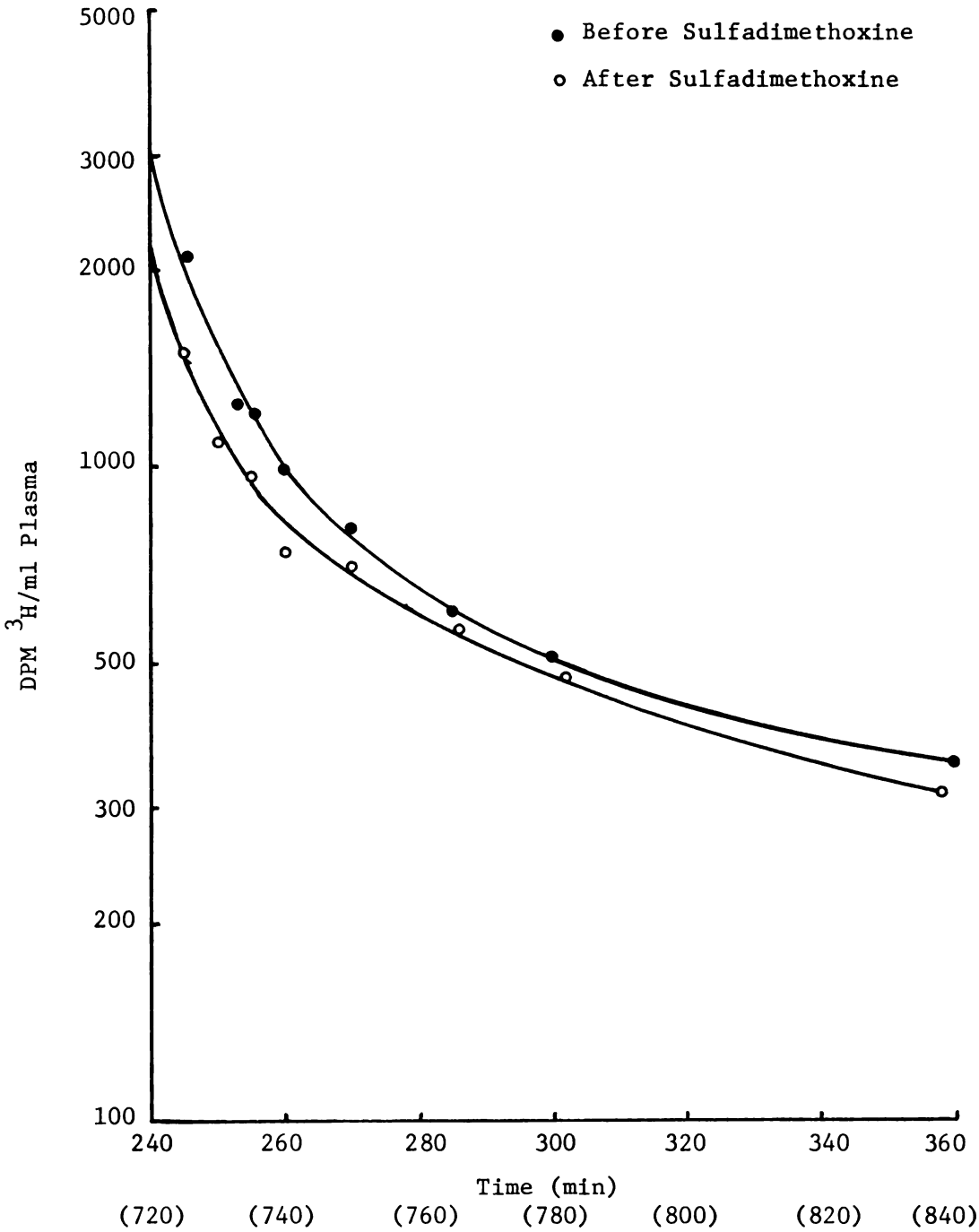


Fig. XLIII: Plasma ^{3}H dpm/ml after a bolus of 2.44 x 10^{7} dpm ^{3}H-hydroxytolbutamide before, and after sulfadimethoxine co-administration during Interaction Study #3 (Sheep #1494 - Experiment XI - 5/22/73)

binding of benzyl penicillin (3.3 mcg/ml). Although no mention is made of the FFA concentration in normal serum, the author indicates that when the FFA concentration is increased by 50% of the original level normally present in serum, the increment of binding is about 12.5%, while it exceeds 20% when the FFA concentration is doubled. The explanation for the observed results by the author is that the FFA changes the affinity and/or binding sites of the protein thus causing an increase in the binding of the benzyl penicillin molecule. The results of FFA analysis of various plasma samples in this present study are recorded in Table XXV. An apparent depression of the FFA levels could be indicative of the antilipolytic effects of tolbutamide reported by Feldman and Lebovity[40]. They report a decrease in FFA after a dose of tolbutamide. The reduction of FFA in this study might account for some of the decreased binding, but the apparent rise in FFA after 9 hrs would probably negate the possibility that lack of FFA accounts for the lower than anticipated binding of both sulfadimethoxine and tolbutamide at Phase 2 (Figure XLII). In vitro work would be required to assess the influence of FFA on the binding of both sulfadimethoxine and tolbutamide.

Plasma ^{3}H dpm-time curves found after the administration of ^{3}H-hydroxytolbutamide (2.44×10^{7} dpm), before and after the sulfadimethoxine administration can be seen in Figure XLIII. As can be seen, the lines appear to both exhibit continual curvature while identical in shape. The curve after sulfadimethoxine appears to be lower thus suggesting that the apparent volume of distribution of hydroxytolbutamide has increased. It is difficult to assess changes in clearance in Figure XLIII because 1) the lines exhibit continual curvature; 2) since in fact only 0.2 ml of plasma was counted, the net radioactivity (cpm observed - cpm blank)

Table XXVI: Kinetic parameter comparison of tolbutamide - Interaction Study #3(5/22/73)

Experiment	Date	$\alpha(hr^{-1})$	$\beta(hr^{-1})$	$t_{\frac{1}{2}\beta}$ (hr)	k_{12} (hr^{-1})	k_{21} (hr^{-1})	k_{10} (hr^{-1})	V_1 (ml)	V_2 (ml)	Vd (ml)	CL (ml/min)
Experiment V*1	6/6/73	1.15	0.73	4.0	0.313	0.745	0.267	8600	3612	12212	38.3
^{14}C-Tolbut.*2	5/22/73	1.73	0.185	3.75	0.50	1.14	0.28	9500	4170	13670	44.3

*1 I.V. bolus of 860 mg tolbutamide in Sheep #1494

*2 Phase 2 of Interaction Study #3

$$\frac{V_1^2}{V_1^1} = \frac{9500}{8600} = 1.10$$

$$\frac{Vd^2}{Vd^1} = \frac{13670}{12212} = 1.12$$

was very low during the latter time points; 3) the I.V. bolus of hydroxytolbutamide appears to provide a poor estimate of clearance (p.87). Yet Figure XLIII suggests that sulfadimethoxine does not change the elimination of hydroxytolbutamide to any great extent.

The administration of ^{14}C-tolbutamide at Phase 2 of the study with the subsequent decay during Phase 2 and Phase 3 resulted in a change in half life from 3.75 hrs (Phase 2) to 5.2 hrs (Phase 3 -Figure XXXVII). The kinetic parameters of Phase 2 are compared to an I.V. bolus of tolbutamide given after this interaction study in Table XXVI. Although the elimination rate constant (k_{10}) and the apparent volumes have not changed greatly, the resultant clearance of ^{14}C-tolbutamide is about 16% higher. If one can assume that Experiment V provides the apparent volume of distribution (Vd) of tolbutamide before sulfadimethoxine, then it becomes apparent that a change in Vd is a poor index of an increase in the fraction unbound in the body. Using the fraction unbound (α) values of Table XXV and the Vd values of Table XXVI, then $\frac{\alpha}{Vd}$ has not remained constant: $\frac{\alpha}{Vd}$ (at 6 hr) = $\frac{0.185}{12212} = 1.5 \times 10^{-5}$; $\frac{\alpha}{Vd}$ (at 19 hr) = 3.5×10^{-5} . This suggests that sulfadimethoxine itself and/or changes produced by it, has caused a significant change in the tissue binding of tolbutamide. It may also indicate that the distribution of the unbound tolbutamide molecule is confined to a certain volume. It should be noted here that the question of the change in half-life of tolbutamide from Phase 2 (3.75 hr) to Phase 3 (5.2 hr) will be discussed later.

Again as in Interaction Study #1, the plasma glucose changes appear to coincide with changes in unbound tolbutamide concentrations. The return to baseline conditions in Phase 1 and 2 must be accounted for by readjustment of the body to the challenge of depressed plasma glucose levels.

Table XXVII: Some clearance values of T and SDM determined during Interaction Study #3

Time	Parameter	Formula	Ro^t	Ro^s	C_{tt}	C_{ut}	C_{ts}	$\frac{\Delta T}{\Delta t}$ **	$\frac{\Delta OH}{\Delta t}$ **	Value (ml/min)
Steady-	CL_{tt}	$\frac{Ro^t}{C_{tt}}$	95	-	40	-	-	-	-	39.6
State	CL_{ut}	$\frac{Ro^t}{C_{ut}}$	95	-	-	7.4	-	-	-	214
Before	CL_{ts}	$\frac{Ro^s}{C_{ts}}$	-	-	-	-	-	-	-	-
Ro^s	CL_{mtt}	$\frac{\frac{\Delta OH}{\Delta t}}{C_{tt}}$	-	-	40	-	-	-	70.1	29.2
(Phase 1)	CL_{mut}	$\frac{\frac{\Delta OH}{\Delta t}}{C_{ut}}$	-	-	-	7.4	-	-	70.1	158
	CL_{ett}	$\frac{\frac{\Delta T}{\Delta t}}{C_{tt}}$	-	-	40	-	-	7.2	-	3
	CL_{eut}	$\frac{\frac{\Delta T}{\Delta t}}{C_{ut}}$	-	-	-	7.4	-	7.2	-	16.2
Steady-	CL_{tt}	$\frac{Ro^t}{C_{tt}}$	95	-	40.6	-	-	*** -	*** -	39
State	CL_{ut}	$\frac{Ro^t}{C_{ut}}$	95	-	-	18.4	-	-	-	86.1
After	CL_{ts}	$\frac{Ro^s}{C_{ts}}$	-	285	-	-	120	-	-	39.6
Ro^s	CL_{mtt}	$\frac{\frac{\Delta OH}{\Delta t}}{C_{tt}}$	-	-	40	-	-	-	55.4	23.1

Table XXVII (continued)

Time	Parameter	Formula	Ro^t	Ro^s	C_{tt}	C_{ut}	C_{ts}	$\frac{\Delta T}{\Delta t}$ **	$\frac{\Delta OH}{\Delta t}$ **	Value (ml/min)
(Phase 2)	CL_{mut}	$\frac{\frac{\Delta OH}{\Delta t}}{C_{ut}}$	-	-	-	18.4	-	-	55.4	50.2
	CL_{ett}	$\frac{\frac{\Delta T}{\Delta t}}{C_{tt}}$	-	-	40	-	-	18.2	-	7.6
	CL_{eut}	$\frac{\frac{\Delta T}{\Delta t}}{C_{ut}}$	-	-	-	18.4	-	18.2	-	16.5

* Abbreviations explained in the Appendix

** Mean of 3.88 and 5.45 hr time point

*** Mean of 15.80 and 18.50 hr time point

T = tolbutamide; SDM or s = sulfadimethoxine; tt = total T; ut = unbound T

e = excreted; m = metabolic

Although the urinary data in Interaction Study #1 were inconclusive, the data in the present study solidify certain previous observations. Figure XXXVIII a and b indicate again that in the absence of sulfadimethoxine about 75% of the infused tolbutamide can be accounted for in terms of excreted tolbutamide and hydroxytolbutamide (Phase 1). After the introduction of the sulfadimethoxine, the rate of excretion of hydroxytolbutamide goes down while that of parent tolbutamide goes up. At Phase 2 some interesting aspects are revealed as summarized in Table XXVII. Various steady-state clearances have been calculated based upon either the total or unbound tolbutamide concentration. It can be seen that the renal clearance of tolbutamide based upon the unbound tolbutamide levels (CL_{eut}) is the same value (16.2 and 16.5 ml/min). This would suggest that the renal clearance is dependent upon the unbound concentration. Despite the lack of data over a large concentration range, regression analysis has been performed, attempting to correlate the rate of urinary tolbutamide excretion with plasma concentrations of total or unbound tolbutamide. As seen in Figures XLIV and XLV, the correlation coefficient (r) using unbound plasma tolbutamide ($r = 0.90$) is better than when total plasma tolbutamide concentrations are used ($r = 0.59$; the plasma values were obtained for midpoint of excretion intervals by linear interpolation). This information adds further weight to the hypothesis that the unbound plasma tolbutamide concentrations determine the rate of urinary excretion of tolbutamide. Therefore, by deduction, the total body clearance of tolbutamide (CL_{tt}) cannot solely be governed by the total tolbutamide plasma concentrations.

The plasma hydroxytolbutamide levels in this study differ considerably from those seen in Interaction Study #1. Figure XLVI

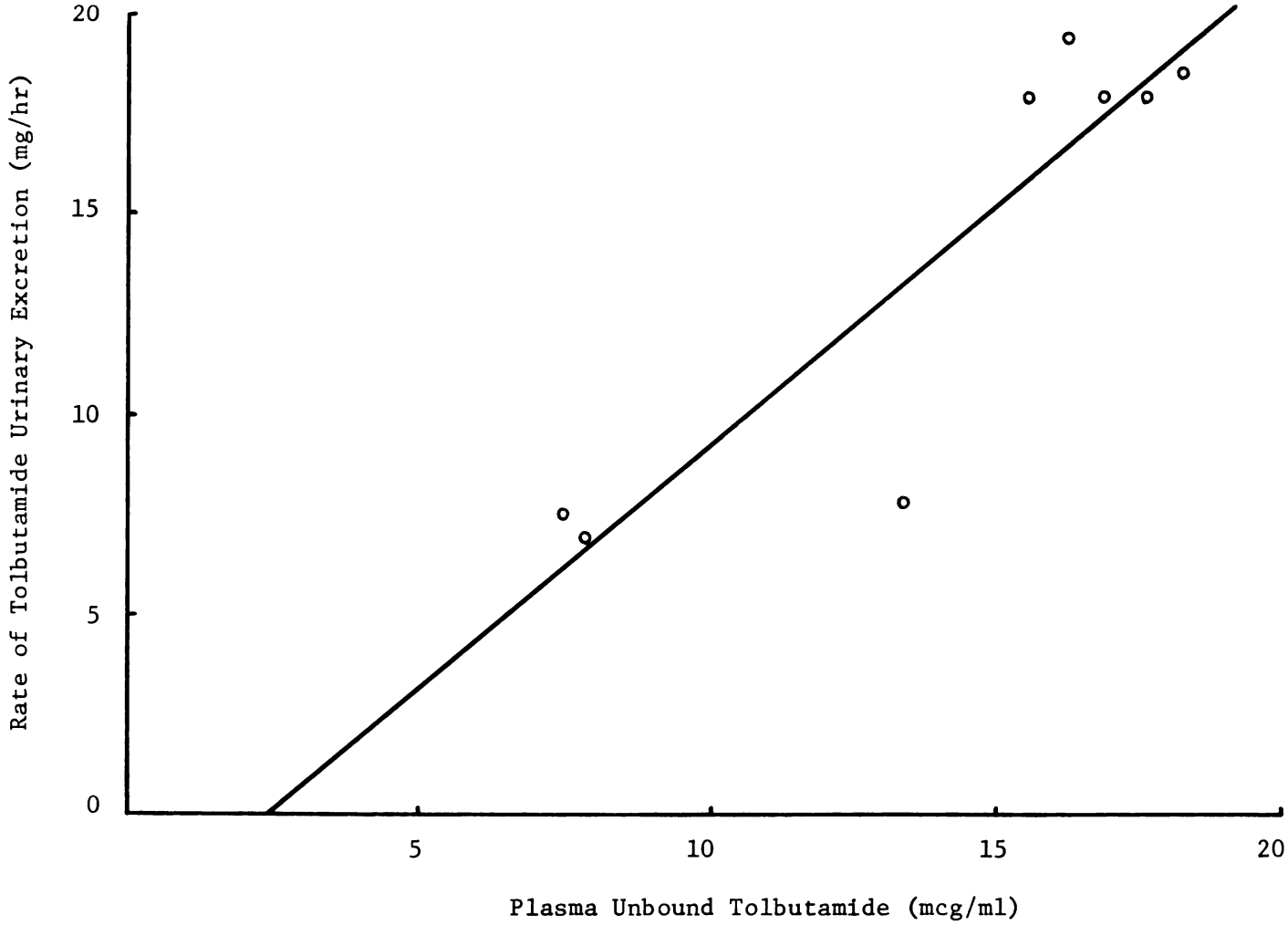


Fig. XLIV: Regression analysis of rate of urinary excretion of tolbutamide on the unbound plasma tolbutamide concentrations of Interaction Study #3 (r = 0.90; plasma values by linear interpolation to the midpoint of excretion interval; standard error of slope and interval is 0.24 and 3.55 respectively).

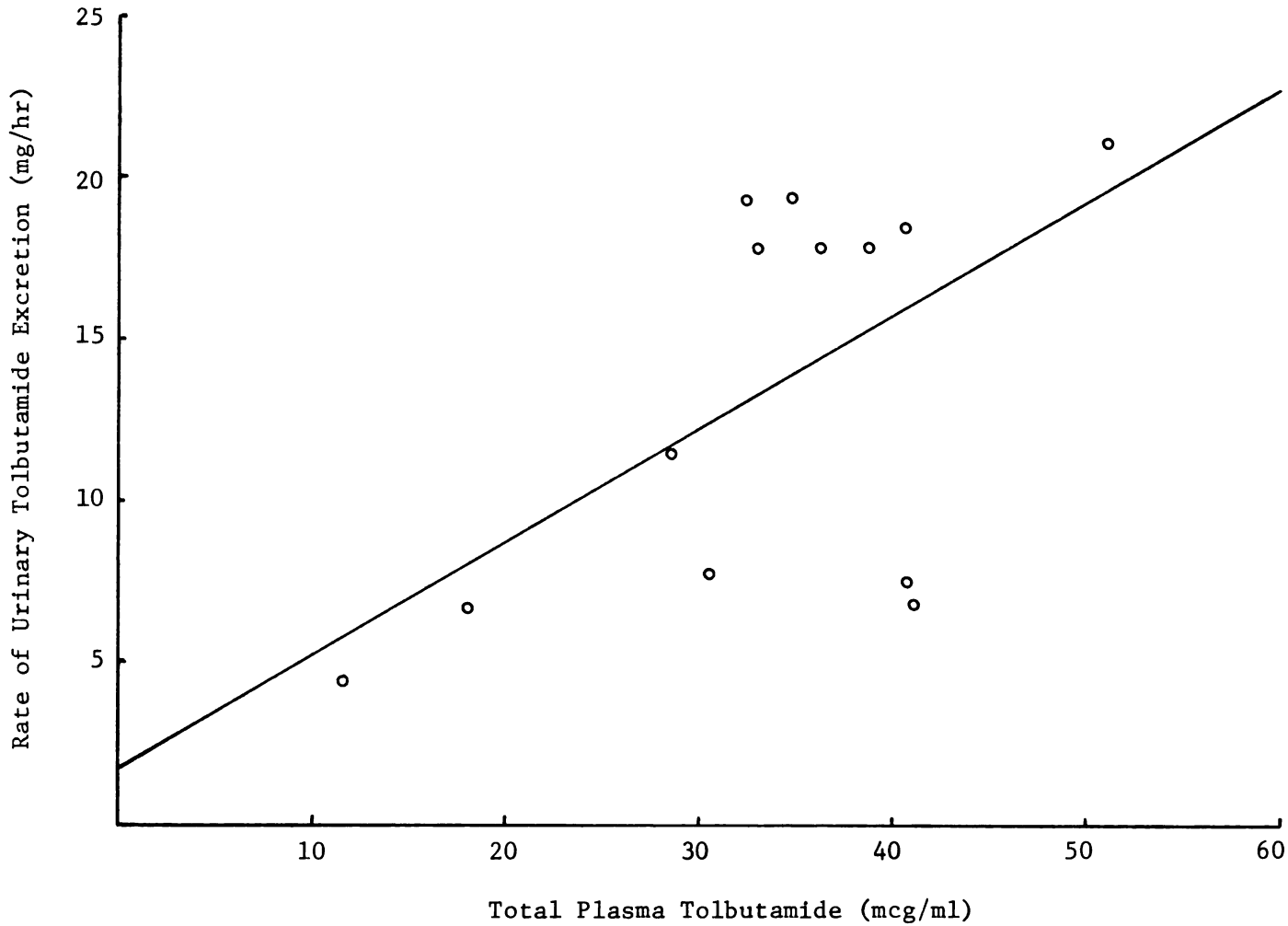


Fig. XLV: Regression analysis of rate of urinary excretion of tolbutamide on the total plasma concentrations of tolbutamide of Interaction Study #3 (r = 0.59; plasma values by linear interpolation to the midpoint of excretion interval; standard error of slope and intercept is 0.15 and 5.15 respectively).

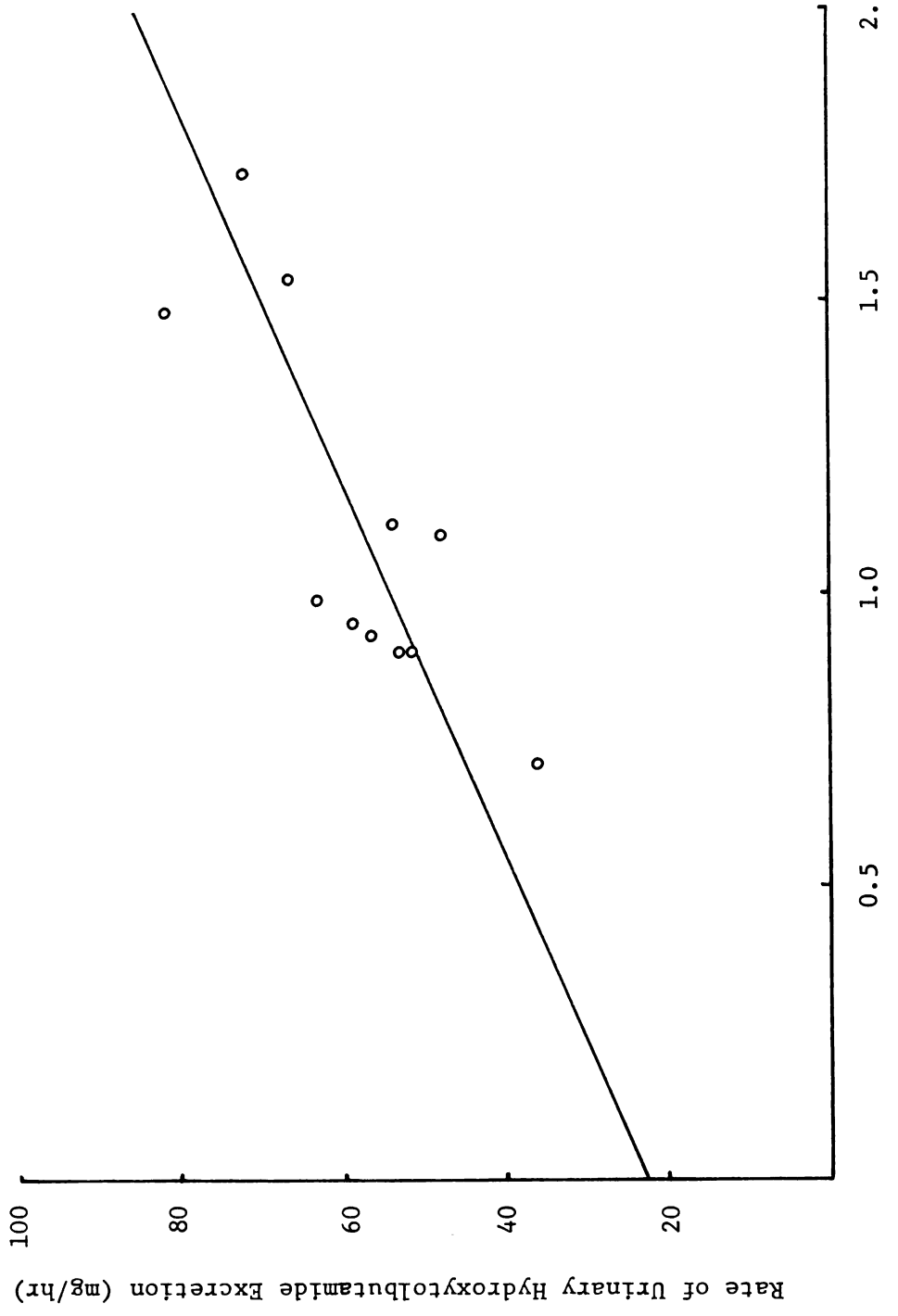


Fig. XLVI: Regression analysis of rate of urinary excretion of hydroxytolbutamide on the plasma concentrations of hydroxytolbutamide of Interaction Study #3 ($r = 0.81$; slope = 522 ml/min; plasma values by linear interpolation to the midpoint of the excretion interval; standard error of slope and intercept is 7.58 and 8.82 respectively).

exhibits the regression analysis of rate of urinary excretion of hydroxytolbutamide on the plasma hydroxytolbutamide concentrations (linear interpolation of plasma data to midpoint of excretion interval). The value of r = 0.81 indicates a reasonable correlation between the two experimental determinations. The regression coefficient (slope) indicates the renal clearance of hydroxytolbutamide to be 522 ml/min. This value is lower than that(738 ml/min) found in Interaction Study #2. However when only steady-state conditions are considered, then the renal clearance of hydroxytolbutamide $\left(\frac{\Delta\ OH}{\frac{\Delta\ t}{C_{OH}}}\right)$ in the absence

$\left(\frac{70.1\ mg/hr}{1.48\ mcg/ml}\right)$ or in the presence of sulfadimethoxine $\left(\frac{55.4\ mg/hr}{1.12\ mcg/ml}\right)$ is 790 and 824 ml/min respectively and agrees with the results in Interaction Study #2 (738 ml/min). The presence of sulfadimethoxine therefore does not appear to influence the renal clearance of hydroxytolbutamide since the difference is within experimental error (5%).

As is readily apparent in Interaction Study #3, the rate of infusion of tolbutamide (R_0^t) at steady-state does not equal the rate of excretion of total tolbutamide equivalents $\left(\frac{\Delta\ OH}{\Delta\ t} + \frac{\Delta\ T}{\Delta\ t}\right)$ with the measureable excretion products only accounting for about 75% of R_0^t. This indicates that another elimination route or product exists which will be designated "x". Since it has been shown in Interaction Study #2 as well as during the I.V. boluses of hydroxytolbutamide that greater than 90% of the administered hydroxytolbutamide can be accounted for as urinary parent compound leads one to suggest that plasma hydroxytolbutamide does not undergo further metabolism or extrarenal elimination (via route "x"). However, the present data unfortunately do not permit further elucidation

of "x". If one can assume unequivocally from Interaction Study #2 that sulfadimethoxine does not alter the renal clearance of hydroxytolbutamide, then the rate of urinary hydroxytolbutamide excretion can be used to predict plasma concentrations of hydroxytolbutamide if one considers the apparent volume of distribution changes of hydroxytolbutamide by sulfdimethoxine to be minor (^{3}H-hydroxytolbutamide data - Fig.XLII). Given the rates of urinary excretion of hydroxytolbutamide at Phase 1 and 2 of 70.1 and 55.4 mg/hr respectively, with a plasma hydroxytolbutamide concentration of 1.48 mcg/ml at Phase 1, one would predict a plasma level of $\frac{55.4}{70.1}$ (1.48) = 1.17 mcg/ml hydroxytolbutamide which is very close to that actually found (1.12 mcg/ml).

As has been discussed earlier (p. 160), the renal clearance of tolbutamide (T) is dependent upon the unbound plasma T concentration (Cut). Given that the extrarenal T clearance (metabolic) is very low (CL_{tt}-CL_{ett}; Table XXVII; p. 158) suggests that the hepatic extraction ratio (extrarenal clearance/hepatic blood flow) is also very low. This possibly indicates that the extraction ratio is limited by the unbound T concentration and/or T is a poor substrate for metabolic conversion. It is felt that the T plasma data seen in Figure XXXVI, p. 140, can be explained if it is assumed that the extrarenal clearance of tolbutamide is also dependent upon the unbound T concentration. With this assumption one would expect the unbound T concentration to return to Phase 1 levels at steady-state in Phase 2. The continual rise of the unbound T concentration to a new elevated steady-state level at Phase 2 is compatable with the results in Interaction Study #1 if one considers sulfadimethoxine to inhibit the clearance of T in addition to acting as a displacing agent. The steady-state data seen at Phase 2 should then merely reflect a

modification of Phase 1 data due to clearance inhibition. The following general approach will be considered for explanation purposes (reader may refer back to Chapter I, pp.16 to 21. The rate of body change of T may be approximated by Equation 19, where CL_{tt} is

$$\frac{-dT}{dt} = k_T T = k_T V dC_{tt} = CL_{tt} C_{tt} \qquad \text{Eq.19}$$

the total body clearance of T and C_{tt} is the total plasma concentration of T. In the presence of inhibitor, Equation 20 results.

$$\frac{-dT}{dt} = \frac{k_T T}{1 + {}^{I}/_{K_I}} = \frac{k_T V dC_{tt}}{1 + {}^{I}/_{K_I}} = \frac{CL_{tt} C_{tt}}{1 + {}^{I}/_{K_I}} \qquad \text{Eq.20}$$

If α is used to denote the fraction of T unbound $(\frac{Cut}{Ctt})$, then the maximum total body clearance observed (clearance based upon unbound T $(\frac{Ro^t}{Cut})$) in the absence of inhibitor ($CL_{max\ obs}{}^1$) would be $\frac{CL_{tt}}{\alpha}$. The substitution of $\frac{CL_{tt}}{\alpha}$ into Equations 19 and 20 results in Equations 21 and 22 respectively.

$$\frac{-dT}{dt} = CL_{max\ obs}{}^1\ Cut \qquad \text{Eq.21}$$

$$\frac{-dT}{dt} = \frac{CL_{max\ obs}{}^1\ Cut}{1 + {}^{I}/_{K_I}} \qquad \text{Eq.22}$$

At steady-state, given a zero-order T infusion of Ro^t, Equations 21 and 22 become Equations 23 and 24 respectively, where

$$Cut = \frac{Ro^t}{CL_{max\ obs}{}^1} \qquad \text{Eq.23}$$

$$Cut = \frac{Ro^t}{\frac{CL_{max\ obs}^1}{1 + {}^I/_{K_I}}} \qquad \text{Eq.24}$$

finally,

$$CL_{max\ obs}^2 = \frac{CL_{max\ obs}^1}{1 + {}^I/_{K_I}} \qquad \text{Eq.25}$$

The maximum total body clearance observed at a steady state of inhibitor ($CL_{max\ obs}^2 = \frac{Ro^t}{Cut^2}$) would be reduction of $CL_{max\ obs}^1$ (absence of inhibitor) by the influence of competitive inhibition. The unbound steady-state T concentration in the presence of inhibitor (Cut^2) could then be higher (same rate of infusion (Ro^t) of T) as designated by Equation 26 where Cut^1 is the steady-state unbound T concentration in the absence of inhibitor.

$$Cut^2 = Cut^1 (1 + {}^I/_{K_I}) \qquad \text{Eq.26}$$

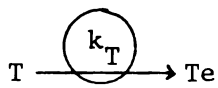
Thus if the concentration of inhibitor (I) present is equal to K_I (concentration of inhibitor necessary to reduce the rate of T elimination by 1/2), then the steady-state unbound T concentration (Cut^2) would be doubled. The presiding $C_{tt}2$ would then be determined by α^2 which in turn would depend on the displacement ability of the inhibitor/displacer upon T. The elevated Cut^2 at steady-state in Phase 2 could thus be a result of inhibition of T elimination by sulfadimethoxine.

Without the availability of Phase 3, it would be impossible to test the proposed mechanism of interaction. The basis for testing the proposed mechanism lies in the observed change in half-life of T from 3.75 hr in Phase 2 to 5.15 hr in Phase 3. However first consideration must be given

as to possible points of interaction by sulfadimethoxine. Figure XLVII exhibits three proposed ways sulfadimethoxine might interact to reduce the elimination rate of T. The circled first order rate constants indicate the routes of inhibition. Tables XXVIII a, b and c summarize some calculations based upon the 3 proposals which will now each be discussed.

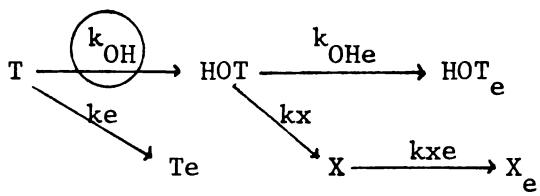
Proposal 1 - sulfadimethoxine (SDM) affects the total clearance of tolbutamide (T). The equations developed in pages 166 to 167 have been used to calculate various unknown parameters in Table XXVIIIa. The total SDM plasma concentration (Cts) at steady-state in Phase 2 has been used to calculate the K_I value (81 mcg/ml) from the $CL_{max\ obs}$ of 86.1 and 214 ml/min in Phases 2 and 1 respectively. This K_I value is then used to calculate a clearance expected (70 ml/min) in Phase 3 knowing the attending SDM plasma concentration (Cts = 168 mcg/ml). It should be noted that this Cts value was selected at the 24 hr. point, about half way between the initiation of SDM-bolus[2] and the termination of SDM-infusion[2], because steady-state SDM plasma levels were not reached in Phase 3. Then, another assumption was made in this as well as the other two proposals - that in the tolbutamide kinetics, clearance = $k_{10}V_1 \simeq \beta Vd$ (total body clearance of tolbutamide from a 2 compartment model in the sheep may be approximated by total body clearance from a one compartment model where $\beta \simeq kd$). This last assumption permitted an appraisal of the change in the volume term when going from Phase 2 to Phase 3. This appraisal was achieved by using Figure XXXVII in which the terminal Phase 3 of the curve was back-extrapolated to 19 hrs. (i.e. time when SDM administration changed), and the change between observed and extrapolated dpm was used as a measure of the change in the volume of distribution of T. It was thus calculated that the apparent volume of distribution of Phase 3 (Vd^3) was 1.1 times that of Phase 2. This information together with an estimate of the maximum clearance

PROPOSAL 1

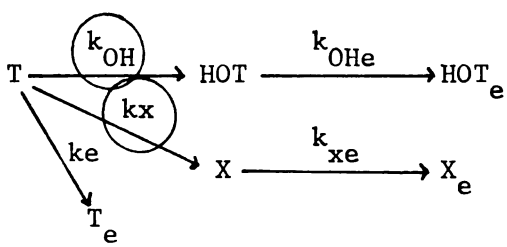


Te = T + HOT + X

PROPOSAL 2



PROPOSAL 3



Note:

T = tolbutamide

HOT = hydroxytolbutamide

X = unknown route of elimination or metablic product of T
(see p.164).

Subscript e denotes excreted

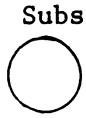
 = points of sulfadimethoxine inhibition

Figure XLVII: Proposed routes by which sulfadimethoxine inhibits the elimination of tolbutamide in the sheep.

Table XXVIIIa: Proposal 1 of interaction study #3

Parameter	Formula	Phase 1	Phase 2	Phase 3
$CL_{max\ obs}$	$\frac{Ro^t}{Cut}$	$\frac{95}{7.4} = 214$	$\frac{95}{18.4} = 86.1$	Formula $\frac{CL_{max\ obs}{}^1}{1+\frac{Cts}{K_I}}$ $\frac{214}{1+\frac{168}{81}} = 70$
Cts	-	-	120	168^{*1}
K_I	$\frac{Cts}{\frac{CL_{max\ obs}{}^1}{CL_{max\ obs}{}^2}-1}$	-	$\frac{120}{\frac{214}{86.1}-1} = 81$	-
Vd	-	Vd^1	Vd^2	$Vd^3 = 1.1Vd^{2*2}$
t1/2 Found	-	-	3.75	5.2
t1/2 Calculated	$t1/2^2$ $\frac{CL_{max\ obs}{}^2(Vd^3)}{CL_{max\ obs}{}^2(Vd^2)}$	-	-	$\frac{(3.75)(86.1)(1.1Vd^2)}{(70)(Vd^2)} = 5.07$

* designations explained in the Appendix

* numerical superscripts refer to the phase (i.e. 1, 2 or 3)

*1 value taken at 24 hr.

*2 obtained by back extrapolation of terminal log-linear portion of curve (Fig.XXXVII). to 19 hr and comparing Cp (19 hr) to back extrapolated value.

Table XXVIIIb: Proposal 2 of interaction study #3

Parameters	Formula	Phase 1	Phase 2	Phase 3
$CL_{max\ obs}$	$\frac{Ro^t}{Cut}$	$\frac{95}{7.4} = 214$	$\frac{95}{18.4} = 86.1$	$CL_{max\ obs}$ 16.5 + 55 = 71.5
$CL_{max\ renal\ obs}$	$\frac{\Delta T}{\Delta t}$ Cut	$\frac{7.2}{7.4} = 16.2$	$\frac{18.2}{18.4} = 16.5$	$= CL_{max\ renal\ obs^2}$ 16.5
$CL_{max\ OH\ obs}$	$CL_{max\ obs} - CL_{max\ renal\ obs}$	214-16.2=197.8	86.1-16.5=69.6	$\frac{CL_{max\ OH\ obs^1}}{1 + \frac{Cts}{K_{I(OH)}}}$ $\frac{197.8}{1 + \frac{168}{65}} = 55$
C_{ts}	-	-	120	168^{*1}
$K_{I(OH)}$	$\frac{Cts}{\frac{CL_{max\ OH\ obs^1}}{CL_{max\ OH\ obs^2}} - 1}$	-	$\frac{120}{\frac{197.8}{69.6} - 1} = 65$	-
Vd	-	Vd^1	Vd^2	$Vd^3 = 1.1Vd^{2*2}$
t1/2 Found	-	-	3.75	5.2
Calculated	$t1/2^2$ $\frac{CL_{max\ obs^2}(Vd^3)}{CL_{max\ obs^3}(Vd^2)}$	-	-	$\frac{(3.75)(86.1)(1.1Vd^2)}{(71.5)(Vd^2)} = 4.97$

* designations explained in the Appendix
* numerical superscripts refer to the Phase (i.e. 1, 2 or 3)
*1,*2 - see Table XXVIIIa

Table XXVIIIc: Proposal 3 of interaction study #3

Parameters	Formula	Phase 1	Phase 2	Phase 3
$CL_{max\ obs}$	$\frac{Ro^t}{Cut}$	$\frac{95}{7.4} = 214$	$\frac{95}{18.4} = 86.1$	Formula $CL_{max\ obs}$ 16.5+39.5+16.1 = 72.1
$CL_{max\ renal\ obs}$	$\frac{\frac{\Delta T}{\Delta t}}{Cut}$	$\frac{7.2}{7.4} = 16.2$	$\frac{18.2}{18.4} = 16.5$	$CL_{max\ renal\ obs}$ 16.5
$CL_{max\ OH\ obs}$	$\frac{\frac{\Delta OH}{\Delta t}}{Cut}$	$\frac{70.1}{7.4} = 158$	$\frac{55.4}{18.4} = 50.2$	$\frac{CL_{max\ OH\ obs}^{1}}{1 + \frac{Cts}{K_{I(OH)}}}$ $\frac{158}{1+\frac{168}{55.9}} = 39.5$
$CL_{max\ X\ obs}$	$CL_{max\ obs} - (CL_{max\ renal\ obs} + CL_{max\ OH\ obs})$	214-(158+16.2) = 39.8	86.1-(16.5+50.2) = 19.4	$\frac{CL_{max\ X\ obs}^{1}}{1 + \frac{Cts}{K_{I(x)}}}$ $\frac{39.8}{1+\frac{168}{114}} = 16.1$
C_{ts}	-	-	120	168^{*1}
$K_{I(OH)}$	$\frac{Cts}{\frac{CL_{max\ OH\ obs}^{1}}{CL_{max\ OH\ obs}^{2}} - 1}$	-	$\frac{120}{\frac{158}{50.2} - 1} = 55.9$	-

Cont'd..

Table XXVIIIc: Proposal 3 of interaction study #3
(cont'd)

Parameters	Formula	Phase 1	Phase 2	Phase 3
$K_{I(x)}$	$\frac{Cts}{\frac{CL_{max\ X\ obs}{}^{1}}{CL_{max\ X\ obs}{}^{2}} - 1}$	-	$\frac{120}{\frac{39.8}{19.4} - 1} = 114$	-
Vd	-	Vd^1	Vd^2	$Vd^3 = 1.1Vd^{2*2}$
t1/2 Found	-	-	3.75	5.3
t1/2 Calculated	$t1/2^2\ \frac{CL_{max\ obs}{}^{2}(Vd^3)}{CL_{max\ obs}{}^{3}(Vd^2)}$	-	-	$\frac{(3.75)(86.1)(1.1Vd^2)}{(72.1)(Vd^2)} = 4.91$

* designation explained in Appendix

* numerical superscripts refer to the Phase (i.e. 1, 2 or 3)

*1, *2 - see Table XXVIIIa.

values of Phases 2 and 3, was then used to calculate the expected half-life of T in Phase 3. As can be seen the value calculated for Proposal 1 (5.07 hr) was very close to that actually found (5.2 hr).

Proposal 2 - sulfadimethoxine (SDM) inhibits only the metabolic clearance of tolbutamide (T) and the sole route of metabolism is to hydroxytolbutamide. The assumptions used in Proposal 1 were used here with one addition, namely, that the renal clearance of T based on unbound plasma T concentration ($CL_{max\ renal\ obs}$) would remain the same in Phase 3 as in Phase 2. A value of 65 mcg/ml (Cts) was calculated for $K_{I(OH)}$ which is lower than the K_I value in Proposal 1. The calculated half-life using this second proposal (4.97 hr) still compared reasonably with that found (5.2 hr).

Proposal 3 - sulfadimethoxine (SDM) inhibits only the metabolic clearance with one K_I value for hydroxytolbutamide formation and another K_I value for the formation of "X" $\left[CL_{max\ X\ obs} = CL_{max\ obs} - (CL_{max\ renal\ obs} + CL_{max\ OH\ obs})\right]$.

The calculations found in Table XXVIIIc indicate that $K_{I(OH)}$ is 55.9 mcg/ml while the $K_{I(x)}$ is 114 mcg/ml. The same assumptions used in Proposal 2 have been utilized in this proposal. Again the half-life calculated (4.91 hr) compares well with that found (5.2 hr).

Three proposals have been forwarded to account for the interaction of SDM and T based upon the propounded mechanism that T elimination (metabolic and renal) depends upon the unbound T concentration and that SDM in addition to being a displacer of protein bound T, acts as an inhibitor of metabolic elimination of T. All 3 proposal adequately explain the events in Phases 2 and 3. The question raised then is which of the 3 proposals best satisfy the available information. Proposal 1 can probably be dismissed because it can be seen in Table XXVII (p.158) that SDM does

not change the maximum observed renal clearance of T (CL_{eut}). Therefore SDM must, according to the propounded mechanism, act via Proposals 2 or 3. Unfortunately, available information does not permit one to accept unequivocally one of Proposals 2 or 3, but is evident that either adequately describes the data.

Only 1 hypothesis has been forwarded and 3 proposed mechanisms within the hypothesis to account for the interaction of SDM and T in the sheep. Since only steady-state values were used in the suggested hypothesis, it was determined to use all data as evidence. Since it has been shown earlier (p. 87) that the renal elimination of hydroxytolbutamide ($\frac{\Delta OH}{\Delta t}$) is rate limited by the metabolic formation of hydroxytolbutamide (HOT) from T then Equation 27 may be written where CL_{mt} is the metabolic clearance of T

$$\frac{\Delta OH}{\Delta t} \cong CL_{mt}Ct \qquad \text{Eq.27}$$

based upon T concentration (Ct). Using Equation 20, one could then write Equation 28 because SDM does not appear to inhibit the renal clearance of

$$\frac{\Delta OH}{\Delta t} \cong \frac{CL_{mt}Ct}{1 + {}^{I}/_{K_I}} \qquad \text{Eq.28}$$

HOT. By rearranging Equation 28 one obtains Equation 29

$$\frac{Ct}{\frac{\Delta OH}{\Delta t}} \cong \frac{1}{CL_{mt}} + \frac{1}{CL_{mt}K_I}(I) \qquad \text{Eq.29}$$

which says that a plot of $\frac{Ct}{\frac{\Delta OH}{\Delta t}}$ vs inhibitor plasma concentration (SDM) should give a straight line with y-intercept of $\frac{1}{CL_{mt}}$ and a positive slope of $\frac{1}{CL_{mt}K_I}$, thus permitting an evaluation of K_I. Using unbound plasma

T (Cut) would give a y-intercept of $CL_{max\ OH\ obs}$ while using total plasma T (C_{tt}) would yield CL_{mtt}. This approach might test whether the metabolic clearance of T depends upon ubound or total T. Figures XLVIII and XLIX represent such least square fitted plots of Interaction Study #3. Figure XLVIII in fact suggests that one should use C_{tt}, but as can be seen, the data is too localized in either case to permit an assessment of the mechanism. Again using the principle that $\frac{\Delta OH}{\Delta t}$ is rate-limited by its formation, regression analysis has been performed on $\frac{\Delta OH}{\Delta t}$ upon unbound plasma T (Cut) or total plasma T (C_{tt}) with the results plotted in Figures L and LI, giving correlation coefficients (r) of -0.72 and 0.92 respectively. This would suggest that the metabolic elimination of T depends upon the total plasma concentration of T. However such a situation could be envisaged if in fact the rate of formation of HOT was dependent upon Cut while displacement and metabolic inhibition of T by SDM took place. A similar hypothesis was recently forwarded by Jähnchen *et al.*[98] who investigated the influence of phenylbutazone on the disposition of dicumarol in rats. In diluted serum (1:10) containing 4.4 mg/L dicumarol, the addition of 80 mg/L phenylbutazone caused a 6-fold increase in unbound dicumarol. However *in vivo* phenylbutazone caused no statistically significant change in dicumarol half-life nor apparent volume of distribution. Although lacking concrete supporting evidence they suggested that the *in vivo* results might reflect a net effect of increased unbound dicumarol concentration (which could cause a more rapid biotransformation) and an acute inhibitory effect of phenylbutazone on the biotransformation of dicumarol. In the present interaction study (#3) one might advance another hypothesis. Since the renal clearance of T is dependent upon unbound T as shown, the metabolic clearance could depend upon the total plasma T with

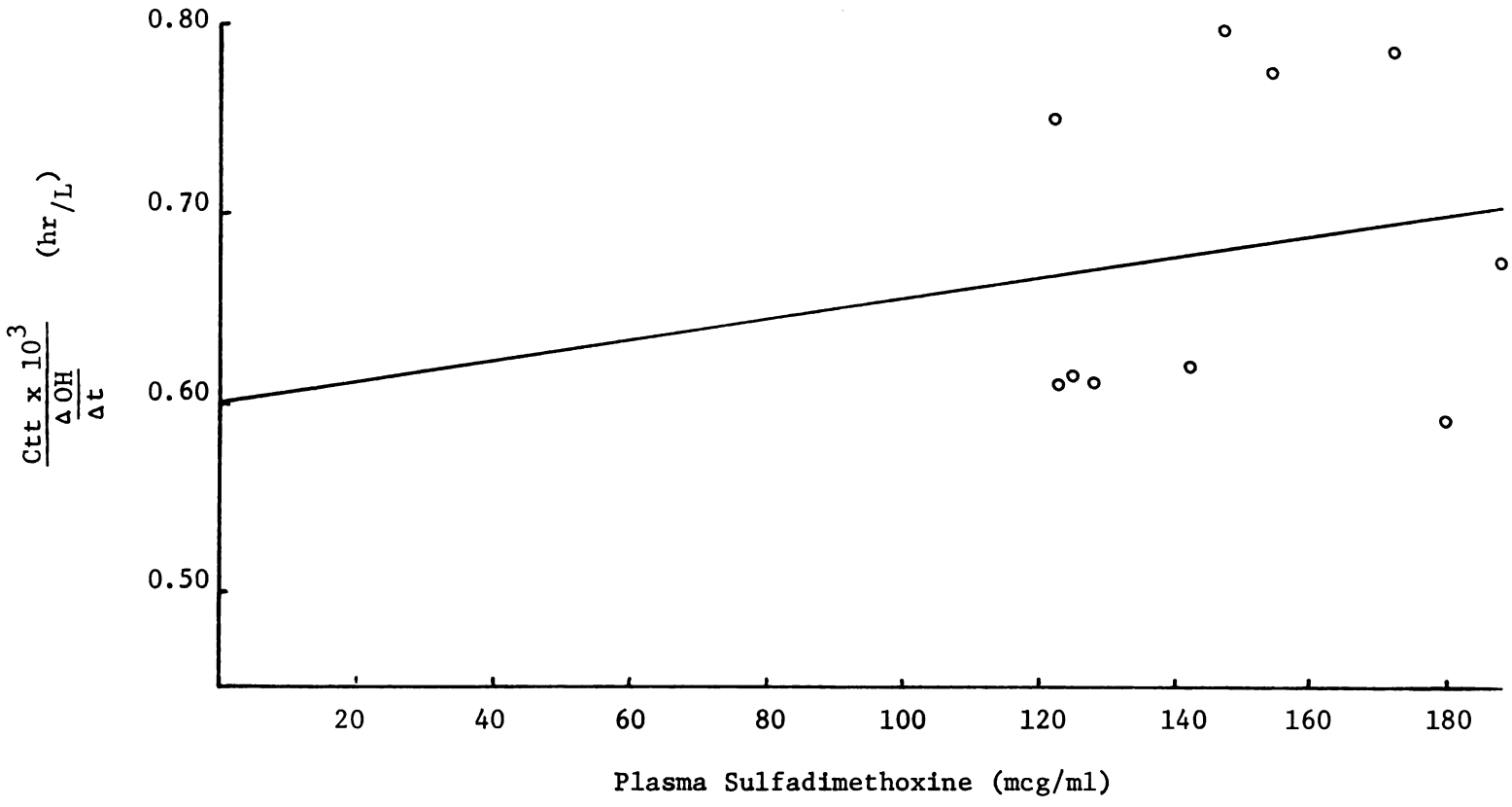


Fig.XLVIII: A plot of total plasma tolbutamide concentrations divided by the rate of hydroxytolbutamide urinary excretion against plasma sulfadimethoxine for Interaction Study #3. (Plasma values by linear interpolation to midpoint of excretion interval; standard error of slope and intercept is 1.19×10^{-6} and 0.18×10^{-3} respectively.)

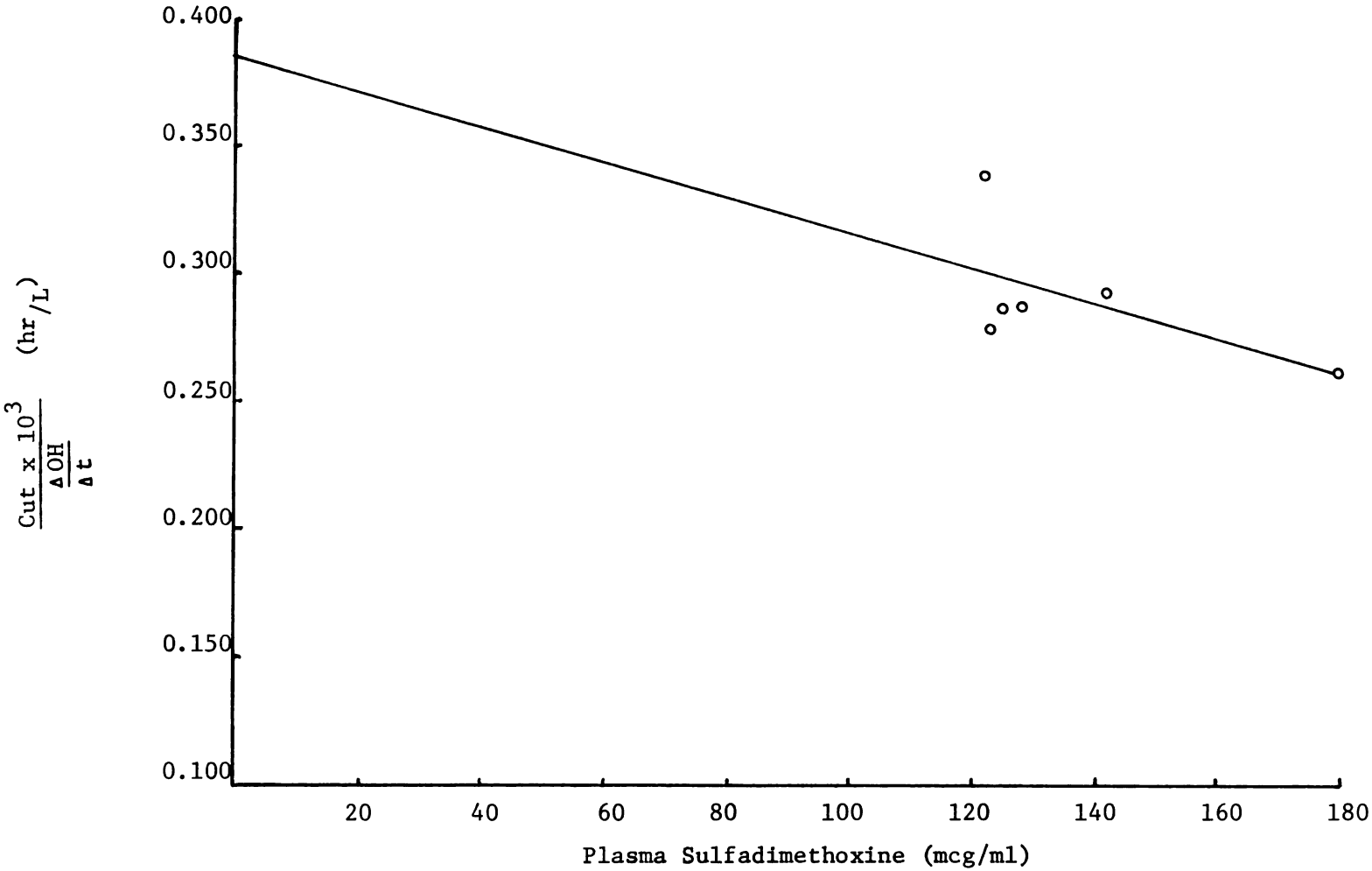


Fig.XLIX: A plot of unbound plasma tolbutamide concentrations divided by the rate of hydroxytolbutamide urinary excretion against plasma sulfadimethoxine for Interaction Study #3. (Plasma values by linear interpolation to midpoint of excretion interval; the standard error of slope and intercept is 4.64×10^{-7} and 0.64×10^{-4} respectively.)

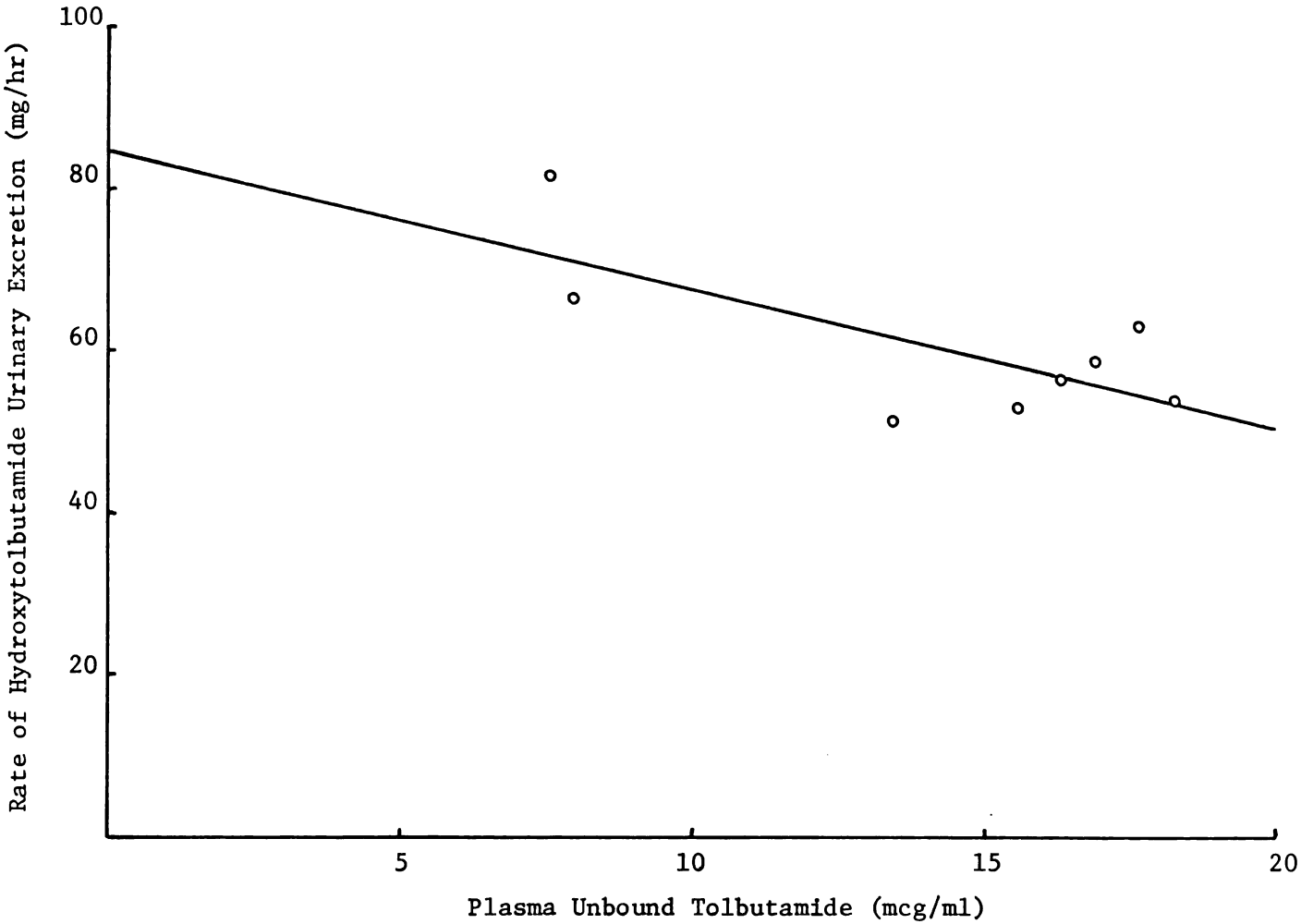


Fig.L: Regression analysis of rate of urinary excretion of hydroxytolbutamide on the unbound plasma concentrations of tolbutamide of Interaction Study #3 (r = -0.72; plasma values by linear interpolation to the midpoint of excretion interval; the standard error of slope and intercept is 0.66 and 9.78 respectively).

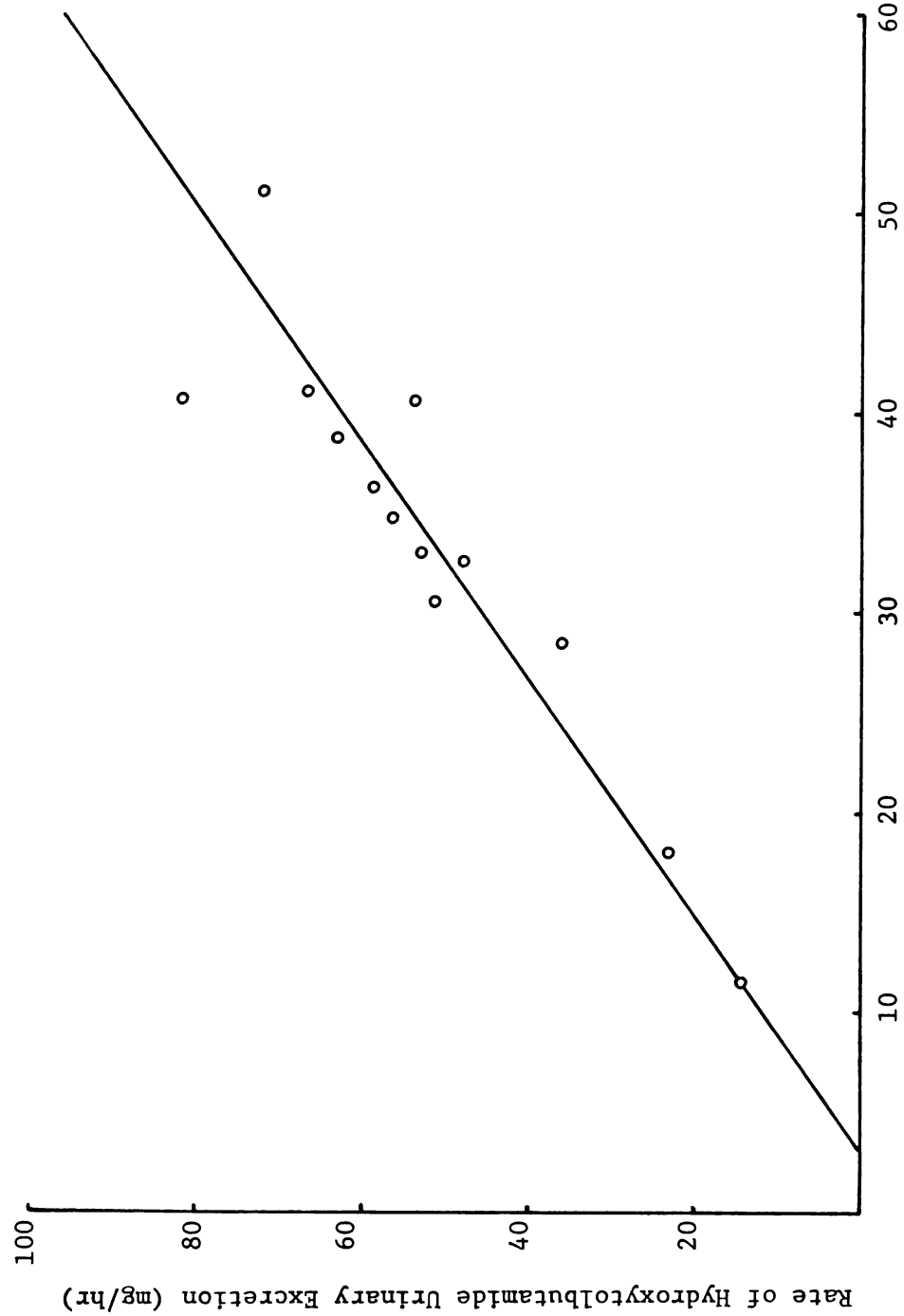


Fig.LI: Regression analysis of rate of urinary excretion of hydroxytolbutamide on the total plasma concentrations of tolbutamide of Interaction Study #3 ($r = 0.92$; plasma values by linear interpolation to the midpoint of excretion interval; the standard error of slope and intercept is 0.21 and 7.40 respectively).

subsequent possible metabolic inhibition of T by SDM. However this hypothesis when carried through calculations as in Proposal 2 does not suggest a change in the half-life of T in Phase 3.

Conclusion:

Interaction study #3 as well as study #1 suggest that a metabolic interaction exists between tolbutamide and sulfadimethoxine in the sheep. However clear evidence for a metabolic interaction can only be obtained from in vitro enzymatic studies. The significance of the interaction might well be questioned. In this study sulfadimethoxine levels were moderate. Using Proposal 3 with the same tolbutamide infusion and assuming that the maximum renal clearance observed remains the same (16.5 ml/min), then the $CL_{max\ obs}$ calculated for a high steady-state plasma sulfadimethoxine level of 300 mcg/ml would be 52.4 ml/min. The corresponding unbound tolbutamide plasma concentration would be expected to have a value of about 30 mcg/ml and the total tolbutamide plasma concentration about 60 to 60 mcg/ml (α = 0.5 to 0.6) depending upon what value of α (fraction unbound) would be obtained at that concentration of sulfadimethoxine in the in vivo setting. As can be seen, the value of total plasma tolbutamide would not be much higher than that found in the absence of sulfadimethoxine. However the unbound level has risen dramatically (4-fold). If in fact the unbound concentration is responsible for therapeutic effect or untoward effects, then such a change (4-fold) might cause an exaggerated therapeutic response (hypoglycemia) or toxic symptoms due to untoward effects. This might be especially true if higher tolbutamide infusion rates than employed here, were used. Fortunately for the sheep, the renal clearance of tolbutamide

(corrected for protein binding) appears to be unaffected by sulfadimethoxine and the renal clearance is dependent upon the unbound concentration of tolbutamide. This reduces the contribution of the metabolic inhibition to the ultimate steady-state unbound tolbutamide concentration, for as the value of α increases, more of the parent tolbutamide is eliminated renally per unit time. The significance of an interaction between tolbutamide and sulfadimethoxine might be much greater in a species like man where supposedly less than 2% of the dose is excreted unchanged. However the significance of the interaction would also depend on the effectiveness of sulfadimethoxine as a metabolic inhibitor of tolbutamide metabolism.

Throughout the calculations of inhibitor constants (K_I), consideration was only given to the total sulfadimethoxine levels. If the metabolic clearance of tolbutamide is dependent upon the unbound concentration, then it might well be the unbound sulfadimethoxine concentration which dictates the degree of metabolic inhibition. Unfortunately the unbound concentrations of sulfadimethoxine are not known in Phase 3 to carry out calculations such as were done for total sulfonamide levels.

CHAPTER IV

SULFADIMETHOXINE DISPOSITION

I. Introduction:

Widely different plasma sulfonamide concentration-time curves were observed after boluses of sulfadimethoxine, when plotted on semi-log paper. A 4 Gm bolus (Fig.XXI, p. 97) seemed to decay log linearly after an initial apparent distribution phase indicative of a two compartment model (Fig.XIV, p. 76) while on a different occasion a 1 Gm bolus exhibited continual curvature. With the suggestion that the clearance of tolbutamide was dependent upon the unbound concentration (Cf) and observing that the clearance of sulfadimethoxine (CL_{ts}) based upon the total plasma concentration (C_{ts}) was similar to that of tolbutamide (Table XXVII, p.158) it was suspected that the clearance of sulfadimethoxine was possibly also dependent upon unbound sulfonamide. Some considerations were then made with a simple 1 compartment model (Fig. XIV, p. 76). Following a single intravenous bolus dose and denoting Ab as the amount of drug in the body, Equation 30 follows. From Chapter I, a drug whose clearance is dependent

$$\frac{-dAb}{dt} = KdAb = KdVdC_t = CLC_t \qquad \text{Eq.30}$$

on the unbound concentration and which obeys Equation 31,

$$CL = \alpha CL_{max} \qquad \text{Eq.31}$$

substituting Equation 31 into Equation 30 yields Equation 32,

$$Kd = \frac{\alpha CL_{max}}{Vd} \qquad \text{Eq.32}$$

This equation indicates that a compound whose clearance is dependent upon Cf would exhibit monoexponential plasma decay provided the ratio $\frac{\alpha}{Vd}$ remains constant. However if α varies with concentration, the product $Cl_{max}\alpha$ and hence clearance (CL = KdVd) would not be constant.

Integrating Equation 30 and evaluating between time zero and infinity results in the familiar Equation 33.

$$\text{Dose} = CL\int_0^\infty Ctdt \qquad \text{Eq.33}$$

A plot of $\int_0^\infty Ctdt$ versus Dose is linear when clearance depends on Ct whereas if clearance is dependent upon Cf, such a plot would yield a non-linear relationship. On the other hand, if clearance does depend on Cf, a linear Dose versus $\int_0^\infty Cfdt$ relationship results which may readily be seen to follow by substituting Equation 31 into Equation 33 and realizing that $\alpha = \frac{Cf}{Ct}$:

$$\text{Dose} = CL_{max}\int_0^\infty Cfdt \qquad \text{Eq.34}$$

Thus Dose and $\int_0^\infty Cfdt$ would be linearly related whereas now Dose versus $\int_0^\infty C_t dt$ would yield a non-linear relationship. An attempt has been made to graphically illustrate the preceding discussion in Figure LII a) - clearance dependent upon the total (Ct) concentration; b) - clearance dependent upon the unbound (Cf) concentration. In case a), a plot of $\int_0^\infty Cfdt$ would result in a non-linear relationship while in case b), a plot of $\int_0^\infty Ctdt$ would result in the non-linear relationship. If only relatively small doses of a compound are used it might be difficult to establish the mechanism of clearance since a plot of either $\int_0^\infty C_{f \text{ or } t}dt$ might appear to give a linear relationship. Yet if a large dose range is used, the mechanism of clearance should become apparent. It should be noted that in the theoretical

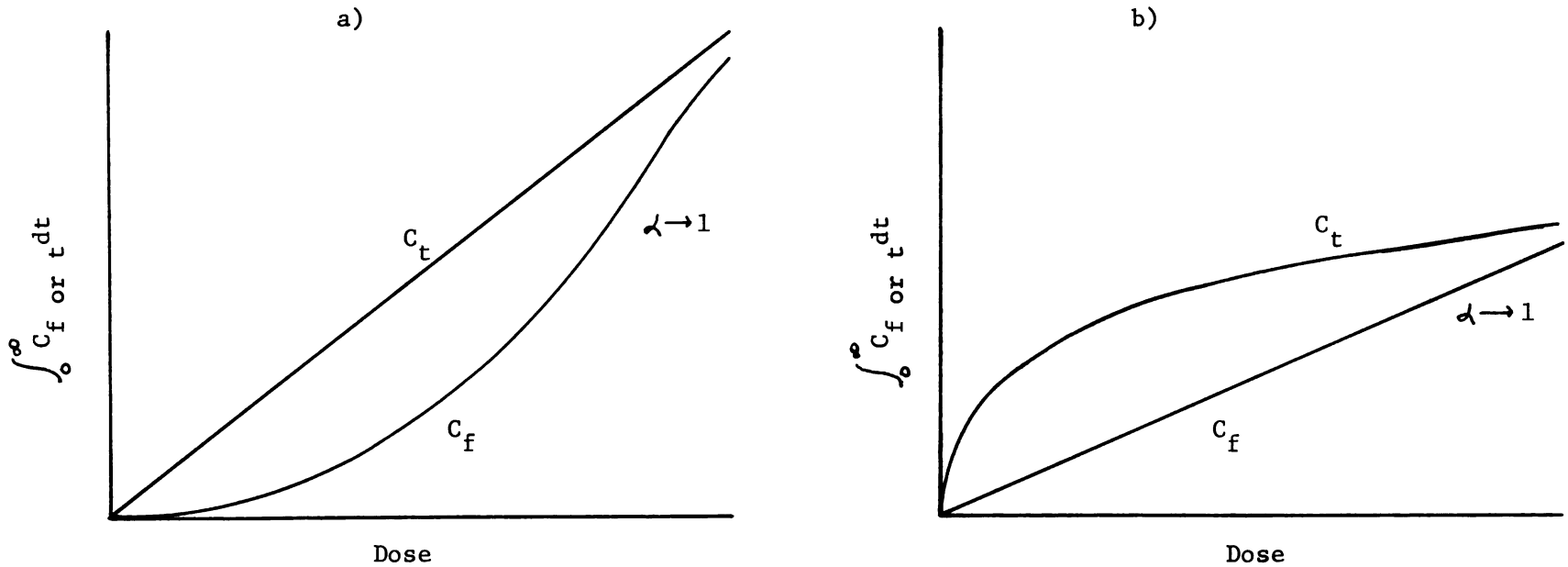


Fig.LII: Theoretical consideration of Dose versus $\int_0^\infty C_{f \text{ or } t} dt$ in which a) clearance is dependent upon the total concentration (C_t) and b) clearance is dependent upon the unbound concentration (C_f).

limiting case where the dose administered is very large so that α approaches 1, both areas (C_f or C_t) will approach the same value. These dose vs area plots may be regarded as a relatively simple test to evaluate the relationship between clearance and concentration. The steady-state approach might also be used to test the mechanism of clearance. In this case Equations 33 and 34 would result in Equations 35 and 36 respectively where Ro is the zero-order rate of administration.

$$Ct_{ss} = \frac{Ro}{CL} \qquad \text{Eq.35}$$

$$Cf_{ss} = \frac{Ro}{CL_{max}} \qquad \text{Eq.36}$$

The same general curves as exhibited in Figure LII would exist if steady-state concentartions (Cf_{ss} or Ct_{ss}) were substituted for $\int_0^\infty C_f$ or $C_t dt$ and Ro for Dose and thus could again test the mechanism of clearance.

Due to the difference in kinetics of tolbutamide observed in the sheep after repeated dosing over a period of time, which could possibly also occur with sulfadimethoxine, it was decided to give two doses of the sulfonamide close together and compare the relationships mentioned in Equations 33 and 34 rather than evaluate previous data obtained when doses were given many weeks apart. Equilibrium dialysis was used to determine the values of C_f at each C_t. However, due to the dilution problems in equilibrium dialysis it was necessary to prepare a calibration curve relating the C_f found after dialysis to the C_f in the original plasma sample.

II Results and Discussion:

Figure LIII illustrates the family of plasma concentration (C_t) - time curves generated after various boluses of sulfadimethoxine in sheep #1494. As can be seen, only Experiment VIII (4 Gm Dose) appears to exhibit a terminal log-linear phase.

The unbound sulfadimethoxine concentrations determined using equilibrium dialysis were converted to the corresponding values in the original plasma sample by means of a calibration curve.The data used to construct the calibration curve are tabulated in Table XXIX and are plotted in Figure LIV. The calibration curve was constructed as follows. A series of known plasma concentrations of sulfadimethoxine were dialyzed (6 hrs) against Krebs-Henseleit Buffer (Chapter II, p. 23) containing 1.124×10^5dpm ^{14}C - sulfadimethoxine. After dialysis was complete the plasma half-cell was reanalyzed for sulfadimethoxine content while the radioactivity measurements were used to calculate the % unbound drug in the equilibrated plasma sample. The relationship between original plasma concentration (o), equilibrated plasma concentration (•) and the % unbound are indicated in Figure LIV by the vertical arrows. For example, after equilibration, plasma which originally contained 400 mcg/ml, then contained 265 mcg/ml sulfadimethoxine, due to the movement of unbound drug into the protein-free half-cell, with a resultant 46.2% unbound sulfadimethoxine. To find the actual extent of unbound sulfadimethoxine at 400 mcg/ml one simply moves vertically from the lower to the upper curve (at 400 mcg/ml) to find that at an equilibrated dialyzed plasma sulfadimethoxine concentration of 400 mcg/ml, 54.5% is unbound. Hence the percent unbound in the original plasma sulfadimethoxine sample (400 mcg/ml) must have been 54.5%. The % difference in the fraction unbound before and after dialysis is smallest at the very

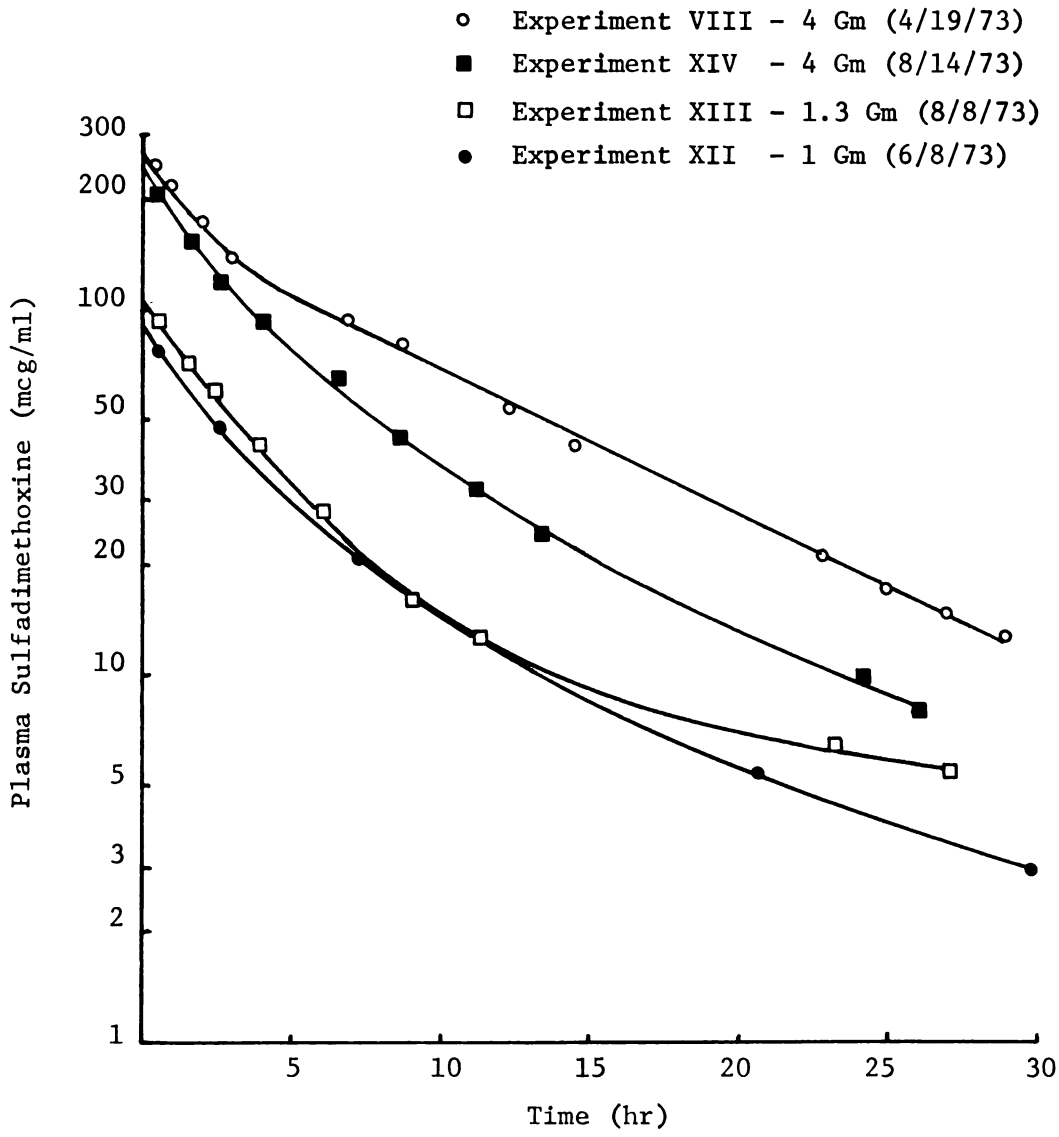


Fig.LIII: Sulfadimethoxine plasma concentrations after administration of various I.V. sulfadimethoxine boluses in Sheep #1494.

Table XXIX: Data for equilibrium dialysis calibration curve (Fig.LIV)

Original Conc.(mcg/ml)	Conc. after dialysis (mcg/ml)*1	% Unbound*2
800	520	59.4
600	390	53.8
400	265	46.2
300	215	40.0
200	150	30.4
100	84	15.2
50	46	7.3
25	25	5.4
12.5	13	4.9

*1 analyzed chemically after equilibrium dialysis (6 hr)

*2 % calculated after equilibrium dialysis using ^{14}C-sulfadimethoxine (1.124×10^5 dpm)

$$\% \text{ unbound} = \frac{\text{dpm buffer side}}{\text{dpm plasma side}} \times 100$$

Note: Plasma obtained from sheep #1494 (8/17/73)

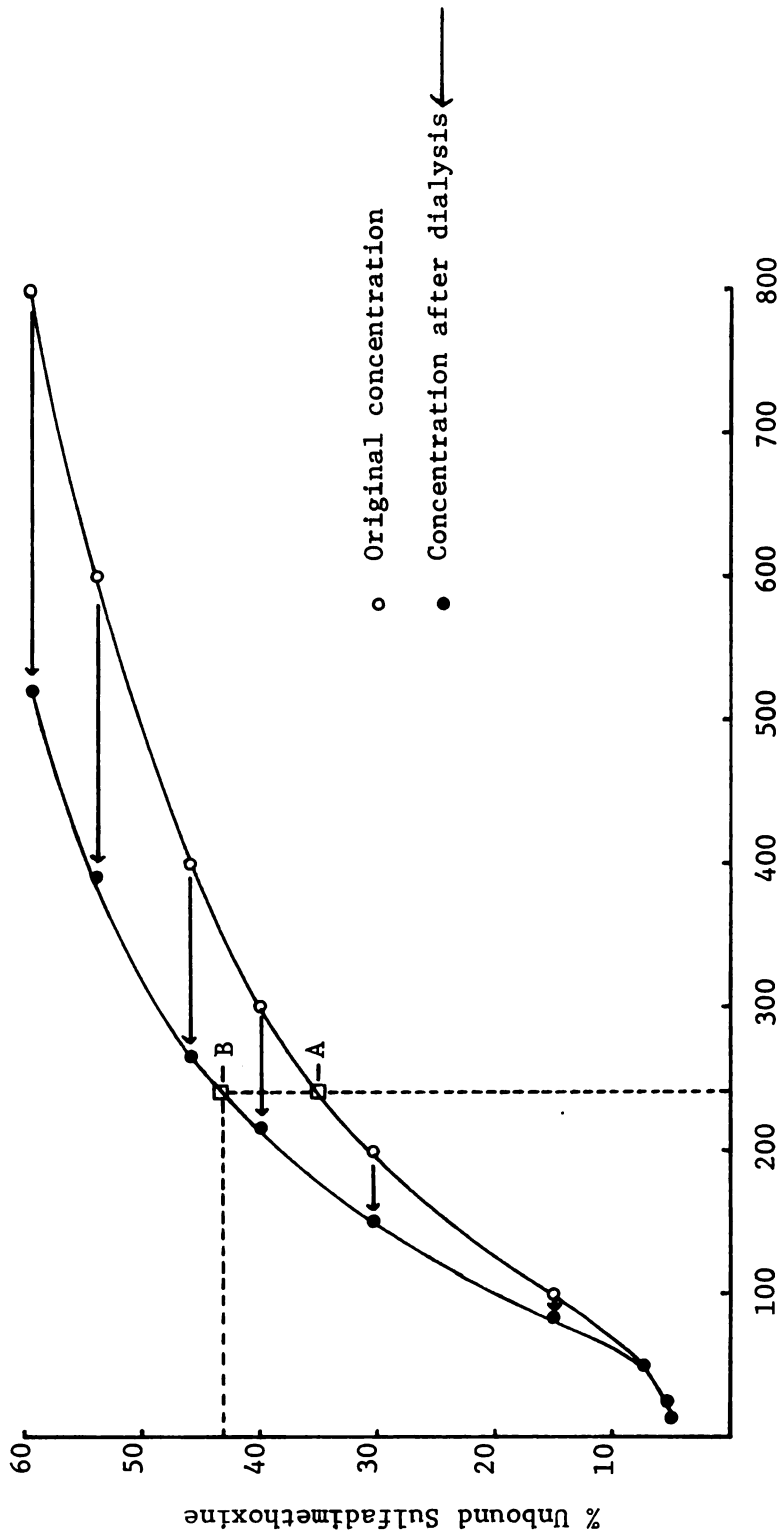


Figure LIV: Equilibrium dialysis calibration curve in sheep plasma 1494B (Table XXIX). The % unbound sulfadimethoxine in plasma containing sulfadimethoxine (e.g. 240 mcg/ml) is found by moving vertically from the lower curve (point A) to the upper curve (point B) to obtain the % unbound (43%).

low and theoretically at the very high concentrations. At the lower end α (the fraction unbound) is very small with a small fraction of the protein-binding sites occupied, and the small movement of drug from the plasma to buffer half-cell does little to alter the degree of protein binding. At the very high concentration α approaches one and for all purposes the effects of protein binding are not evident.

Unfortunately, after the plasma samples of the two boluses were analyzed, different % unbound values were obtained than expected as seen in Table XXX. As a result it was necessary to construct a calibration curve for each sheep plasma of the corresponding experiment. The samples of plasma collected after the bolus of sulfadimethoxine provided their own calibration curves as seen in Figure LV in the following way: the initial total plasma sulfadimethoxine concentration was known; the radioactivity, after dialysis, provided C_f (fraction of total radioactivity found in both half-cells, present in the buffer half-cell multiplied by initial sulfadimethoxine total concentration); the radioactivity, after dialysis, provided the % unbound in the equilibrated cells (radioactivity in buffer half-cell divided by radioactivity in plasma half-cell); total sulfadimethoxine concentration in plasma half-cell after dialysis was found by subtracting C_f from the initial total plasma sulfadimethoxine concentration. As can be seen in Figure LV the plasma samples from the two experiments appeared to bind sulfadimethoxine differently. In order to ascertain the % unbound for the maximum initial sulfadimethoxine plasma concentrations in each experiment an estimated extrapolation was made of each upper curve. This then permitted the calculation of C_f (adjust) in Table XXX as described earlier in Figure LIV. In order to assess the areas of C_t and C_f (adjust) in Table XXX it was necessary to know time zero concentrations, C_t^o and C_f^o (adjust) respectively. Due to the continual curvature of both log C_t

Table XXX: Equilibrium dialysis of I.V. boluses of sulfadimethoxine in Sheep #1494

Experiment*1	Time(hr)	Ct(mcg/ml)*2	After dialysis Cf(mcg/ml)	%Unbound Found	%Unbound*3 Expected	Cf adjust(mcg/ml)*4
XIII	0.65	92.4	13.1	16.6	13.7	21.5
(8/8/73)	1.54	71.2	7.9	11.1	10.3	9.3
	2.42	60.0	-	-	-	-
	3.97	42.5	3.4	8.0	6.8	3.4
	6.11	28.2	1.8	6.4	5.6	2.0
	9.13	16.2	-	-	-	-
	11.38	12.8	0.6	5.0	5.0	0.6
	23.25	6.5	0.3	4.6	5.0	0.3
	27.13	5.5	0.3	4.7	5.0	0.3
Area*5		670.1				68.3
XIV	0.51	206.8	53.1	34.6	30.8	80.7
(8/14/73)	1.71	153.9	34.0	28.3	23.7	53.2
	2.65	120.6	-	-	-	-
	4.06	93.6	12.3	15.1	13.7	17.1
	6.57	64.8	6.0	10.3	9.7	7.4
	8.59	45.0	3.1	7.4	7.2	3.5
	11.23	32.4	-	-	-	-
	13.46	24.2	1.3	5.4	5.0	1.3
	24.22	10.0	0.5	4.7	5.0	0.5
	26.02	8.0	0.3	4.3	5.0	0.3
Area*5		1353.8				259.5

*1 Experiment XIII = 1.3 Gm; Experiment XIV = 4.0 Gm.
*2 Initial total plasma sulfadimethoxine (before dialysis).
*3 from Figure LIV.
*4 from appropriate calibration curve (Figure LV).
*5 Area = $\sum\left[(\sqrt{C^1C^2})t_2-t_1;\ \frac{C}{K}\text{ after last data point}\right]$; C_t^o & C_f^o estimated by back extrapolation. Units - (mcg/ml).hr.

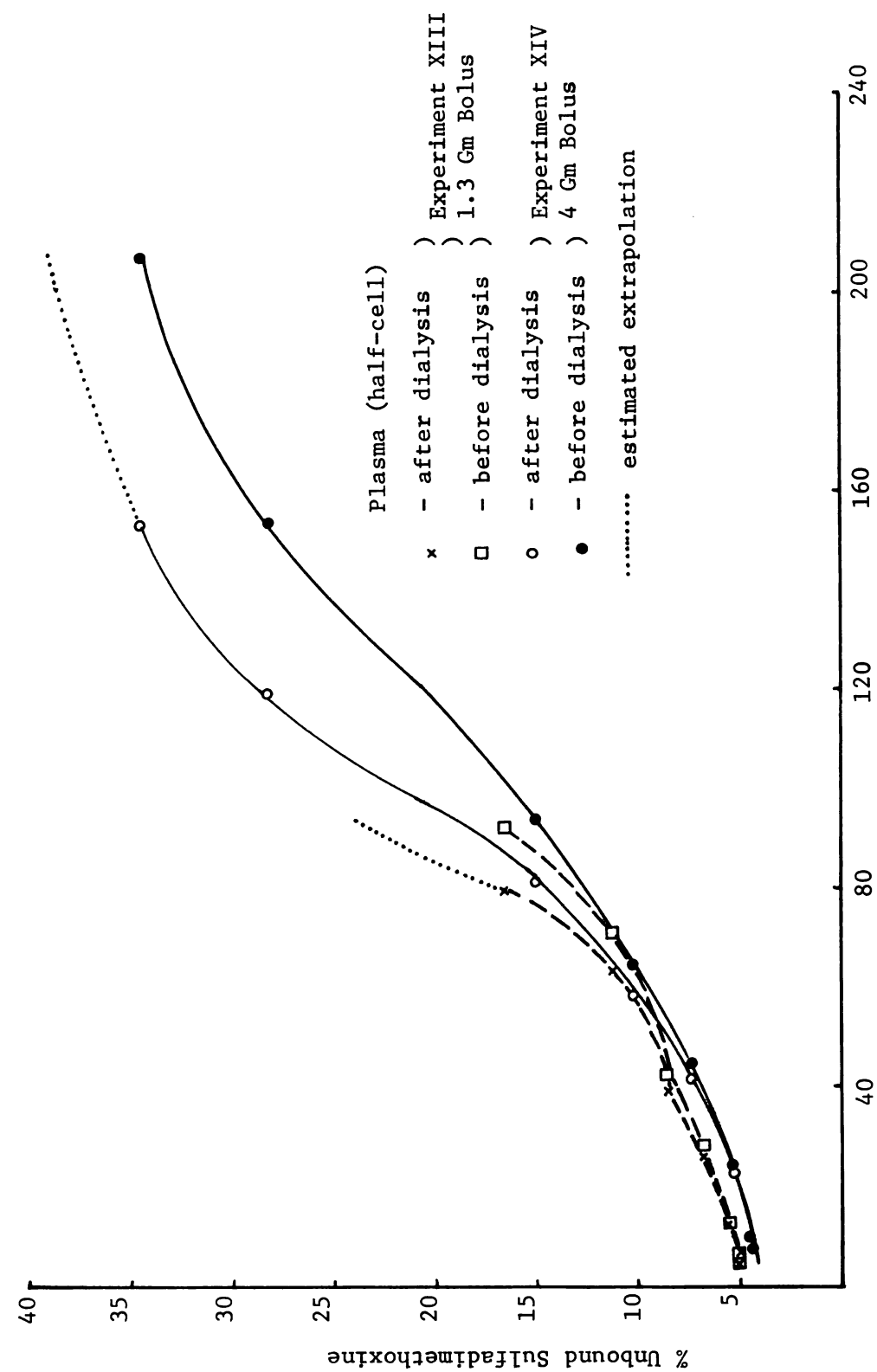


Fig. LV: Equilibrium dialysis calibration curve for plasma samples obtained after the I.V. administration of sulfadimethoxine to sheep #1494. Movement vertically from the lower curve to the upper curve provides the % unbound sulfadimethoxine in the appropriate plasma, at a given initial total sulfadimethoxine concentration.

and log C_f with time, no simple solution exists for C_t^o and C_f^o (adjust). In order to estimate these values, a linear back extrapolation was made using the two earliest C_t and C_f values in time, and although they undoubtedly are not the actual values, they represent an approximation.

In order to attempt an explanation for the differences seen in Figures LIV and LV, Rosenthal plots were made using the data of Tables XXIX and XXX. The results are seen in Figure LVI. It is evident for the three plasma samples of sheep #1494, that sulfadimethoxine is not bound to only one site. As a result it is difficult to assess possible differences in the proteins. However since the slopes are all practically the same in the initial region (low b) while the intercepts appear to vary somewhat, it may be that nPt (the total molar concentration of primary binding sites) is different. Protein analysis on these plasma samples, although not performed, might have established a difference in albumin or other protein concentrations thus accounting for apparent differences in n Pt. It would appear that not enough low concentrations of sulfadimethoxine were employed, when initially assessing the binding of sulfonamide in plasma HB, and in fact K and nPt for the primary site are different than proposed in Figure XXV (Chapter III, p.112). The values for K and nPt of the primary site reported (from Figure XXV) were $1.54 \times 10^4 M^{-1}$ and $8 \times 10^{-4} M$ while they may be closer to $1.1 \times 10^5 M^{-1}$ and $2.0 \times 10^{-4} M$ respectively based upon Figure LVI.

The results of the calculated areas of Table XXX have been plotted in Figure LVII. The line describing the Dose vs $\int_o^\infty C_f dt$ relationship practically passes through zero thus suggesting that the clearance of sulfadimethoxine is dependent upon the unbound concentration (CL_{max} = 235 ml/min) and follows the theoretical considerations of Figure LIIb. Provided

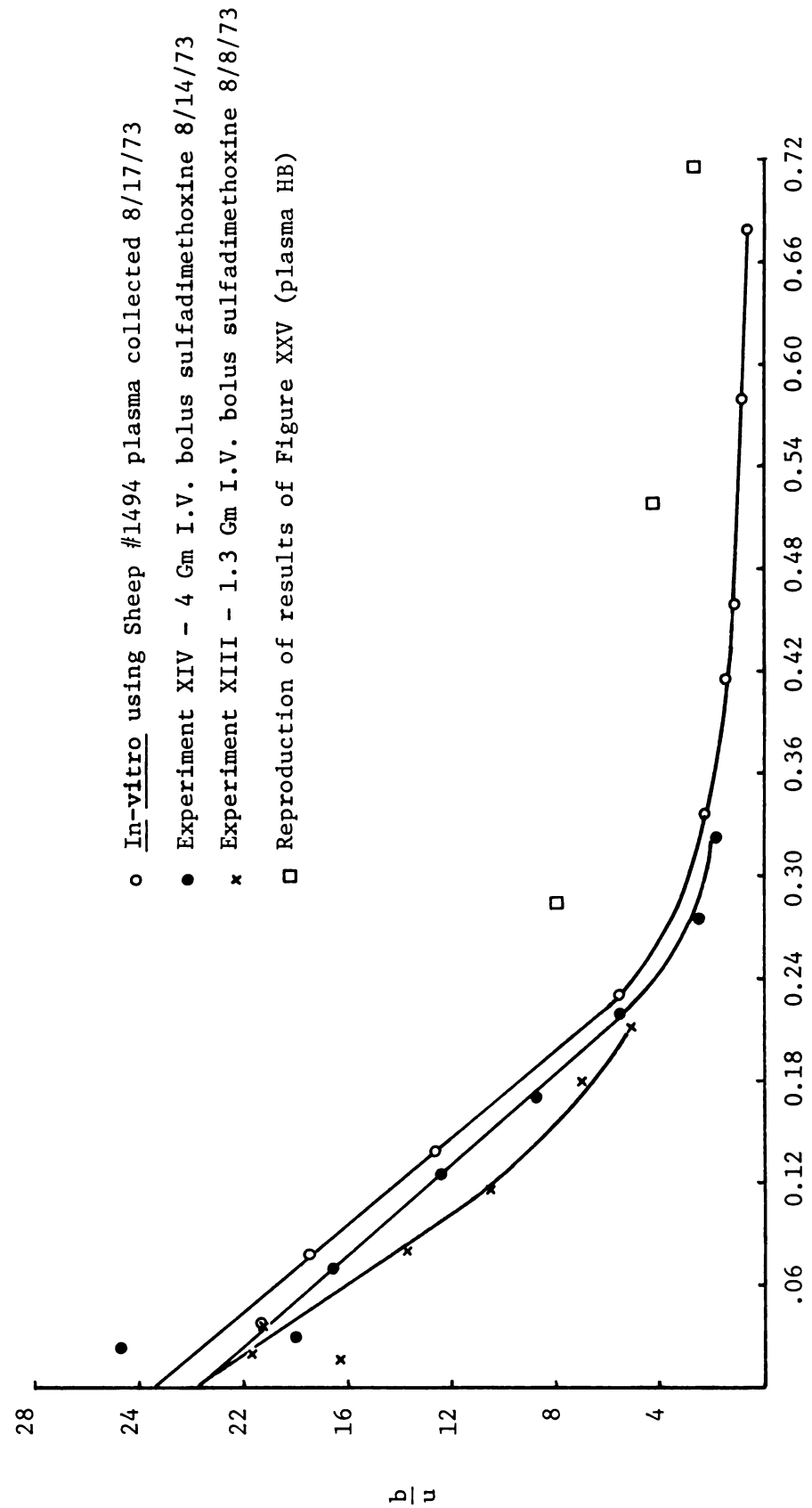


Fig.LVI: Rosenthal plots of the binding of sulfadimethoxine to plasma of sheep #1494.

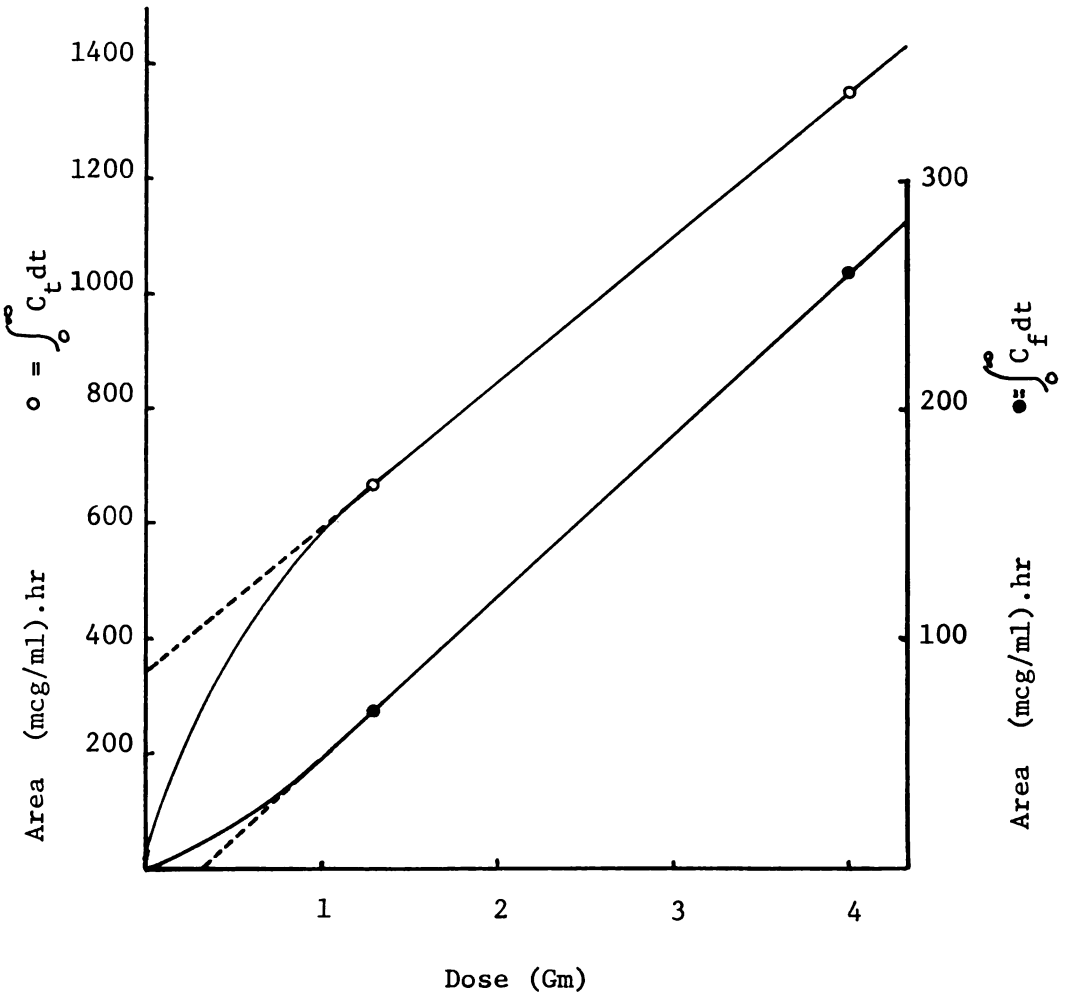


Fig.LVII: Dose vs area after sulfadimethoxine bolus in sheep #1494

sulfadimethoxine is excreted unchanged in the sheep, this CL_{max} would yield an apparent extraction ratio of about 0.3 (30% of plasma delivered to the kidney is cleared of unbound sulfadimethoxine). Further, it is interesting to note that the ratios of areas (C_t to C_f) at the 1.3 and 4 Gm I.V. dose are 9.8 and 5.2 respectively demonstrating convergence of the curves with increasing dose after some unknown maximum ratio. This again follows the theoretical considerations of Figure LIIb.

Coffey[99] who gave a theoretical consideration on the effects of protein binding of drugs on areas under plasma concentration-time curves developed Equation 37 which is similar to that of Equation 34 (p.184), where V_1 is the volume of the plasma compartment and k_2 the unbound (free)

$$\frac{D}{V_1 k_2} = \int_0^\infty C_f dt \qquad \text{Eq.37}$$

drug elimination rate constant. However the author failed to extend his discussion to the use of Equation 37 to establish the method of elimination of a compound whereas in this chapter it has been shown that obtaining areas for both C_f and C_t and preparing plots as indicated (Figure LVII) may provide a quick method of assessing the mode of clearance of a compound. It should be noted that when the overall elimination of a compound is composed of various routes, then the possibility exists that the mode of clearance (based upon C_t or C_f) for all the routes may not be the same. As a result neither Dose vs $\int_0^\infty C_f dt$ nor $\int_0^\infty C_t dt$ will exhibit linearity over a wide dose range.

The bolus of sulfadimethoxine which appeared to have a terminal log-linear phase (Experiment VIII) was also evaluated for unbound levels and the results are seen in Figure LVIII. As can be seen, the near linearity of the terminal phase of the total (C_t) curve, while λ changes (until about 22 hrs)

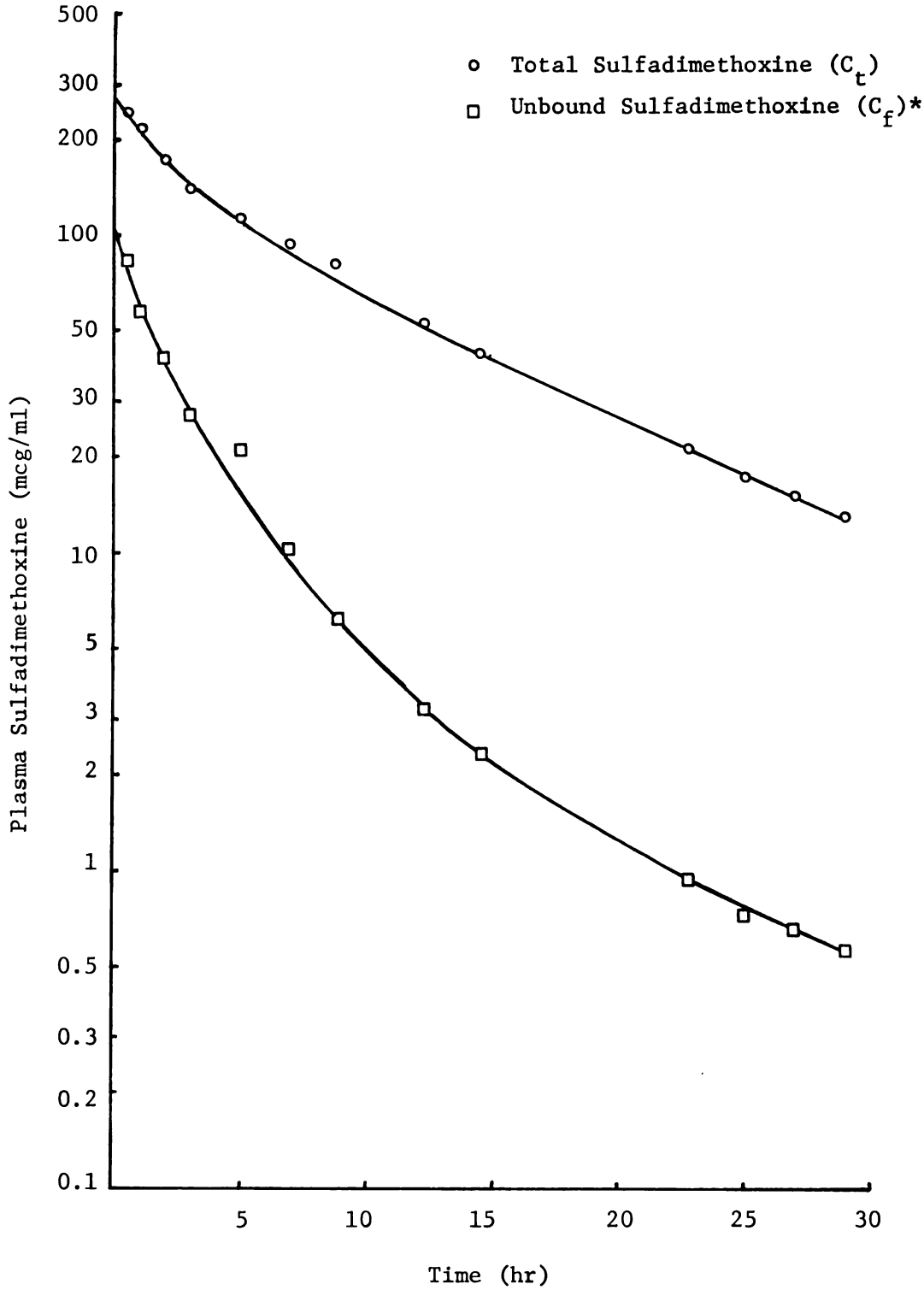


Fig.LVIII: Total and unbound plasma sulfadimethoxine after 4 Gm I.V. bolus of sulfadimethoxine in sheep #1494 (Experiment VIII; 4/19/73).

* found by equilibrium dialysis

suggests the $\frac{\alpha}{Vd}$ remains fairly constant (Equation 32, p.183). After 22 hrs, α appears to be constant and follows the theoretical considerations of Coffey[99] who indicated that at sufficiently low concentrations $\frac{Ct}{Cf}$ would become constant. The results seen in Figure LVIII match general predictions provided by two publications in which consideration was given to the shape(s) of the total plasma concentration-time curve(s) given that elimination was proportional to the unbound concentration of administered drug. Coffey et al[101] used predominantly a one compartment model with binding in that compartment for their predictions, while Krueger-Thiemer et al[100] considered predominantly a two compartment model for their predictions in which binding took place in the central compartment (plasma volume from which elimination took place) and unbound drug could equilibrate instantaneously with the second compartment (residual body water). Both publications however indicate that a decrease in initial dose would result in total plasma concentration-time curves exhibiting increased linearity. However for sulfadimethoxine in the sheep (Figure LIII, p.188) this does not appear to be the case. It may be that unbound sulfadimethoxine distributes non-instantaneously into two apparent volumes (two compartment model) and that binding takes place in both compartments. This distribution characteristic could account for the lack of linearity of total concentration-time curves at the lower doses employed.

The fact that $\frac{\alpha}{Vd}$ appears to remain relatively constant in Figure LVIII may be something unique to this specific bolus, since Figure LIII indicates that the other 4 Gm bolus (Experiment XIV) does not exhibit the same linearity, nor do the lesser doses. The results in Interaction Study #3 (Chapter III, p. 157) suggest that $\frac{\alpha}{Vd}$ does not remain constant for

tolbutamide although Vd retains a similar value. It is interesting to note that in the work of Jaehnchen et al[98], in which the interaction of phenylbutazone and dicumarol was studied in rats, phenylbutazone caused a change in α for dicumarol with no statistically significant change in Vd thus indicating that $\frac{\alpha}{Vd}$ did not remain constant for dicumarol. As a result it may be that Vd has a limiting value for certain compounds (e.g. extracullular volume), although this is questionable since it would appear obvious that the action of compounds like tolbutamide and dicumarol occurs intracellularly.

The question arises finally whether tolbutamide is capable of influencing the disposition of sulfadimethoxine. Although this aspect has not been studied, it should be pointed out that work by Anton[102], who among other things looked at the effect of tolbutamide on the binding of sulfadimethoxine to undiluted human plasma, demonstrated that the % unbound after the addition of tolbutamide (100 and 300 mcg/ml) changed from a control value of 3% to 6 and 17% respectively (100 mcg/ml sulfadimethoxine). Thus tolbutamide is capable of displacing sulfadimethoxine from plasma proteins and could influence the elimination of the sulfonamide. Since the concentrations of tolbutamide employed in the interaction studies were relatively low (less than 65 mcg/ml) one might not expect a change in elimination kinetics of sulfadimethoxine. It was observed that the plasma kinetics after administration of sulfadimethoxine in Interaction Study #1 (Chapter III, p.120) were similar to those seen after the I.V. administration of sulfadimethoxine alone (Experiment VIII, Figure LII, p.185).

III Conclusion:

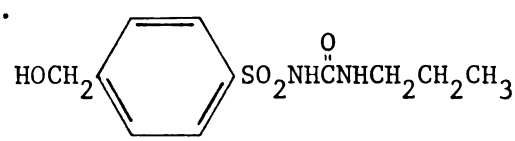
The elimination of sulfadimethoxine in the sheep appears to be dependent upon the unbound plasma concentration with an appropriate total body clearance of 235 ml/min (based upon unbound plasma concentration). It has been demonstrated that the mode of clearance of a compound may be evaluated by performing area analysis of the total and unbound concentrations after a series of I.V. boluses.

CHAPTER V

OVERVIEW

The purpose of this research project was to investigate whether protein displacement was a viable explanation for certain drug-drug interactions. The hypothesis forwarded was that the presence of a steady-state of displacer should cause no change in the steady-state unbound levels of the primary protein-bound agent administered, if the clearance of the primary agent was dependent upon the unbound concentration. Tolbutamide and sulfadimethoxine, the primary agent and displacer respectively were selected as model compounds. The project required the development of a chronic animal preparation. The sheep model proved to be very satisfactory although the areas of externalization of its catheters proved to be susceptible to infection. As a result when future studies of this type are performed, it would be advisable to evaluate organ functions such as kidney and liver (using appropriate tests or internal marker compounds), registering any changes which might be very important in kinetic investigations of this type.

The methods of analysis of the compounds used were generally satisfactory although a better internal standard should be obtained for the gas chromatographic assay of plasma hydroxytolbutamide before the internal standard is added and thus potential errors exist. A possible compound might be hydroxytolbutamide with the n-butyl side chain replaced by an n-propyl chain.



The assay for sulfadimethoxine in sheep urine needs further development due to interference of some endogenous component. Possibly a fluorometric or gas chromatogrpahic method would be suitable.

The administration of tolbutamide in the sheep requires further investigation to elucidate the fate of the unaccountable 25% of the dose. This will be most easily carried out with radioactively tagged tolbutamide. Logical compounds might be conjugates, sulfonamides or dealkylated sulfonylureas.

Of the protein binding determination methods, ultrafiltration proved to be simple and fast but required a large volume ($\sim$ 4 ml) of plasma. When only small plasma volumes are available, the Dianorm equilibrium dialysis system is useful but the dilution aspect requires construction of a calibration curve to obtain the % unbound in the pre-dialyzed plasma sample. The Dianorm system used would be even better if the plasma half-cell were kept at 1 ml but the buffer half-cell were remanufactured to hold only 0.2 ml. This should increase the region where dilution effects are insignificant by a factor of 5 (Figure LIV, Chapter IV, p. 190).

It has been shown that hydroxytolbutamide has a diuretic effect. As indicated during its discussion, further work is needed to establish whether the effect is representative of other sheep or even other species. Also, the possible diuretic effect of tolbutamide, carboxytolbutamide and other similar compounds requires co-investigation with such a study providing possible valuable structure-activity relationships.

It is interesting to find that the clearance (CL) of both tolbutamide and sulfadimethoxine are about the same in the sheep. It is more interesting to find that the clearance dependent upon the unbound concentration (CL_{max}) of tolbutamide (Interaction Study #3; 215 ml/min) and sulfadimethoxine (Chapter IV: 235 ml/in) are about the same. It leads one to suggest that compounds with a low CL value and exhibiting marked plasma protein binding may be clearance limited by their protein binding. In this present study,

more doses of sulfadimethoxine are needed to confirm the observation of clearance dependent upon the unbound plasma concentration. An unfortunate aspect of this study is the lack of administration of various sizes of I.V. bolus of tolbutamide in order to establish unequivocally that its total body clearance is dependent upon the unbound concentration.

Unfortunately the drug interaction studies have been carried out in only one sheep. Of major concern is the lack of adequate controls, i.e. steady-state tolbutamide followed by saline administration only (at the pH of the sulfadimethoxine solutions). Also, although steady-states, which demonstrate sensitivity to changes, were predominantly evaluated, it would be advisable to administer tolbutamide at steady-state but co-administer sulfadimethoxine at slow changing but increasing rates (provide accumulation) in order to test more rigorously the equations of inhibition developed (Chapter III, p. 175).

In the light of the proposed hypothesis stated at the outset of this overview, this study was not completed. Possibly indocyanine green might serve to demonstrate displacement without metabolic inhibition. Yet, the study illustrated that measuring total drug concentrations is not sufficient when evaluating drug interactions. The project also illustrated how interactions of medicinal agents might be studied and how the ensuing complexities might possibly be analyzed. It also illustrated that mere analysis of the chemical entities administered or their resultant biotransformation products may not lead to an explanation regarding the extent of interaction but requires an evaluation of changes in endogenous compounds which potentially represent an integral part of the interaction. The beneficial aspects to the study of drug interactions lie not only in the answers to the mechanisms of the interactions themselves, but on the addition of information concerning the in vivo effects/changes which are part of the spectrum of drug actions.

BIBLIOGRAPHY

1. Stockley, I.H., "Mechanisms of drug interaction in the pharmacokinetic phase of drug activity.", Am. J. Hosp. Pharm. 27, 977-85 (1970).

2. Lasagna, L., "The diseases drugs cause.", Perspectives Biol. Med. 7, 457-70. (1963-1964)

3. Stockley, I.H. "Mechanisms of drug interaction. 1. Basic Principles.", Pharm. J. 206, 377-81 (1971).

4. Hartshorn, E.A., "Drug interactions. III. Classes of Drugs. Interactions of Cardiac Drugs.", Drug Intel. Clin. Pharm. 4, 272-5 (1970).

5. Parker, W.J., "Alcohol-drug interactions.", J. Amer. Pharm. Ass. 10, 664-5, 668-73, 677 (1970).

6. Hussar, D.A., "Hypoglycemic agents. Their interactions.", J. Amer. Pharm. Ass. 10, 619-24 (1970).

7. King, T.M. and Burgard, J.K., "Drug interaction.", J. Obstet. Gynecol. 98, 128-34 (1967).

8. Cucinell, S.A., "Phenobarbital. Interactions with other drugs.", (Woodbury, D.M.), Antiepileptic Drugs, Raven Press: New York, N.Y. (1972), pp. 319-27.

9. Mannering, G.J., "Drug interactions. Principles and problems in relation to drugs of abuse.", (Epstein, S.S.), Drugs Abuse: Their Genet. Other Chron. Non-psychiatric Hazards, Symp., MIT: Cambridge, Mass. (1969, Pub. 1971), pp. 52-75.

10. Rosenoer, V.M. and Gill, G.M., "Drug interactions in clinical medicine.", Med. Clin. N. Amer. 56, 585-97 (1972).

11. Brown, S.S., "Interactions of drugs and foreign compounds.", Foreign Compd. Metab. Mammals. 2, 456-74 (1972).

12. Martin, E.W., "Hazards of Medication.", J.B. Lippincott and Co.: Philadelphia, Pa. (1971).

13. Hanston, P.D., "Drug Interactions.", 2nd Ed., Lea and Febiger: Philadelphia, Pa. (1973).

14. Kunin, C. and Finland, M., "Clinical pharmacology of the tetracyline antibiotics.", Clin. Pharmacol. Ther. 2, 51-69 (1961).

15. Hart, S.L. and McColl, I., "The effect of purgative drugs on the intestinal absorption of glucose.", J. Pharm. Pharmacol. 19, 70-1 (1967).

16. Aggeler, P.M., O'Reilly, R.A., Leong, L. and Kowitz, P.E., "Potentiation of anticoagulant effect of warfarin by phenylbutazone.", New Eng. J. Med. 276, 496-501 (1967).

17. Anton, A.H., "The effect of disease, drugs, and dilution on the binding of sulfonamides in human plasma.", Clin. Pharmacol. Ther. 9, 561-7 (1968).

18. Cucinell, S.A., Conney, A.H., Sansur, M.S., and Burns, J.J., "Drug interactions in man. 1. Lowering effect of phenobarbital on plasma levels of bishydroxycoumarin (dicumarol) and diphenylhydantoin (dilantin).", Clin. Pharmacol. Ther. 6, 420-9 (1965).

19. Olesen, O.V., "Disulfiramum (Antabuse) as inhibitor of phenytoin metabolism.", Acta Pharmacol. Toxicol. 24, 317-22 (1966).

20. Hansen, J.M., Kristensen, M., Skovsted, L. and Christensen, L.K., "Dicoumarol-induced diphenylhydantoin intoxication", Lancet 2, 265-6 (1966).

21. Beckett, A.H. and Rowland, M., "Urinary excretion kinetics of amphetamine in man.", J. Pharm. Pharmacol. 17, 628-39 (1965).

22. Milne, M.D., "Influence of acid-base balance on efficacy and toxity of drugs.", Proc. Roy. Soc. Med. 58, 961-3 (1965).

23. Field, J.B., Ohta, M., Boyle, C. and Remer A., "Potentiation of acetohexamide hypoglycemia by phenylbutazone.", New Eng. J. Med. 277, 889-94 (1967).

24. Gundersen, T., Odegaard, K. and Gjessing, H.C., "Treatment of gonorrhoea by one oral dose of ampicillin and probenecid combined.", Brit. J. Vener. Dis. 45, 235-7 (1969).

25. Goodman, L.S. and Gilman, A., "The Pharmacological Basis of Therapeutics.", 4th Ed. (1970) MacMillan: New York, N.Y. (a) Ch. 21-25. (b) Ch.71. (c) pp. 831-85.

26. Inglis, J.M. and Barrow, M.E.H., "Premedication - a reassessment.", Proc. Roy. Soc. Med. 58, 29-32 (1965).

27. Morrelli, H.F. and Melmon, K.L., "The clinician's approach to drug interactions.", Calif. Med. 109, 380-9 (1968).

28. Roberts, J., Ito, R., Reilly, J. and Cairoli, V.J., "Influence of reserpine and Beta TM 10 on digitalis induced ventricular arrythmia.", Circ. Res. 13, 149-58 (1963).

29. Mukherjee, D., "Studies on the effect of free fatty acid on binding of benzylpenicillin by serum proteins.", J. Antibiot.23, 348-53 (1970).

30. Branch, R.A., Shand, D.G., Wilkinson, G.R. and Nies, A.S., "The reduction of lidocaine clearance by dl-propranolol: An example of hemodynamic drug interaction.", J. Pharmacol. Exp. Ther. 184, 515-9 (1973).

31. Caraway, W.T. and Kammeyer, C.W., "Chemical interference by drugs and other substances with clinical laboratory test procedures.", Clin. Chem. Acta 41, 395-434 (1972).

32. Singh, H.P., Hebert, M.A. and Gault, M.H., "Effect of some drugs on clinical laboratory values as determined by the Technicon SMA 12/60." Clin. Chem. 18, 137-44 (1972).

33. Young, D.S., Thomas, D.W., Friedman, R.B. and Pestaner, L.C., "Effects of drugs on clinical laboratory tests.", Clin. Chem. 18, 1041-303 (1972).

34. Sotaniemi, E. and Palva, I.P., "The use of polypharmaceutical drug therapy in a medical ward.", Ann. Clin. Res. 4, 158-64 (1972).

35. Macleod, A.G., "Diabetes", The Upjohn Company: Kalamazoo, Mich. (1969).

36. Banting, F.G. and Best, C.H., "The internal secretion of the pancreas.", J. Lab. Clin. Med. 7, 251-66 (1922).

37. Oakley, W.G., Pyke, D.A. and Taylor, K.W., "Clinical Diabetes and its Biochemical Basis.", Blackwell Scientific Publ.: Oxford (1968).

38. Von Mering, J. and Minkowski, O., "Diabetes Mellitus nach Pankreasextirpation.", Arch. Exp. Path. Pharmakol. 26, 371-87 (1889).

39. Grodskey, G.M., Landahl, H., Cury, D., and Bennett, L.L., "A two-compartment model for insulin secretion.", Int. Symp. on Early Diabetes, Marbella, Academic Press: New York, N.Y. (1969).

40. Feldman, J.M. and Lebovitz, H.E., "Appraisal of the Extrapancreatic actions of sulfonylureas.", Arch. Int. Med. 123, 314-22 (1969).

41. Hasselblatt, A. and Bludau, W., "Dosisabhaengigkeit von serumkonzentration und blutzuckerwirkung des N-4-methyl-benzolsulfonyl-N-butylharnstoff (Rastinon, D860, Artosin) bei intravenoesen dauerinfusionen.", Klin. Wochschr. 36, 157-63 (1958).

42. Baird, J.D. and Duncan, L.J.P., "An analysis of the hypoglycemic response to tolbutamide.", Scot. Med. J. 2, 341-50 (1957).

43. Wittenhagen, G., Mohnike, G. and Langenbeck, W., "Beitrage zu Abbau und ausscheidung von N-(4-methyl-benzolsulfonyl)- N^1 -butyl-harnstoff (D860).", Z. Physiol. Chem. 316, 157-63 (1959).

44. Mohnike, G. Wittenhagen, G. and Langenbeck, W., "Ueber das Ausscheidsprodukt von N-(4-methyl-benzolsulfonyl)-N^1-butylharnstoff beim Hund.", Naturwissenshaft. 45, 13 (1958).

45. Thomas, R.C. and Ikeda, G.J. "The metabolic fate of tolbutamide in man and in the rat.", J. Med. Chem. 9, 507-10 (1966).

46. Tagg, J., Yasula, D.M., Tanabe, M. and Mitoma, C., "Metabolic Studies of tolbutamide in the rat.", Biochem. Pharmacol. 16, 143-53 (1967).

47. Larsen, J.A. and Madsen, J., "Inhibition of Ethanol Metabolism by oral antidiabetics.", Proc. Soc. Exptl. Biol. Med. 109, 120-2 (1962).

48. Matin, S. and Rowland, M., To be published.

49. Schultz, E. and Schmidt, F.H., "Blutzuckersenkende Wirkung vong hydroxytolbutamid beim menschen.", Klin. Wschr. 48, 759-60 (1970).

50. Roggin, G.M. and Iber, F., "The duration of accelerated drug removal after heavy alcohol ingestion.", Clin. Res. 17, 309 (1969).

51. Kater, R.M.H., Tobon, F. and Iber, F.L., "Increased rate of tolbutamide metabolism in alcoholic patients.", J.A.M.A. 207.

52. Kater, R.M.H., Roggin, G., Tobin, F., Zieve, P. and Iber, F., "Increased rate of clearance of drugs from the circulation of alcoholics.", Am. J. Med. Sci. 258, 35-9 (1969).

53. Landon, J., Wynn, V. and Samols, E., "The effect of anabolic steroids on blood sugar and plasma insulin levels in man.", Metabolism 12, 924-35 (1963).

54. Christensen, L.K. and Skovsted, L., "Inhibition of drug metabolism by chloramphenicol.", Lancet 2, 1397-9 (1969).

55. Kristensen, M. and Hansen, J.M., "Potentiation of the tolbutamide effect by dicumarol.", Diabetes 16, 211-4 (1967).

56. Solomon, H.M. and Schrogie, J.J., "Effect of Phenyramidol and bishydroxycoumarin on the metabolism of tolbutamide in human subjects.", Metabolism 16, 1029-33 (1967).

57. Spurny, O.M., Wolf, J.W. and Devins, G.S., "Protracted tolbutamide-induced hypoglycemia.", Arch. Int. Med. 115, 53-6 (1965).

58. Christensen, L.K., Hansen, J.M. and Kristensen, M., "Sulfaphenazole-induced hypoglycemic attacks in tolbutamide-treated diabetics.", Lancet 2, 1298-1301 (1963).

59. Slade, I.H. and Josefa, R.N., "Fatal hypoglycemic coma from the use of tolbutamide in elderly patients: Report of two cases.", J. Am. Geriat. Soc. 15, 948-50 (1967).

60. Welch, R.M., Harrison, Y.E., Conney, A.H. and Burns, J.J., "An experimental model in dogs for studying interactions of drugs with bishydroxycoumarin.", Clin. Pharmacol. Ther. 10, 817-25 (1969).

61. Wishinsky, W., Glasser, E.J. and Perkal, S., "Protein interactions of sulfonylurea compounds.", Diabetes (suppl.) 11, 18-25 (1962).

62. Cherner, R., Groppe, C.W. Jr. and Rupp, J.J., "Prolonged tolbutamide-induced hypoglycemia," J.A.M.A. 185, 883-4 (1963).

63. Mahfouz, M., Abdel-Maguid, R. and El-Dakhakhny, M., "Potentiation of the hypoglycemic action of tolbutamide by different drugs.", Arznein.-Forsch. 20, 120-2 (1970).

64. Matin, S.B., Karam, J. and Rowland, M., J. Pharmacokinetics and Bioceutics. Submitted for publication.

65. Mennear, J.H. and Gossel, T.A., "Interactions between diphenylhydantoin and tolbutamide in mice.", Toxicol. Appl. Pharmacol. 24, 309-16 (1973).

66. Pang, S., Personal communication from our laboratory.

67. Kristensen, M. and Christensen, L.K., "Drug induced changes of the blood glucose lowering effect of oral hypoglycemic agent.", (Loubatieres, A. and Renold A.E.) Pharmacokinetics and Mode of Action of Oral Hypoglycemic Agents. III Capri Conference. Casa Editrice: Milan, Italy (1969) pp.116-136.

68. McQueen, E.G. and Wardell, W.M., "Drug displacement from protein binding: Isolation of a redistributional drug interaction In Vivo.", Br. J. Pharmac. 43, 312-24 (1971).

69. Reichelderfer, G., Personal communication from our laboratory.

70. Anton, A.H., "The effect of disease, drugs, an dilution on the binding of sulfonamides in human plasma.", Clin. Pharmacol. Ther. 9, 561-7 (1968).

71. Spingler, H., "Ueber Eine Moeglichkeit zur colorimetrischen bestimmung von N-(4-methyl-benzolsulfonyl)-N^{1}-butyl- harnstoff in serum.", Klin. Wochenschr. 35, 533-5 (1957).

72. Forist, A.A., Miller, W.L., Krake, J. and Struck, W.A., "Determination of plasma levels of tolbutamide (1-butyl-3-p-tolylsulfonylurea, Orinase).", Proc. Soc. Exp. Biol. Med. 96, 180-3 (1957).

73. Sabih, K. and Sabih, K., "Gas chromatographic method for determination of tolbutamide and chlorpropamide.", J. Pharm. Sci. 59, 782-4 (1970).

74. Prescott, L.F. and Redman, D.R., "Gas-liquid chromatographic estimation of tolbutamide and chlorpropamide in plasma.", J. Pharm. Pharmac. 24, 713-6 (1972).

75. Matin, S.B. and Rowland, M., "Determination of tolbutamide and chlorpropamide in biological fluids.", J. Pharm. Pharmac. 25, 186-8 (1973).

76. Matin, S.B. and Rowland, M. To be published.

77. Nelson, E., "Reilly, I. and Chulski, T., "Determination of carboxytolbutamide in urine.", Clin. Chim. Acta 5, 774-6 (1960).

78. Bratton, A.C. and Marshall, E.K., "A new coupling component for sulfanilamide determination.", J. Biol. Chem. 128, 537-50 (1939).

79. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., "Protein measurement with the folin phenol reagent.", J. Biol. Chem. 193, 265-75 (1951).

80. Dole, V.P., "A relation between non-esterified fatty acids in plasma and the metabolism of glucose.", J. Clin. Invest. 35, 150-4 (1956).

81. Goldstein, A., "Interactions of drugs and plasma proteins.", Pharmacol. Rev. 1, 102-65 (1949).

82. Meyer, M.C. and Guttman, D.E., "The binding of drugs by plasma proteins.", J. Pharm. Sci. 57, 895-918 (1968).

83. Krebs, H.A. and Henseleit, K., "Untersuchungen Ueber die Harnstoff-bildung im tierkoerper.", Z Physiol. Chem. 110, 33-66 (1932).

84. Kushinsky, S. and Paul, W., "Use of a self-cleaning oven to clean liquid scintillation vials and other glassware.", Anal. Biochem. 30, 465-7 (1969).

85. Portmann, G.A., "Pharmacokinetics.", (Swarbrick, J.), Current Concepts in the Pharmaceutical Sciences, Biopharmaceutics, Lea and Febiger: Philadelphia (1970) pp. 1-56.

86. Kaneko, J.J. and Cornelius, C.E., "Clinical Biochemistry of Domestic Animals.", 2nd Ed., Academic Press: New York (1970).

87. Howanitz, J. and Miller, M., "Diuretic action of three sulfonylurea drugs.", Ann. Int. Med. 78, 541-4 (1973).

88. Linkenheimer, W.H. and Stolzenberg, S.J., "Pharmacologic characteristics of 4 sulfonamides in sheep.", Am. J. Vet. Res. 26, 1095-1102 (1965).

89. Rosenthal, H.E., "A graphic method for the determination and presentation of binding parameters in a complex system.", Anal. Biochem. 20, 525-32 (1967).

90. Kurz, H., "Differentiation between drug-binding plasma proteins.", Arch. Pharmakol. 269, 426-7 (1971).

91. Rudman, D., Bixler III, T.J. and Del Rio, A.E., "Effect of free fatty acids on binding of drugs by bovine serum albumin, by human serum albumin and by rabbit serum.", J. Pharmacol. Exptl. Therap. 176, 261-72 (1971).

92. Colowick, S.P. and Kaplan, N.O., Methods in Enzymology, Volume III, Academic Press: New York (1957), pp. 450-1.

93. Lenz, P.H. and Passannante, A.J., "Rapid glucose oxidase-peroxidase ultramicro method for determination of blood glucose.", Clin. Chem. 16, 426-30 (1970).

94. Guyton, A.C., Textbook of Medical Physiology, W.B. Saunders Co.: Philadelphia (1961) p.451.

95. Dulin, W.E., Morley, E.H. and Nezamis, J.E., "Role of the adrenal in response of rats to Orinase,", Proc. Soc. Exp. Biol. Med. 93, 132-6 (1956).

96. Rowland, M., "Influence of route of administration on drug availability.", J. Pharm. Sci. 61, 70-4 (1972).

97. Fujita, T.,"Hydrophobic bonding of sulfonamide drugs with serum albumin.", J. Med. Chem. 15, 1049-56 (1972).

98. Jähnchen, E., Wingard, L.B. Jr. and Levy, G., "Effect of phenylbutazone on the distribution, elimination and anticoagulant action of dicumarol in rats.", J. Pharmacol. Exp. Ther. 187, 176-84 (1973).

99. Coffey, J.J., "Effects of protein binding of drugs on areas under plasma concentration-time curves.", J. Pharm. Sci. 61, 138-9 (1972).

100. Krueger-Thiemer, E., Diller, W. and Buenger, P., "Pharmacokinetic models regarding protein binding of drugs.", Antimicrob. Agents Chemother. 1965, 183-91 (1966).

101. Coffey, J.J., Bullock, F.J. and Schoenemann, P.T., "Numerical solution of nonlinear pharmacokinetic equations: Effects of plasma protein binding on drug distribution and elimination,", J. Pharm. Sci. 60, 1623-8 (1971).

102. Anton, A.H., "The effect of disease, drugs, and dilution on the binding of sulfonamides in human plasma.", Clin. Pharmacol. Ther. 9, 561-7 (1968).

APPENDIX

Vd	=	volume of distribution of a one compartment model; Vd_{ss} of a two compartment model.
Kd	=	first order rate constant of mass elimination from the one compartment model.
A	=	initial concentration (at time zero) of feathered, rapidly eliminating phase of a biexponential plasma concentration-time curve.
α	=	rate constant of rapidly eliminating phase of a biexponential plasma concentration-time curve; in protein binding: unbound concentration divided by total concentration in plasma.
B	=	initial concentration (at time zero) of the slowly eliminating phase of a biexponential plasma concentration-time curve.
β	=	rate constant of slowly eliminating phase of a biexponential plasma concentration-time curve.
$t_{\frac{1}{2}}$	=	half-life
V_1	=	volume of central compartment of 2 compartment model.
V_2	=	volume of peripheral compartment of 2 compartment model.
k_{12}, k_{21}	=	first order rate constants describing mass transfer between the central and peripheral compartments of the 2 compartment model.
k_{10}	=	first order rate constant of mass elimination from the central compartment of the 2 compartment model.
CL	=	total body clearance
CL_b	=	blood clearance
CL_p	=	plasma clearance
V_p	=	plasma volume
Cp	=	total plasma concentration of compound
Cf	=	unbound plasma concentration of compound
λ	=	concentration ratio of compound between blood and plasma
H	=	hematocrit

Kp = apparent partition coefficient of the compound between plasma and erythrocytes.

Ro^t = rate of tolbutamide infusion

Ro^s = rate of sulfadimethoxine infusion

Ctt = total tolbutamide plasma concentration

Cut = unbound tolbutamide plasma concentration

Cts = total sulfadimethoxine plasma concentration

$\frac{\Delta T}{\Delta t}$ = rate of urinary excretion of tolbutamide

$\frac{\Delta OH}{\Delta t}$ = rate of urinary excretion of hydroxytolbutamide

CL_{tt} = clearance based upon total tolbutamide plasma concentration.

CL_{ut} = clearance based upon unbound tolbutamide plasma concentration.

CL_{mtt} = metabolic (hydroxytolbutamide) clearance based upon total tolbutamide plasma concentration.

CL_{mut} = metabolic (hydroxytolbutamide) clearance based upon unbound tolbutamide plasma concentration.

CL_{ett} = renal tolbutamide clearance based upon total tolbutamide plasma concentration.

CL_{eut} = renal tolbutamide clearance based upon unbound tolbutamide plasma concentration.

CL_{ts} = clearance based upon total sulfadimethoxine plasma concentration.

X = unknown metabolite(s) or route of elimination of tolbutamide.

K_I = total plasma sulfadimethoxine concentration required to reduce the clearance by 1/2.

Ro^{OH} = rate of formation of hydroxytolbutamide

C_{OH} = plasma concentration of hydroxytolbutamide.

CL_{OH} = total body clearance of hydroxytolbutamide

Experiment I: Sheep - #368; Dose - 900 mg Tolbutamide; Date - 2/18/72

C_p (mcg/ml)*	t (hr)
140	0.17
115	0.50
95	1.00
80	1.99
63	3.00
46	3.99
33	6.00
10	15.50
5	21.00

* Tolbutamide concentration in plasma

Experiment II: Sheep - #1494; Dose - 865 mg Tolbutamide; Date - 2/21/73

t(hr)	C_p(mcg/ml)*1	C_{OH}(mcg/ml)*2
0.25	82	2.5
0.50	81	2.8
0.75	75	3.3
1.00	67	3.2
2.00	51	2.6
5.00	26.5	1.1
8.00	12.5	0.6
11.00	6.6	-
14.00	3.8	-

*1 - tolbutamide plasma concentration

*2 - hydroxytolbutamide plasma concentration

Rate of Tolbutamide urinary excretion

t(hr)	urine(ml)	Cu(mcg/ml)*1	$\frac{\Delta Ae}{\Delta t}$ (mg/hr)*2	midpoint (hr)
0.26	17	40	2.6	0.13
0.52	22.5	300	26.0	0.39
0.76	17.5	250	18.3	0.64
1.02	25	225	21.7	0.99
2.01	130	191	25.1	1.52
5.01	282	118	11.1	3.51
8.02	327	61	6.6	6.51
11.05	308	39	4.0	9.53
14.05	311	22	2.3	12.55

*1 Tolbutamide concentration in urine

*2 rate of excretion

Experiment III: Sheep - #1494; Dose - 900 mg Tolbutamide; Date - 4/26/73

t(hr)	Cp(mcg/ml)*	Plasma Glucose (mg%)
0	-	40
0.25	-	20
0.50	84	26
0.82	-	10
1.00	71	23
1.50	68	31
2.00	68	39
3.00	65	40
4.00	59.5	39
6.00	53.5	38
7.40	47.5	-
10.50	38	-

* total tolbutamide plasma concentration

Experiment IV: Sheep - #1494; Dose - 860 mg Tolbutamide; Date - 6/6/73

Cp(mcg/ml)*	t(hr)
60	1.0
32	3.5
15.5	7.8
6.5	12.7

* tolbutamide concentration in plasma

Experiment V: Sheep - #1494; Dose - 900 mg Tolbutamide; Date - 6/10/73

Cp(mcg/ml)*	t(hr)
88	0.28
75	0.50
67.5	1.00
46.2	2.60
22.6	6.63
1.0	20.00

* tolbutamide concentration in plasma

Experiment VI: Sheep - #368; Dose - 250 mg Hydroxytolbutamide;
Date - 4/10/72

Cp(mcg/ml)*	t(min)
9.19	5.2
6.67	10.1
4.81	15.0
3.70	20.1
1.91	30.0
1.23	40.1
1.06	50.0
0.57	67.6
0.32	90.1

* hydroxytolbutamide concentration in plasma

Experiment VII: Sheep - #1494; Dose - 250 mg Hydroxytolbutamide;
Date - 4/24/73

Cp(mcg/ml)*	t(min)	Plasma Glucose (mg%)
0	0	49
10.32	4.9	48
4.16	10.1	48
3.30	15.0	47
1.68	20.0	44
1.32	24.9	46
0.97	30.2	46
0.58	40.0	45
0.37	50.0	48
0.23	60.0	42

* hydroxytolbutamide concentration in plasma

Experiment VIII: Sheep - #1494; Dose - 4 Gm Sulfadimethoxine;
Date - 4/19/73

Cp(mcg/ml)*1	t(hr)	Plasma Glucose (mg%)	Cu(mcg/ml)*2 [%]
0	0	55	-
-	0.25	43	-
248	0.5	51	84.2 [34.0]
220	1.0	53	57.8 [26.3]
176	2.0	51	41.3 [23.5]
140	3.0	51	27.4 [19.6]
114	5.0	55	21.2 [18.6]
96	6.9	-	10.3 [11.3]
81	8.8	-	6.23 [7.7]
54	12.3	-	3.24 [6.0]
43	14.5	-	2.32 [5.4]
21.5	22.8	-	0.93 [4.3]
17.5	25.0	-	0.72 [4.1]
15	27.0	-	0.66 [4.4]
13	29.0	-	0.56 [4.3]

*1 total plasma concentration of sulfadimethoxine

*2 unbound plasma concentration determined by equilibrium dialysis not corrected for sample dilution

Experiment IX: Sheep - #1494; Interaction study #1; Plasma data (3/19/73

Time (hr)	Total T (mcg/ml)	Unbound T (mcg/ml)	Glucose (mg%)	SDM (mcg/ml)	HO-T (mcg/ml)
0	-	-	41	-	-
0.5	58.3	-	20	-	1.0
1.0	68.8	12.5	23	-	1.1
1.5	60.5	-	31	-	1.2
2.0	60.8	12.1	38	-	1.0
2.5	60.0	-	37	-	1.1
3.0	64.5	14.5	35	-	1.1
3.5	50.8	-	36	103	1.3
4.0	50.3	26.0	32	155	1.4
5.0	46.3	21.3	31	225	1.1
6.0	48.8	31.6	29	288	1.5
7.1	54.5	-	24	233	1.4
8.0	57.0	-	28	205	1.4
9.1	64.3	32.4	37	170	1.2
10.0	62.3	-	37	148	1.6
11.0	65.5	-	33	133	1.5
12.0	70.0	28.0	40	125	1.5
13.2	66.3	-	32	115	1.6
14.5	67.8	30.6	-	98	-
15.0	-	-	34	-	-

T = tolbutamide

SDM = sulfadimethoxine

HO-T = hydroxytolbutamide

Experiment IX: Sheep - #1494; Interaction study #1; Urinary excretion data (3/19/73) (cont'd)

Collection Time (hr)	Midpt. (hr)	T (mg/hr)	HO-T (mg/hr)	Total T Equiv. (mg/hr)*2
0 - 0.5	0.25	1.9	2.3	4.1
0.5 - 1.0	0.75	4.9	26.7	30.2
1.0 - 2.0	1.50	3.4	37.5	38.8
3.0 - 4.0*1	3.50	6.5	48.2	52.0
4.0 - 4.5	4.25	10.0	39.0	46.9
4.5 - 5.0	4.75	12.0	32.4	42.6
5.0 - 6.5	5.75	9.8	28.5	36.7
6.5 - 7.5	7.00	10.7	30.8	39.8
7.5 - 10.0	8.75	12.3	34.7	45.1
10.0 - 11.0	10.50	19.7	44.7	61.9
11.0 - 14.6	12.80	16.3	56.2	69.4

*1 2.0 - 3.0 poor urine collection

*2 data value has been corrected for molecular weight (hydroxytolbutamide x $\frac{270}{286}$)

T = tolbutamide

HO-T = hydroxytolbutamide

Experiment X: Sheep - #1494; Interaction study #2; Plasma data;
5/22/73

Time (hr)	Hydroxytolbutamide (mcg/ml)	Sulfadimethoxine (mcg/ml)
0.25	2.23	-
0.50	2.73	-
0.75	2.73	-
1.25	3.50	-
1.50	3.50	-
1.75	3.10	-
2.00	3.20	-
2.29	3.06	117
2.50	3.24	-
2.75	2.86	149
3.00	2.86	-
3.25	3.06	179
3.50	3.14	-
3.75	3.50	206
4.04	3.40	-
4.33	3.20	237
4.54	3.46	-
4.75	-	261
5.00	1.44	228
5.31	0.68	209
5.51	-	208
5.75	-	198
6.00	-	188
18.87	-	48
22.28	-	35
25.13	-	28

Experiment X: Sheep - #1494; Interaction study #2; Urinary excretion data (cont'd)

Midpoint Time (hr)	Excretion Rate (mg/hr)
0.13	11.5
0.39	111
0.63	135
1.02	140
1.75	144
2.25	148
2.63	148
2.88	154
3.13	155
3.38	148
4.04	149
4.66	150
5.15	75
5.41	35
5.62	22

Experiment XI: Part a):

Sheep - #1494; Interaction study #3; Plasma data; 6/12/73

Time (hr)	Ctt (mcg/ml)*1	Cut (mcg/ml)*2	C_{OH} (mcg/ml)*3	Cts (mcg/ml)*4	Glucose (mg%)	FFA (mcM/ml)*5
0					50	0.66
0.165					45	
0.33					37	
0.50					37	
1.00	51		1.72		30	
2.00	(46)*1	8.40			43	0.51
3.00	43					
3.97					46	
4.10	40.5		1.53			
4.26	40.5					
4.50	41.5					
6.00	40.0	7.40	1.48		45	0.42
6.05	29.5	15.32		620		
6.06	27.0	14.40		525		
6.13	26.0	13.40		324		
7.01	30.0	12.80	0.90	190	37	0.33
8.03	31.0				33	
9.04	(32)*6	15.20		154	35	0.44
10.00	33.0		0.90		46	
11.00				130	47	

Cont'd ..

Experiment XI: Sheep - #1494; Interaction study #3; Plasma data (cont'd)
(cont'd)

Time (hr)	Ctt (mcg/ml)*1	Cut (mcg/ml)*2	C_{OH} (mcg/ml)*3	Cts (mcg/ml)*4	Glucose (mg%)	FFA (mcM/ml)*5
11.96					46	
13.03	36.0			125		
13.97	(37)*6	17.20			50	
14.75	38.0		0.98			
14.98				123		
16.07					42	
17.60				124	40	
18.51	40.6		1.12			0.52
19.00	(38)*6	18.40		120	47	
19.24	37.5			227		
19.52	40.5			208		
19.75	39.5		1.23			
19.97	36.2	14.80		196	43	
20.49	33.0					
21.00	32.0		1.07			
22.00	28.4			176		
24.01	22.5		0.53	168		
27.01	13.5			156		
28.48	12.0			152		
29.60	10.0			132		
30.51	8.5			118		

Cont'd..

Experiment XI: Sheep - #1494; Interaction study #3; Plasma data (cont'd)
(cont'd)

Time (hr)	Ctt (mcg/ml)*1	Cut (mcg/ml)*2	C_{OH} (mcg/ml)*3	Cts (mcg/ml)*4	Glucose (mg%)	FFA (mcM/ml)*%
32.58	6.1			100		
34.54				82		
36.00				67		
38.33				55		
50.55				20		
53.00				17		
56.00				14		

*1 total plasma tolbutamide
*2 unbound plasma tolbutamide
*3 plasma hydroxytolbutamide
*4 total plasma sulfadimethoxine
*5 non-esterified fatty acids
*6 estimated

Experiment XI: Sheep - #1494; Interaction study #3; Plasma data (cont'd) after ^{14}C-tolbutamide bolus (2.47 x10^8 dpm)

Time (hr)	Dpm ^{14}C/ml
14.25	21050
14.59	16729
14.75	15702
14.98	14773
16.07	10939
17.60	8298
18.51	6862
19.00	6155
19.24	5569
19.52	5602
19.75	5547
19.97	5260
20.49	4862
21.00	4409
22.00	3967
24.01	3006
27.01	2022
28.48	1646
29.60	1448
30.51	1315
32.58	961

Experiment XI: Sheep - #1494; Interaction study #3; Urinary data (cont'd)

Collection Time (hr)	Midpoint (hr)	$\frac{\Delta T}{\Delta t}$ (mg/hr)*1	$\frac{\Delta OH}{\Delta t}$ (mg/hr)*2	$\frac{\Delta \text{Tolb.Eq.}}{\Delta t}$ (mg/hr)*3
0 - 2.00	1.00	21.1	72.1	89.1
3.00 - 4.75*4	3.88	6.9	66.7	69.9
4.75 - 6.10	5.45	7.5	81.8	84.7
6.10 - 9.00	7.55	7.8	51.5	56.5
9.00 - 11.00	10.00	17.9	53.2	68.1
11.00 - 12.50	11.75	19.4	56.7	72.9
12.50 - 14.00	13.25	17.9	59.0	73.6
14.00 - 17.60	15.80	17.9	63.3	77.7
17.60 - 19.50	18.55	18.5	54.0	69.5
19.50 - 22.00	20.75	19.4	48.1	64.8
22.00 - 24.00	23.00	11.5	36.1	45.6
24.00 - 27.00	25.50	6.7	23.2	28.6
27.00 - 30.50	28.75	4.4	14.4	18.0
32.50 - 36.00*4	34.25	1.4	7.1	8.1

*1 rate of tolbutamide excretion

*2 rate of hydroxytolbutamide excretion

*3 rate of tolbutamide equivalents excreted corrected for molecular weight. $\left(\frac{\Delta T}{\Delta t} + \left(\frac{\Delta OH}{\Delta t} \times \frac{270}{286}\right)\right)$

*4 follows an interval of loss of unanalyzed urine.

Experiment XI: Sheep #1494; Interaction study #3; Plasma data (cont'd) after ^{3}H-hydroxytolbutamide bolus (2.44 x 10^{7}dpm)

A) Before Sulfadimethoxine (Phase 1)

Time (min)	Dpm ^{3}H/ml
246	2113
253	1250
256	1215
260	986
270	810
285	599
300	511
360	352
660	158

B) After Sulfadimethoxine (Phase 2)

Time (min)	Dpm ^{3}H/ml
725	1496
730	1092
735	968
740	739
750	704
766	563
782	475
838	317

Experiment XII: Sheep - #1494; Dose - 1 Gm Sulfadimethoxine;
Date - 6/8/73

Time (hr)	Cp (mcg/ml)*
0.52	77.5
2.58	48.8
7.25	21
20.65	5.5
29.80	3

* sulfadimethoxine plasma concentration

Experiment XIII: Sheep - #1494; Dose - 1.3 Gm Sulfadimethoxine;
Date - 8/8/83

Time (hr)	Cp (mcg/ml)*
0.65	92.4
1.54	71.2
2.42	60
3.97	42.5
6.11	28.2
9.13	16.2
11.38	12.8
23.25	6.5
27.13	5.5

* sulfadimethoxine plasma concentration

Experiment XIV: Sheep - #1494; Dose - 4 Gm Sulfadimethoxine;
Date - 8/14/73

Time (hr)	Cp (Mcg/ml)*
0.51	206.8
1.71	153.9
2.65	120.6
4.06	93.6
6.57	64.8
8.59	45
11.23	32.4
13.46	24.2
24.22	10
26.02	8

* sulfadimethoxine plasma concentration