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KIDNEY-DIRECTED PRODRUGS

City University of New York PH.D. 1980

KIDNEY-DIRECTED PRODRUGS

by

Haruko Mizoguchi

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New York

1980

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

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ABSTRACT

Kidney-Directed Prodrugs

by

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Prodrugs are inactive precursors of drugs which may be converted to the active drugs in enzyme catalyzed reactions. This conversion can be expected to occur at the site of localization of the required enzyme or enzyme system. The present work describes the synthesis of prodrugs which are converted to the active drugs by kidney-specific enzymes, such as γ-glutamyl transpeptidase, aromatic L- amino acid decarboxylase and N-acylamino acid deacylase. It was initiated with the aim of directing drug action in a specific manner to the kidney, and is based on the finding that γ-glutamyl derivatives of amino acids and other compounds containing an amino function, are selectively accumulated and metabolized in the kidney. The following prodrugs were synthesized and tested for kidney-directed action: γ-glutamyl derivatives of 3,4-dihydroxyphenylalanine (DOPA) and dopamine as kidney-specific dopamine precursors and γ-glutamyl sulfamethoxazole and its N-acyl derivatives as models of kidney-directed antibacterial agents. Administration of γ-glutamyl-DOPA to mice by intraperitoneal injection led to a great accumulation of free dopamine in the kidney as a consequence of the sequential action of γ-glutamyl transpeptidase and aromatic L-amino acid decarboxylase, two enzymes which are highly concentrated in the kidney. Twenty minutes after the administration of the prodrug, the

concentration of dopamine in the kidney was five times higher than after an equimolar dose of L-DOPA. The concentration of dopamine in the heart and other tissues was only a small fraction of that observed in the kidney, reflecting the kidney-selectivity of the prodrug. Infusion of L-γ-glutamyl-DOPA to rats produced a 60% increase in renal plasma flow at a dose of 10 nmole/g/30 min. By contrast the same dose of L-DOPA had no effect on renal plasma flow; only after doubling the dose of this drug a small increase in renal plasma flow was noticed. The dose of γ-glutamyl-dopamine required to produce an increase in renal plasma flow was five times greater than that after γ-glutamyl-DOPA. In dogs in which renal blood flow was measured directly with an electromagnetic flow probe placed around the renal artery, a single injection of L-γ-glutamyl-DOPA produced a prolonged increase in renal blood flow. This was accompanied by high urinary excretion of free dopamine. When the drug was infused directly into the renal artery in the dog, an immediate increase of urine output was observed on the side of infusion. A tendency toward an increase in glomerular filtration rate and sodium excretion was also observed in rats. No increases in systemic blood pressure, cardiac output, heart rate and blood sugar levels were observed after doses which caused an increase in renal blood flow, indicating that the systemic effects of the prodrug are minimal. A small pressor response was observed in rats only when the dose of L-γ-glutamyl-DOPA was 20-times higher than the dose required to increase renal blood flow. It is suggested that L-γ-glutamyl-DOPA may be of therapeutic value as a kidney-selective vasodilator.

γ-Glutamyl and N-acyl-γ-glutamyl derivatives of sulfamethoxazole (SM) were synthesized and tested as models of kidney-selective prodrugs

with antibacterial activity. In vitro γ-glutamyl derivatives of SM are cleaved in kidney homogenates by γ-glutamyl transpeptidase at a greatly higher rate than in other tissues. Surprisingly, however, in vivo there was only a small preferential accumulation of SM in the kidney with respect to other tissues, after intraperitoneal administration to mice of the γ-glutamyl prodrugs. Relatively high concentrations of SM were found in other tissues, apparently because of rapid cleavage of the γ-glutamyl derivatives even in tissues with a low γ-glutamyl transpeptidase activity. This can be explained by the fact that γ-glutamyl-SM is an excellent substrate for γ-glutamyl transpeptidase and is thus cleaved at a high rate even in the presence of low concentrations of the enzyme. By contrast the release of SM in kidney homogenates from N-acyl- γ-glutamyl derivatives of SM proceeds at a much slower rate than the release of SM from γ-glutamyl derivatives. This process requires the action of two enzymes, an N-acylamino acid deacylase and γ-glutamyl transpeptidase, both highly concentrated in the kidney. In vivo, a kidney-selective accumulation of SM was obtained 20 minutes after intraperitoneal administration of N-acyl-γ-glutamyl derivatives. The concentration of SM was 2.2 times higher after N-chloroacetyl-L-γ-glutamyl-SM than after an equimolar dose of SM; the concentration in other organs was only 2% of that in the kidney with somewhat higher concentrations in the liver. High kidney-selectivity was also observed after the N-acetyl and N-butyryl-L- γ-glutamyl derivatives of SM, although absolute kidney concentrations were lower. It is suggested that γ-glutamyl and N-acyl- γ-glutamyl derivatives of a variety of drugs might potentially be useful when limitation of drug action to the kidney and urinary tract is a desired objective. Such a limitation of activity

would be of particular benefit with drugs having a general systemic toxicity.

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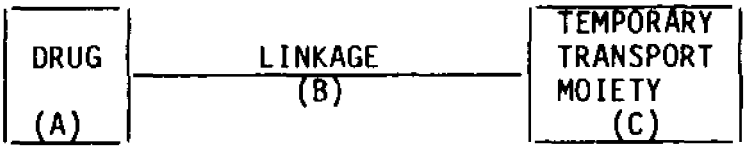
LIST OF ABBREVIATIONS

ADH	Antidiuretic hormone
C_{Cr}	Clearance of creatinine
C_{PAH}	Clearance of p-aminohippurate
DA	Dopamine
DOPA	3,4-Dihydroxyphenylalanine
DOPAC	3,4-Dihydroxyphenylacetic acid
GFR	Glomerular filtration rate
PAH	p-Aminohippurate
RBF	Renal blood flow
RPF	Renal plasma flow
SM	Sulfamethoxazole
$T_{m\ PAH}$	Transport maximum of PAH
UF	Urine flow

INTRODUCTION

Prodrugs are inactive derivatives of pharmacologically active compounds which upon introduction into a biological system generate the parent compounds. These compounds then exert their pharmacological action at the sites of the generation. Prodrugs can also be viewed as temporary transport forms of drugs as shown in the schematic diagram of Stella(1975).



PRODRUG

Numerous barriers exist which may prevent the direct use of a drug. Some of these common barriers are poor bioavailability, lack of solubility and lack of chemical stability. These barriers to the use of a parent compound (A) may be overcome by introducing the temporary transport moiety (C). After overcoming the barrier, the transport form can be converted to the parent compound (A). The conversion involves the breaking of the linkage (B) either by an enzymatic or non-enzymatic process. The prodrug approach has met with considerable success in improving bioavailability of a large number of drugs (Sinkula, 1975). For example, esterification of ampicillin has been found to improve the absorption of ampicillin from the gastrointestinal tract (Daehne et al., 1970).

A logical extension of the prodrug concept is to utilize a prodrug as a way to deliver a drug selectively to a target organ or tissue. Such organ-selective delivery of a drug should greatly increase its concentration in the target organ with the possible exclusion of other sites.

It can also be expected to minimize undesirable systemic effects and drug-induced toxicity. It is well established that morphological differentiation of various organs and tissues is accompanied by a biochemical and metabolic differentiation. One expression of such a differentiation is the presence in an organ of high concentrations of certain enzymes or enzyme systems that are absent or exhibit only low activity in other tissues. This differentiation can be utilized for the purpose of increasing the selectivity of drug action. If the conversion of a prodrug to the active drug is dependent on an organ-specific enzyme or enzyme system, this conversion can be expected to occur only in the organ which has the required enzyme system. There are many organ specific enzyme systems which can be potentially useful in this approach. The work presented below is an attempt to utilize the highly concentrated localization of γ -glutamyl transpeptidase and some other enzymes in the kidney for the purpose of directing drug action toward this organ.

Recent studies have shown that the kidney is highly active in the accumulation and metabolism of γ -glutamyl derivatives of amino acids and peptides (Orlowski and Wilk, 1976,1978$_b$). This activity is apparently related to the presence of a high concentration of γ -glutamyl transpeptidase [EC 2.3.2.2.] in the kidney (Goldbarg et al., 1960; Orlowski and Szewczuk, 1961). This organ is also rich in aminopeptidase A [EC 3.4.11.7] (Glenner et al., 1962$_b$), aromatic L-amino acid decarboxylase [EC 4.1.1.26] (Dietrich, 1953; Clark et al., 1954; Davis and Awapara, 1960) and several acylases capable of hydrolysing N-acylamino acids (Birnbaum et al., 1952; Fones and Lee, 1953; Endo, 1978$_b$).

These findings induced us to explore the possibility of using prodrugs susceptible to the action of these enzymes and therefore likely to

be selectively converted to the active drugs in the kidney. Two types of prodrugs were synthesized; derivatives of sulfamethoxazole (a sulfonamide) and derivatives of dopamine. Sulfonamides are especially suited for the preparation of prodrugs. They are relatively stable molecules, containing an aromatic amino group. This group can be easily substituted by a γ-glutamyl or other groups in a bond susceptible to cleavage in an enzyme catalyzed reaction. Furthermore, after release of the free sulfonamides their concentration and distribution can be followed conveniently by reliable methods available for their quantitative determination (Bratton and Marshall, 1939; Goldbarg and Rutenburg, 1958). Selective accumulation of sulfonamides in the kidney is also of considerable clinical interest since these drugs are frequently used in the treatment of urinary tract infections.

Kidney-specific dopamine precursors also have potential therapeutic significance. This catecholamine causes renal vasodilatation when given intravenously. This action, however, is accompanied by side effects due to stimulation of α - and β -adrenergic receptors. A kidney vasodilator free of these side effects therefore has potential clinical significance.

γ -Glutamyl transpeptidase

γ -Glutamyl transpeptidase is a membrane bound glycoprotein that catalyzes both transpeptidation (reaction 1) and hydrolysis (reaction 2) of γ -glutamyl derivatives (Hanes et al., 1952; Revel and Ball, 1959; Szewczuk and Baranowski, 1963; Orlowski and Meister, 1965; Zelazo and Orlowski, 1976). γ -Glutamyl derivatives are derivatives of glutamate in which the γ-carboxyl group participates in an amide or peptide bond with a compound containing an amino function.

γ-Glutamyl amino acid$_1$ + amino acid$_2$

$$\longrightarrow$$

γ-glutamyl amino acid$_2$ + amino acid$_1$ (1)

γ-Glutamyl amino acid + H_2O

$$\longrightarrow$$

glutamate + amino acid (2)

The extent of contribution made by each of these two reactions is dependent on pH. At pH above 8.0, the transpeptidation reaction predominates while the significance of the hydrolytic reaction increases with decreasing pH toward physiological values (Karkowsky et al., 1976).

The substrate specificity of γ-glutamyl transpeptidase with respect to both γ-glutamyl acceptor and donor is broad. Therefore not only L-amino acids but several dipeptides are active as γ-glutamyl acceptors (Tate and Meister, 1974,1975; Zelazo and Orlowski, 1976). The specificity is not limited to L- γ-glutamyl stereoisomers. Release of p-nitroaniline from D- γ-glutamyl compounds has also been reported. The rate of these reactions is however much slower than that with the corresponding L-stereoisomers (Orlowski and Meister, 1965).

Biochemical and histochemical studies have shown that γ-glutamyl transpeptidase is highly concentrated in the kidney. If γ-glutamyl transpeptidase activity in human kidney is taken as 100, the relative activity in the pancreas, which is the second organ after the kidney with the highest enzymatic activity is 8.3. Other organs exhibit only traces of activity (Orlowski and Szewczuk, 1961). Kidney is also the organ with the highest γ-glutamyl transpeptidase activity in mice, rats, guinea pigs, rabbits and dogs (Goldbarg et al., 1960). Histochemical studies

have shown that γ-glutamyl transpeptidase is localized in the brush border of the proximal tubules, epithelium of the ascending limb of Henle's loop and medullary vascular bundles (Albert et al., 1961; Glenner et al., 1962_a).

In accord with the histochemical results, the presence of γ-glutamyl transpeptidase activity has been demonstrated in isolated brush borders (Glossmann and Neville, 1972; George and Kenny, 1973). Although the exact localization of this enzyme in the tubular membrane is not known, there is evidence suggesting that the active site of the enzyme faces the luminal side. Purified brush border membrane vesicles exhibiting γ-glutamyl transpeptidase activity can be obtained from rat kidney. Using these vesicles, Horiuchi and co-workers (1978) have shown that an S-acetyl dextran derivative of glutathione is capable of inhibiting the activity of the membrane bound γ-glutamyl transpeptidase. Since the S-acetyl dextran polymer cannot penetrate across the membrane, its inhibition of the enzyme activity suggests that the active site of γ-glutamyl transpeptidase is accessible from the outer surface of brush border membranes.

The ability of kidney to accumulate and metabolize γ-glutamyl derivatives is believed to be related to the high activity of γ-glutamyl transpeptidase in this organ. Consistent with this view is the observation that the intraperitoneal administration of γ-glutamyl-L-2-aminobutyrate in mice produces a significant increase in the concentrations of glutamate and its metabolites in the kidney. In contrast, there is no significant increase in these metabolite concentrations in the liver. Moreover considerably higher concentrations of L-2-aminobutyrate are obtained in the kidney after γ-glutamyl-L-2-aminobutyrate than after

administration of the free amino acid. Qualitatively similar results are obtained after γ-glutamylphenylalanine, γ-glutamylmethionine, γ-glutamylglycylglycine and glutathione (γ-glutamylcysteinylglycine). These findings demonstrate that the addition of a γ-glutamyl moiety to an amino acid facilitates the delivery of this amino acid to the kidney (Orlowski and Wilk, 1976).

Further investigation of the metabolism of γ-glutamyl derivatives of amino acids by Orlowski and Wilk (1978_b) has shown that these derivatives enter into kidney cells at least in part in an intact form. The following experiments support this conclusion. Administration of L- γ-glutamyl-L-2-aminobutyrate produces significant synthesis of ophthalmic acid (L-γ -glutamyl-2-aminobutyrylglycine) in the kidney. Two pathways are possible for the biosynthesis of ophthalmic acid from L- γ-glutamyl-L-2-aminobutyrate. In the first, the dipeptide after entering the cell reacts directly with glycine in an enzymatic reaction catalyzed by glutathione synthetase [EC 6.3.2.3] (reaction 3) (Meister, 1974).

L- γ-Glutamyl-L-2-aminobutyrate + glycine + ATP

$$\xrightarrow{\text{glutathione synthetase}}$$

ophthalmic acid + ADP + Pi (3)

In the second pathway, L-γ -glutamyl-L-2-aminobutyrate is first cleaved to L-glutamate and L-2-aminobutyrate by the action of γ -glutamyl transpeptidase. Following this cleavage, the biosynthesis of ophthalmic acid proceeds in two steps (Cliffe and Waley, 1958,1961) catalyzed in sequence by γ -glutamylcysteine synthetase [EC 6.3.2.2] and glutathione synthetase (reaction 4 and 3).

L-Glutamate + L-2-aminobutyrate + ATP

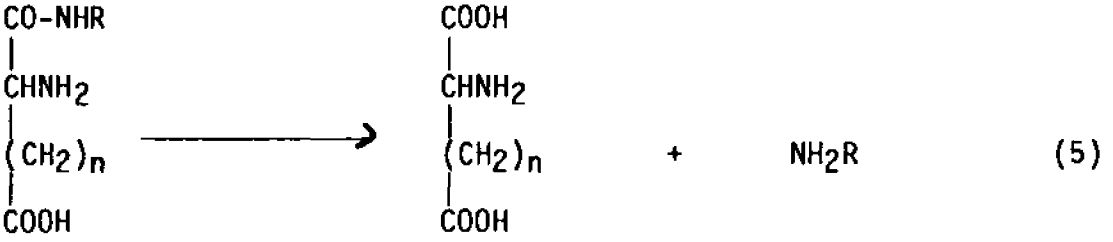
$$\xrightarrow{\text{γ-glutamylcysteine synthetase}}$$

L- γ-glutamyl-L-2-aminobutyrate + ADP + Pi (4)

The two pathways of ophthalmic acid biosynthesis can be distinguished from each other by measuring the specific radioactivity of the tripeptide formed from γ-glutamyl-L-2-aminobutyrate labelled in the glutamate moiety. If the synthesis *in vivo* proceeds directly from the dipeptide (reaction 3), no dilution of the radioactivity by endogenous glutamate should occur. On the other hand, if the second pathway is operating, then the labelled glutamate cleaved from the dipeptide would be expected to be diluted by the endogenous glutamate pool. Consequently the specific radioactivity of ophthalmic acid will be lower than the specific activity of the labelled γ-glutamyl-L-2-aminobutyrate. The finding that the specific radioactivity of ophthalmic acid formed in the kidney from L-γ -[^{14}C]-glutamyl-L-2-aminobutyrate is identical to that of the dipeptide precursor indicates that ophthalmic acid is synthesized directly from L-γ -glutamyl-L-2-aminobutyrate and glycine (Orlowski and Wilk, 1978$_b$). Furthermore it suggests that γ -glutamyl derivatives of amino acids enter the kidney cells in an intact form.

Aminopeptidase A

Aminopeptidase A catalyzes hydrolysis of peptide bonds in which the α-carboxyl group of an N-terminal L-dicarboxylic amino acid is involved (reaction 5) (Glenner and Folk, 1961; Glenner et al., 1962$_b$).



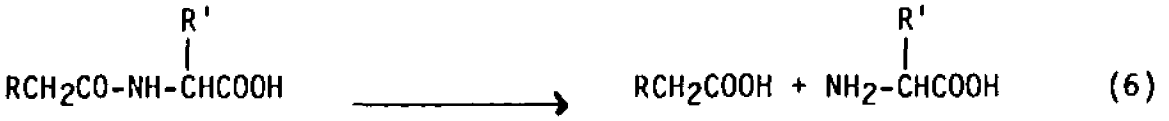
(5)

where n= 1 or 2

The enzyme is activated by Ca^{2+} (Glenner and Folk, 1961). High aminopeptidase A activity is found in rat kidney where the activity is localized to glomeruli (Glenner et al., 1962_b). Brain capillaries (Orlowski and Wilk, 1978_a), pancreas, duodenum (Glenner and Folk, 1961; Glenner et al., 1962_b) and serum (Nagatsu et al., 1965, 1970) also exhibit considerable enzymatic activity.

Acylase

Mammalian tissues contain a number of enzymes which are capable of hydrolyzing N-acyl amino acids. In the kidney, three amino acid acylases differing in substrate specificity have been identified (Greenstein and Winitz, 1961). These enzymes catalyze the general reaction (reaction 6).



(6)

Acylase I — R = Cl, H, NH_2; R'= L-amino acid side chain

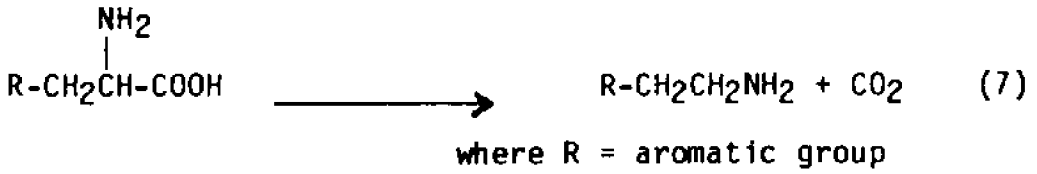
Acylase II — R = Cl, H; R'= $-CH_2COOH$

Acylase III — R = Cl, H; R'= L-aromatic amino acid side chain

Renal acylase I [EC 3.5.1.14] catalyzes hydrolysis of various α-N-acylated L-amino acids (Birnbaum et al., 1952; Rao et al., 1952, 1953; Fones and Lee, 1953). The enzyme requires the presence of a hydrogen atom on the peptide nitrogen and a free terminal α-carboxyl group (Fu and Birnbaum, 1953; Rao et al., 1953). There is a rough correlation between the rate of hydrolysis and the electronegativity of the acyl moiety of a substrate. For a given α-amino acid residue, the rate of hydrolysis decreases with the various N-acyl substituents in the order of trifluoroacetyl > chloroacetyl > acetyl > formyl (Fudor et al., 1950; Fones and Lee, 1953). Birnbaum and colleagues (1952) have demonstrated the existence of another acylase which is designated as acylase II [EC 3.5.1.15]. This enzyme has high specificity for N-acylated L-aspartic acid, a substrate poorly hydrolyzed by acylase I. The activities of both acylase I and acylase II are highest in the kidney (Greenstein and Winitz, 1961; Lorentz et al., 1975). The third acylase which is tentatively defined as acylase III (Greenstein and Winitz, 1961) has been partially purified from rat kidney (Endo, $1978_{a,b}$). Acylase III acts preferentially on N-acyl L-aromatic amino acids. As with other acylases, kidney exhibits the highest acylase III activity among various organs examined. Within the kidney, the cortex shows two to three times higher activity than the medulla (Endo, 1978_a).

Aromatic L-amino acid decarboxylase

Aromatic L-amino acid decarboxylase is an enzyme which catalyzes decarboxylation of aromatic L-amino acids (reaction 7).



Pyridoxal phosphate is required as a cofactor in this enzymatic reaction (Awapara et al., 1962; Christenson et al., 1970). The enzyme is stereospecific for L-isomers of aromatic amino acids (Blaschko, 1942). Aromatic L-amino acid decarboxylase is capable of catalyzing decarboxylation of a large number of aromatic L-amino acids including 3,4-dihydroxyphenylalanine, 5-hydroxytryptophan, tryptophan, tyrosine, phenylalanine as well as a variety of aromatic α-methyl amino acids (Weissbach et al., 1960; Lovenberg et al., 1962; Sourkes, 1966; Dairman et al., 1973). Determination of the activity of aromatic L-amino acid decarboxylase with 3,4-dihydroxyphenylalanine as substrate in kidney, liver, small intestine, brain, heart, lung, spleen and adrenals of rat, rabbit and guinea pig has identified the kidney as the organ with the highest activity in all the species (Davis and Awapara, 1960). This enzyme was localized with a specific immunofluorescence test in the endothelium of the distal and proximal tubules and in glomeruli of the kidney (Goldstein et al., 1972, 1973).

Pharmacology of dopamine

1. Effect on renal blood flow

A dopamine-induced increase in renal blood flow has been observed in rats (Brennan et al., 1977), dogs both anesthetized (McNay et al., 1965; Morimoto, 1967) and unanesthetized (Meyer et al., 1967) and in man (McDonald et al., 1964; Hollenberg et al., 1973). Dopamine (DA) also increases renal blood flow in man under a variety of clinical conditions, including patients with congestive heart failure (Rosenblum et al., 1970; Abrahamsen et al., 1974), hypertension (Breckenridge et al., 1971; Orme et al., 1973), and cirrhosis (Barnardo et al., 1970; Bennett et al., 1975). A similar increase in renal blood flow after DA is seen in animals undergoing experimentally induced shock (Carvalho et al., 1969; Rao and Bhagat, 1978; Neiberger and Passmore, 1979). That this increase involves a direct effect on the kidney, was shown by administering DA directly into the renal artery. A dose related and unilateral increase in renal blood flow on the side of the drug administration was observed in dogs (McNay et al., 1965; Meyer et al., 1967).

DA causes vasodilation and an increase in renal blood flow through its action on specific DA receptors in the renal vasculature. A similar DA-induced vasodilation has been reported in mesenteric (Eble, 1964; Yeh et al., 1969), cerebral (von Essen, 1972; Edvinsson et al., 1978) and coronary arteries (Schuelke et al., 1971). Several reviews covering the subject of vascular DA receptors have appeared (Goldberg, 1972,1975$_{a,b}$; Goldberg et al., 1977,1978; Pendleton and Setler, 1977).

Evidence supporting the conclusion that renal vasodilation is mediated by specific DA receptors comes from studies with various antagonists. It was shown that the renal vasodilating action of DA is distinct

from stimulation of β-receptors or release of known vasodilators such as acetylcholine, histamine and prostaglandins of the A, B and E type. Thus propranolol in doses which block isoproterenol-induced increase in renal blood flow has no effect on that of DA (McNay et al., 1963; McNay and Goldberg, 1966). Atropine (McNay et al., 1963; McNay and Goldberg, 1966), diphenhydramine (McNay et al., 1963) and indomethacin (Dressler et at., 1975; Pendleton and Woodward, 1976) are also ineffective in blocking DA induced renal vasodilation. Moreover a neurogenic mechanism does not appear to be involved since denervation of the kidney (McNay et al., 1963; McGiff and Burn, 1967), treatment with hexamethonium (Morimoto, 1967) or pretreatment with reserpine (McNay and Goldberg, 1966) or with bretylium (McGiff and Burns, 1967) does not antagonize the vasodilating action of DA. Recently, however, Lokhandwala and associates reported that DA attenuates the renal vasoconstrictor response to sympathetic nerve stimulation (Lokhandwala and Buckley, 1977; Lokhandwala and Jandhyala, 1979). The extent of the contribution made by such attenuation to the overall renal vasodilating action of DA is not known.

It appears unlikely that DA induced vasodilation is mediated by its metabolites. Known metabolites of DA are inactive as renal vasodilators (McNay and Goldberg, 1966). Although tetrahydropapaveroline, a condensation product of DA causes renal vasodilation, this can be blocked by β-antagonists (McNay and Goldberg, 1966),

DA induced renal vasodilation is antagonized by phenothiazines and haloperidol in doses that do not affect isoproterenol or bradykinin induced vasodilation (Yeh et al., 1969; Goldberg and Yeh, 1971). Such selective antagonism is also seen with sulpiride (Kohli et al., 1978_b) and metoclopramide (Day and Blower, 1975; Kohli et al., 1978_b) which are

also known central DA antagonists. Bell and associates (1974) have demonstrated a blockade of DA induced increase in renal blood flow by ergometrine, an ergot alkaloid. In addition, apomorphine (Bell et al., 1974) and the structurally related alkaloid bulbocapnine (Setler et al., 1975; Pendleton and Woodward, 1976) antagonize the renal action of DA. With respect to vascular DA receptors, apomorphine appears to act as a partial antagonist.

Not only is DA induced renal vasodilation specifically attenuated by DA antagonists but there is also a strict structural requirement for such 'DA-like' action. 'DA-like' action is defined as that action of a compound which produces renal but not femoral vasodilation after phenoxybenzamine and propranolol treatment and is specifically antagonized by DA antagonists (Goldberg et al., 1968). The initial study examining forty four phenylethylamines and apomorphine has shown only N-methyldopamine and apomorphine to possess 'DA-like' action (Goldberg et al., 1968). Recent studies, however, show that a number of N-n-propyl derivatives of DA are capable of elevating renal blood flow which is attenuated by haloperidol or metoclopramide but not by propranolol, pyrilamine, metiamide, atropine or hexamethonium (Volkman et al., 1977; Ginos et al., 1978; Kohli et al., 1978_a). Other agents that are known to exhibit vascular DA agonist action include 6-propyl-norapomorphine (Crumly et al., 1976), SK&F 38393 (Pendleton et al., 1978) and 2-amino-6,7-dihydroxy-1,2, 3,4-tetrahydronaphthalene (Volkman et al., 1977).

Further support for the involvement of specific DA receptors is obtained from studies utilizing isolated arterial strips. DA has been shown to produce a dose-dependent relaxation in isolated renal arterial

strips pretreated with phenoxybenzamine and contracted with K^+ or prostaglandin F_2 . This DA-induced relaxation is not blocked by β -antagonists (Goldberg et al., 1973; Toda and Goldberg, 1973; Goldberg and Toda, 1975) but antagonized specifically by droperidol (Toda and Hatano, 1979).

2. Effect on other renal functions

In addition to the renal vasodilating action, DA has been reported to increase glomerular filtration rate in dogs (Meyer et al., 1967), normal man (McDonald et al., 1964) and in patients with hypertension (Breckenridge et al., 1971; Orme et al., 1973), congestive heart failure (McDonald et al., 1964; Beregovich et al., 1974), and cirrhosis (Peschl, 1978). Moreover this agent promotes greater Na^+ and in some cases K^+ excretion and increases urinary output in animals (Meyer et al., 1967; Morimoto, 1968; Brotzu, 1970). The natriuretic and diuretic response induced by DA is also observed in patients with hypertension (Orme et al., 1973; Velasco et al., 1974), congestive heart failure (Goldberg et al., 1963; Beregovich et al., 1974) and cirrhosis (Espiritu et al., 1972; Bennett et al., 1975).

3. Effect on blood pressure

The effect of DA on blood pressure is complex since it reduces the resistance in some vascular beds and increases it in others (Eble, 1964; Ross and Brown. 1967). Moreover even in a given vascular bed, the effect on vascular resistance differs depending on the dose of DA administered. In the canine renal vasculature for example, intraarterial injection of DA up to 6 μg causes a dose-dependent increase in the blood flow. A

dose of 12 μg produces a transient initial vasoconstriction followed by vasodilation. Vasoconstriction predominates when the dose is increased further (McNay et al., 1965). Correspondingly in dogs, the intravenous administration of DA in doses of up to about 1 μg/kg causes a decrease in blood pressure. Larger doses produce a biphasic effect consisting of an initial pressor effect followed by a decrease in blood pressure. At doses exceeding 10 μg/kg, the pressor effect predominates (McDonald and Goldberg, 1963; Sampson et al., 1974; Setler et al., 1975).

Following α-blockade, the pressor action of DA is reversed and a dose-dependent decrease in blood pressure is observed (McDonald and Goldberg, 1963; Sampson et al., 1974). The pressor action is not altered by pretreatment with reserpine, 6-hydroxydopamine (Neuvonen and Westerman, 1973), or uptake blockers such as desipramine and protryptiline (Rubenson, 1971; Neuvonen and Westerman, 1973) suggesting a direct action on α-receptors. However since cyproheptadine has been shown to block DA induced contraction in isolated canine vessels, a possible role of the serotonergic mechanism cannot be disregarded. The depressor effect of DA is not blocked by β-antagonists, atropine, diphenhydramine or hexamethonium but attenuated by haloperidol (Sampson et al., 1974), bulbocapnine (Pendleton et al., 1975), ergometrine, apomorphine (Bell et al., 1974) and metoclopramide (Day and Blower, 1975). Therefore the effect of intravenously administered DA on systemic blood pressure appears to be related to a balance between the vasoconstricting and vasodilating action of this agent, the former probably mediated by α-receptors and the latter by DA receptors.

Since higher doses of DA may elevate blood pressure and possibly lower renal blood flow by increasing renal vascular resistance, the

maximal dose of DA that can be administered clinically is limited (Reid and Thompson, 1975). Furthermore because of its vasoconstricting action, this agent has a potential for producing gangrene. In man, DA-induced gangrene, which in one case resulted in amputation of a hand, has been reported by several groups (Alexander et al., 1975; Greene and Smith, 1976; Ebels and van der Heide, 1977; Stetson and Reading, 1977). In this respect, it is of interest that the combined use of DA with α-antagonists (Hsieh and Goldberg, 1979) or with prostaglandin A (Robie et al., 1974; Vincenti and Goldberg, 1978) was shown to prevent an excessive rise in blood pressure while maintaining DA induced renal blood flow elevation.

4. Effect on cardiac functions

DA is a cardiostimulator having both positive inotropic and chronotropic actions. In intact dogs, 1 to 10 μg/kg/min of DA causes an increase in stroke volume without affecting end-diastolic volume, heart rate or mean aortic blood pressure. Since preload, afterload and heart rate remained unchanged, it was concluded that more efficient ventricular emptying was responsible for the observed rise in stroke volume. In the same study, DA also increased systolic ejection rate and circumferential shortening rate suggesting action on myocardial fiber shortening (Black and Rolett, 1966). A DA induced change in cardiac contractility was also indicated by an increase in ventricular contractile force in intact dogs (McDonald and Goldberg, 1963) and lung-heart preparations in which the heart rate was kept constant by electrical stimulation (Holmes and Fowler, 1962). Further support for a positive inotropic effect of DA was obtained by the observation that it increases

the velocity of left ventricular pressure rise, $\frac{dp}{dt}$, during isovolumic contraction (Black and Rolett, 1966; Vatner et al., 1973). The positive inotropic effect of this agent has also been demonstrated in isolated atria (Lee and Yoo, 1964; Chiba, 1975) and in isolated ventricular strips during isometric contraction (Endoh et al., 1976; Mugelli et al., 1977).

The positive chronotropic effect of DA is observed in intact animals (McDonald and Goldberg, 1963; Morimoto, 1967). In these animals, however, its direct action on heart rate may have been complicated by the reflex change in response to DA induced alteration in systemic blood pressure. To avoid this complication, the effect of DA was studied in vagotomized dogs (Privitera et al., 1969), spinal cats (Farmer, 1966), dog lung-heart preparations (Bejrablaya et al., 1958) and in isolated right atria (Chiba, 1975). An increase in heart rate was found in all the cases. It is interesting to note that a low dose of DA preferentially affects cardiac contractility with little or no effect on heart rate (McDonald and Goldberg, 1963; Black and Rolett, 1966).

That the positive inotropic and chronotropic effects of DA are mediated by β-adrenergic receptors is supported by the finding that β-antagonists block these effects (McDonald and Goldberg, 1963; Black and Rolett, 1966; Rolett and Black, 1966; Ross and Brown, 1967). Pretreatment with reserpine partially reduces the DA induced increase in myocardial contractility (Lee and Yoo, 1964; Mugelli et al., 1977) and heart rate (Bejrablaya et al., 1958; Farmer, 1966). The positive inotropic effect is also attenuated by desmethylimipramine (Chiba, 1975), cocaine and bretylium (Lee and Yoo, 1964) while the chronotropic effect is attenuated by cocaine (Farmer, 1966). Therefore both a direct action on β-receptors and indirect action by releasing norepinephrine from the

nerve terminals appear to be involved in its stimulation of the cardiac performance.

The cardiostimulatory action of DA may have both beneficial and detrimental consequences. The ability of DA to induce positive inotropy together with greater renal perfusion is unique among the sympathomimetic amines. As such it has been used successfully in treatment of shock, acute heart failure during and after cardiac surgery, and congestive heart failure (Goldberg et al., 1977). On the other hand, as any cardiostimulator DA is capable of inducing arrhythmia. Thus cases of supraventricular tachycardia (Talley et al., 1969) and ventricular premature contractions (McDonald et al., 1964; Rosenblum et al., 1972) have been reported after DA in man. This agent has also been demonstrated to produce ventricular fibrillation in cats anesthetized with sensitizing agents such as cyclopropane and halothane (Katz et al., 1967). DA, being an inotropic agent, may also increase myocardial oxygen consumption and lactate production (Mueller et al., 1978; Vasu et al., 1978). Consequently this compound carries a potential for precipitating angina pectoris (McDonald et al., 1964) and worsening of myocardial infarction (Reid et al., 1972).

MATERIALS AND METHODS

A. Materials

Chemicals

Chemicals were obtained from the following sources: L-glutamic acid, D-glutamic acid, L-3,4-dihydroxyphenylalanine, dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid and homovanillic acid from Sigma Chemicals Co. (St. Louis, Mo.), phthalic anhydride and hydrazine hydrate from Fisher Scientific Co. (Springfield, N.J.), acetic anhydride and creatinine from Fisher Scientific Co. (Fairlawn, N.J.), triethylamine, butyric anhydride and chloroacetyl chloride from Eastman Kodak Co. (Rochester, N.Y.), sodium aminohippurate from Merck Sharp & Dohme (West Point, Pa.), Glucostat Reagent Set from Worthington Biochemical Corp. (Freehold, N.J.), 3,4-dihydroxyphenylpropionic acid, 4-hydroxy-3-methoxycinnamic acid from Aldrich Chemical Co. (Milwaukee, Wisc.), pentafluoropropionic anhydride from Pierce Chemical Co. (Rockford, Ill.), 2,2,3,3,3-pentafluoro-1-propanol and 1-chloro-1,1,3,3,3-pentafluoro-2-propanol from Peninsula Chemical Research Co. (Gainesville, Fla.), aluminum oxide Woelm basic activity grade 1 from ICN Inc. (Cleveland, Ohio). Dowex-1 (AG-1X4, 200-400 mesh) was purchased from Bio-Rad laboratories (Richmond, Calif.). All coated gas-chromatographic packings were obtained from Applied Science Laboratories (State College, Pa.).

Glycyl-[1-^{14}C]-p-aminohippurate (specific activity 43 mCi/mmole) and inulin-[^{14}C]-carboxylic acid (specific activity 13.3 mCi/mmole) were purchased from New England Nuclear (Boston, Mass.).

All other reagents not specifically mentioned were of reagent grade purity and were obtained from Fisher Scientific Co. (Springfield, N.J.) or Sigma Chemical Co. (St. Louis, Mo.).

Sulfamethoxazole and α-methyl-dopamine hydrobromide were generous gifts from Hoffmann-La Roche, Inc. (Nutley, N.J.).

Syntheses

The synthetic procedures are outlined in Figure 1 and the products are listed in Figure 2-4.

1. Synthesis of phthaloyl glutamic anhydride

Phthaloyl-L-glutamic anhydride was obtained by the method of Sheehan and Bolhofer (1950) as modified by King et al. (1957). L-Glutamic acid (29.4 g, 0.2 mole) and phthalic anhydride (29.6 g, 0.2 mole) were ground to a fine powder and heated to 135-145°C for 20 minutes. The clear melt was then cooled to 100°C and 35 ml of acetic anhydride were added. The mixture was heated for 3 minutes at 100°C and then 105 ml of xylene were added. After the mixture was cooled overnight at 0°C, 29 g (0.11 mole) of phthaloyl-L-glutamic anhydride were obtained.

Phthaloyl-D-glutamic anhydride was synthesized by substituting D-glutamic acid for its L-isomer in the procedure described above.

2. L-γ-Glutamyl-DOPA

L-DOPA (1.97 g, 0.01 mole) was dissolved in 50 ml of 0.5 M Na_2CO_3 under nitrogen. The flask was cooled in an ice bath to 0-5°C and 5.2 g (0.02 mole) of phthaloyl-L-glutamic anhydride dissolved in 30 ml of dry dioxane were added dropwise with stirring. The mixture was stirred for an additional 20 minutes and then acidified to approximately pH 1.0 by the addition of 6 N HCl. The mixture was extracted with several portions of ethyl acetate and the pooled extracts were dried with anhydrous sodium sulfate. Ethyl acetate was removed from the extract by flash evaporation

and the residue was dissolved in 50 ml of methanol. Three milliliters of hydrazine hydrate (99%) were added to the methanol solution and the mixture was left for 2 days at 26°. Methanol was then removed by flash evaporation and the residue was suspended in 50 ml of water. The suspension was acidified to pH 3.0 by the dropwise addition of 1 N HCl. The precipitated white solid (phthaloyl hydrazide) was removed by filtration and the filtrate was adjusted back to pH 5.0. The solution was then applied at 4°C to the top of a Dowex-1 (acetate) column (2.5 x 45 cm). The column was washed with 100 ml of 0.01 M acetic acid and then eluted with a linear gradient established between 2 liters of 0.01 M acetic acid and 2 liters of 2 M acetic acid. Fractions of approximately 20 ml were collected. The presence in the eluate of ninhydrin-positive material was determined by a spot test on Whatman No.1 filter paper. The product of the reaction emerged from the column when approximately 2 liters of the eluant passed through the column. The fractions containing γ-glutamyl-DOPA were pooled and acetic acid was completely removed by flash evaporation under reduced pressure at 37°C. An amorphous white solid was obtained. The yield was 1.4 g (43%) L- γ-glutamyl-DOPA.

$C_{14}\ O_7\ N_2\ H_{18} \cdot H_2O$

Calculated	: C48.84, H5.85, N8.14
Found	: C49.39, H6.02, N7.42

D- γ-Glutamyl-DOPA was synthesized similarly except that phthaloyl-D-glutamic anhydride was used.

3. L- γ-Glutamyl-DA

1.9 g (0.01 mole) of dopamine hydrochloride was dissolved in 40 ml dioxane. 4 g (0.015 mole) of phthaloyl-L-glutamic anhydride were then added and followed by dropwise addition of 1 ml triethylamine. The mixture was heated to 60°C and maintained at this temperature for 15 min with constant mixing. Dioxane was then removed by flash evaporation and the residue was dissolved in 50 ml methanol. 1.5 ml of hydrazine hydrate was added and the mixture was left under nitrogen for two days at 25°C. Methanol was then removed by flash evaporation and the residue was suspended in 25 ml of water. The suspension was acidified to pH 3 with 1 N HCl and allowed to stand for 1 hour at 25°C. The white precipitate was then removed by filtration and the filtrate was applied at 4°C to the top of a Dowex-1 (acetate) column (2.5 x 45 cm). The column was washed with 1 liter of 0.01 M acetic acid. Fractions of approximately 15 ml were collected. The presence in the eluate of ninhydrin-positive material was determined by a spot test on Whatman No.1 filter paper. The absorbance at 280 nm was also measured. The product emerged after approximately 500 ml of the eluent was passed through the column. The fractions containing γ-glutamyl-DA were pooled and acetic acid was completely removed by flash evaporation under reduced pressure at 37°C. The yield of L- γ-glutamyl-DA was 0.8 g (29%).

$C_{13}\ H_{18}\ O_5\ H_2$

Calculated	:	C55.32, H6.43, N9.93
Found	:	C54.40, H6.53, N9.91

D- γ-Glutamyl-DA was synthesized following the same procedure using phthaloyl-D-glutamic anhydride.

4. L- γ- and L- α-Glutamyl-sulfamethoxazole

Sulfamethoxazole (4.1 g, 0.016 mole) was suspended in 40 ml of glacial acetic acid and 4.54 g (0.0175 mole) of phthaloyl glutamic anhydride were added. The mixture was heated at 60°C for 30 minutes and the solvent was removed by flash evaporation. The residue was suspended in ethyl acetate and the solvent was removed under reduced pressure. This process was repeated several times until most of the acetic acid was removed. The remaining material was dissolved in methanol (40 ml) and 1.6 ml of hydrazine hydrate and 1.6 ml triethylamine were added. The mixture was allowed to stand for 48 hours at 26°C and the precipitate which formed was removed by filtration. The filtrate was evaporated under reduced pressure and the residue was dissolved in 50 ml of water (a small amount of an insoluble material was removed by filtration). The aqueous solution was applied to the top of a Dowex-1-acetate column (AG-1X4, 200-400 mesh, 3 x 33 cm). The column was washed with 500 ml of water and elution was started with 0.1 M acetic acid. Fractions of 20 ml were collected. The effluent was tested for ninhydrin-positive material by a spot test on Whatman No.1 filter paper using a 0.2% solution of ninhydrin in acetone. Free glutamate emerged in fractions 70 to 90 and was followed by L-α -glutamyl-sulfamethoxazole in fractions 100 to 170. The column was then washed with 2 liters of 0.3 M acetic acid and L-γ -glutamyl-sulfamethoxazole was eluted from the column with 2.5 liters of 0.5 M acetic acid. Fractions containing the desired products were pooled and evaporated under reduced pressure. 4.36g (71%) of the γ-glutamyl and 0.47 g (7.7%) of the α-glutamyl derivative were obtained. Recrystallization was carried out by dissolving the compounds in a small amount of 0.1 N HCl and adjusting to pH 4 to 5 by the addition of NaOH.

The compounds gave single ninhydrin-positive spots on descending chromatography on Whatman No.1 filter paper. In the solvent system 1-butanol-pyridine-water (1:1:1), the Rf values for the γ-glutamyl and α-glutamyl derivatives were 0.73 and 0.63 respectively. The corresponding. Rf values in 1-butanol-acetic acid-water (60: 15:25) were 0.78 and 0.66.

		C_{15} H_{18} N_4 O_6 S
Calculated	:	C47.11, H4.74, N14.65
Found	:	C47.35, H4.85, N14.03 (L- γ-glutamyl-sulfamethoxazole)
		C46.96, H5.29, N13.34 (L- α-glutamyl-sulfamethoxazole)

The D-stereoisomer of γ-glutamyl-sulfamethoxazole was prepared by the procedure described above using as starting materials phthaloyl-D-glutamic anhydride and sulfamethoxazole.

		C_{15} H_{18} N_4 O_6 S
Calculated	:	C47.11, H4.74, N14.65
Found	:	C47.34, H4.92, N14.34

5. N-Acetyl-L- γ-glutamyl-sulfamethoxazole

1.53 g (4.0 mmole) of L- γ-glutamyl-sulfamethoxazole were dissolved in 20 ml of 0.5 M Na_2CO_3. The solution was cooled in ice and 5 mmole of acetic acid anhydride were added dropwise during a period of 15 minutes with continuous stirring. The mixture was stirred for an additional 20 minutes and then adjusted to about pH 1 by the addition of 3 N HCl. The mixture was extracted with several portions of ethyl acetate in a separatory funnel. The organic phase was collected and dried over

anhydrous sodium sulfate. The removal of the solvent under reduced pressure yielded a crystalline product (1.12 g, 66%).

$C_{17}\ H_{20}\ N_4\ O_7\ S$

Calculated	:	C48.11,	H4.75,	N13.20
Found	:	C47.99,	H4.76,	N12.19

6. N-Butyryl-L- γ-glutamyl-sulfamethoxazole

This compound was prepared from butyric anhydride and L- γ-glutamyl-sulfamethoxazole using the procedure described for the N-acetyl analog. The compound was crystallized from a mixture of ethyl acetate and hexane.

$C_{19}\ H_{24}\ N_4\ O_7\ S$

Calculated	:	C50.43,	H5.35,	N12.38
Found	:	C50.30,	H5.65,	N11.99

7. N-Chloroacetyl-L- γ-glutamyl-sulfamethoxazole

L- γ-Glutamyl-sulfamethoxazole (1.91 g, 5 mmole) was dissolved in 40 ml of 0.5 M Na_2CO_3 and cooled in ice. Chloroacetyl chloride (0.75 ml, 10 mmole) was added dropwise with continuous stirring. After 20 minutes the solution was acidified with 3 N HCl to a pH of about 1.0 and extracted with several portions of ethyl acetate in a separatory funnel. The organic phase was dried over anhydrous sodium sulfate and concentrated by flash evaporation. The remaining oily material was dried with 2-propanol. The product was dissolved in acetone. After addition of ether a white solid was obtained (1.45 g, 63%)

$C_{17}\ H_{19}\ N_4\ O_7\ S\ Cl$

Calculated	:	C44.50,	H4.17,	N12.21
Found	:	C44.94,	H4.56,	N11.70

8. Glycyl-L-γ -glutamyl-sulfamethoxazole

N-Chloroacetyl-L-γ -glutamyl-sulfamethoxazole (0.5 g) was dissolved in 10 ml methanol and cooled to -10°C. A slow stream of ammonia gas was introduced until saturation was obtained. The flask was stoppered and left for 48 hours at 25°C. After removal of excess solvent by flash evaporation a white solid separated (0.32 g, 67%).

$C_{17}\ H_{21}\ N_5\ O_7\ S$

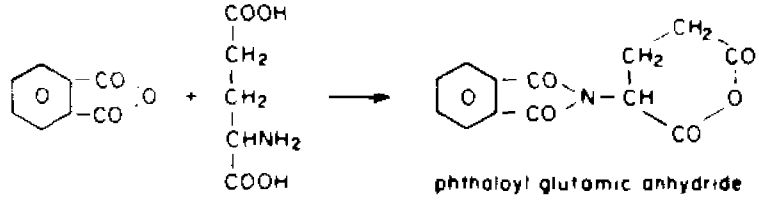
Calculated	:	C46.47, H4.81, N15.94
Found	:	C45.08, H5.06, N15.51

Figure 1

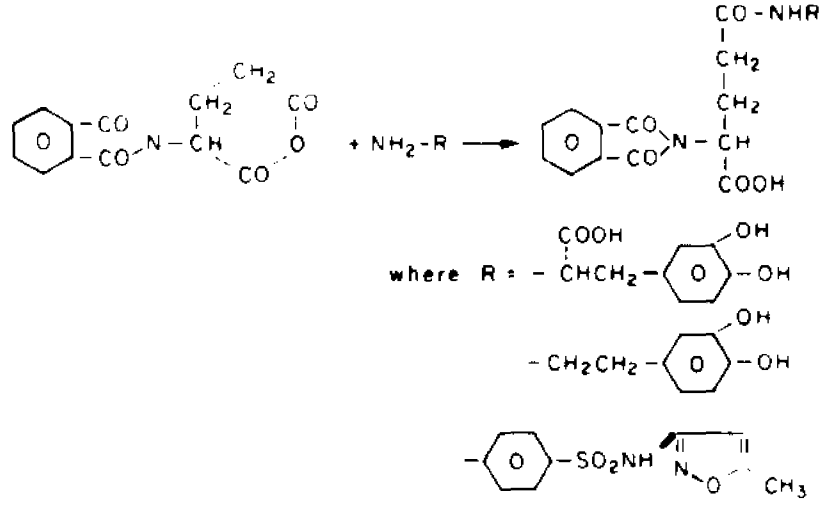
General synthetic scheme for γ-glutamyl compounds

GENERAL SYNTHETIC SCHEME FOR γ-GLUTAMYL COMPOUNDS

1 Synthesis of phthaloyl-L-glutamic anhydride



2 Condensation reaction



3 Removal of a phthaloyl group

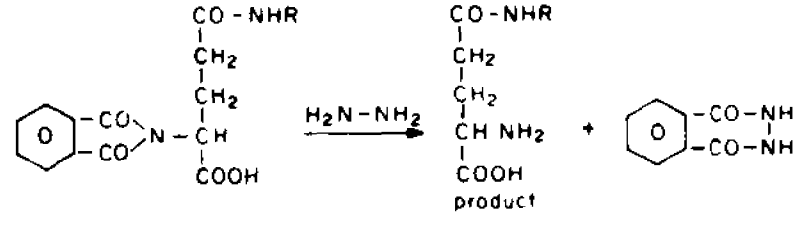
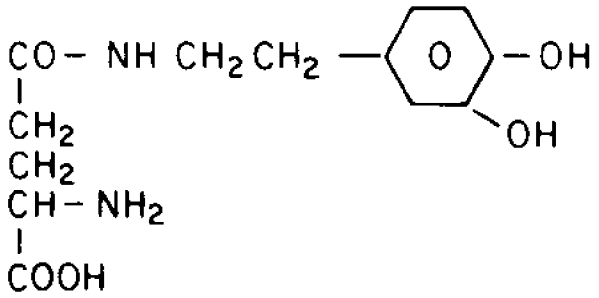
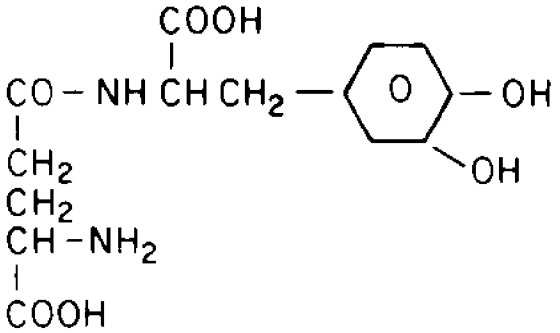


Figure 2

Derivatives of dopamine and DOPA



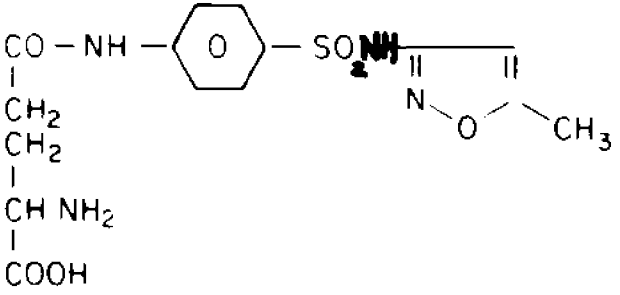
γ - Glutamyl-dopamine



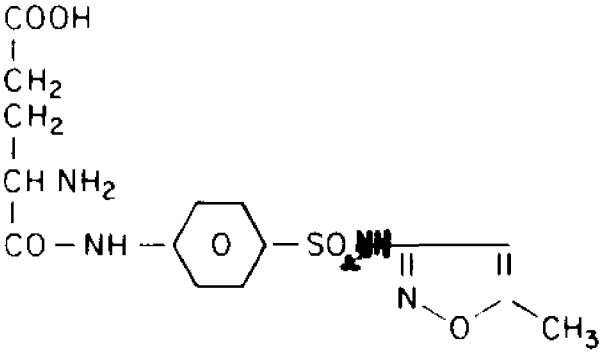
γ - Glutamyl-DOPA

Figure 3

Derivatives of sulfamethoxazole



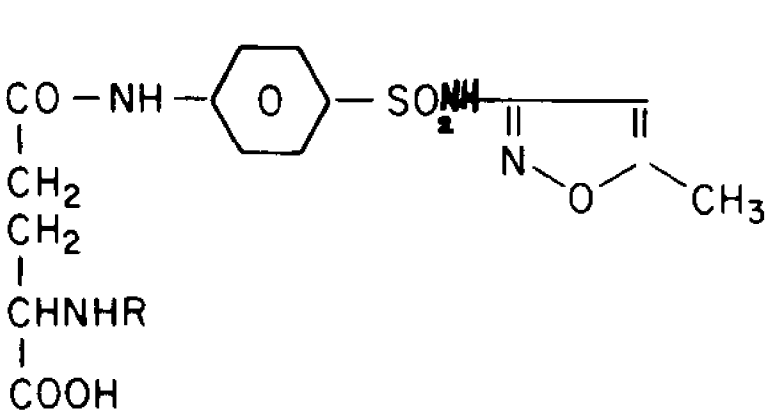
γ - Glutamyl-sulfamethoxazole



α - Glutamyl-sulfamethoxazole

Figure 4

N-Acyl-γ -glutamyl derivatives of sulfamethoxazole



N - Acyl-γ-glutamyl-sulfamethoxazole

R = $-C(=O)-CH_3$

$-C(=O)-CH_2CH_2CH_3$

$-C(=O)-CH_2Cl$

B. Methods

Animals

Male Swiss Albino mice (20 - 35 g), Sprague Dawley rats (200 - 325 g) and male Hartley Albino guinea pigs (300 - 400 g) were housed in the animal facility of the Mount Sinai School of Medicine. Animals were fed a commercial Purina Laboratory Chow diet and water ad libitum unless otherwise specified.

Measurement of tissue levels of DA after administration of DA and its precursors

Solutions of γ-glutamyl-DOPA, γ-glutamyl-DA and L-DOPA were prepared in normal saline. Male Swiss albino mice weighing 20 to 25 g received an intraperitoneal injection of γ-glutamyl-DOPA (0.5 μmole/g), γ-glutamyl-DA (0.5 μmole/g) or L-DOPA (0.5 μmole/g). Twenty minutes after drug administration the animals were decapitated and the following tissues were removed for study: kidney, heart, liver, brain, lung, duodenum, pancreas, spleen and muscle. In a second experiment the time course of DA accumulation in the kidney was followed by decapitating mice 10, 20 and 60 minutes after drug administration. Tissues were homogenized in 5 volumes of cold 1 N HCl and centrifuged, and an aliquot was removed for analysis of DA by a gas chromatographic procedure (Wilk and Stanley, 1977).

An aliquot of the supernatant is added to a 15-ml centrifuge tube containing 100 mg of alumina (Woelm basic activity grade I), 4 ml of 0.5 M Tris-chloride buffer (pH 8.5), 0.1 ml of 10% EDTA (pH 7.2) and 50 ng of α-methyl-DA as internal standard. The tube is stoppered and gently shaken for 3 minutes to adsorb the catecholamines. After

centrifugation the supernatant is removed by aspiration. The alumina is then washed three times with 5 ml of H_2O followed by centrifugation and removal of the H_2O. The catecholamines are eluted with 1 ml of 0.25 M acetic acid in methanol. After centrifugation the eluate is transferred to a 3-ml ground glass stoppered silane-treated centrifuge tube containing 10 µl of a 1.25 mg/ml solution of sodium diethyldithiocarbamate as an antioxidant. The contents are evaporated to dryness under a stream of dry nitrogen gas and the residue is reacted with 50 µl of pentafluoropropionic anhydride in 100 µl of ethyl ether for 5 minutes at room temperature. The reagents are then removed by evaporating under a gentle stream of nitrogen and the derivatives are dissolved in 0.5 ml of toluene for chromatographic analysis. Two standards containing 50 ng of α-methyl-DA and 100 ng of DA are processed simultaneously. Three microliters of each sample are injected on a 3% SE-30 column coated on Gas Chrom Q 100/120 mesh at a temperature of 140°C and a flow rate of 15 ml/min of nitrogen. The derivatives are detected by an electron capture detector fitted with a 150 mCi tritium foil (Packard 7400 series gas chromatograph).

Measurement of total DA in urine

Urine samples were acidified with concentrated HCl to pH 1. The test tubes containing acidified urine were placed in boiling water for 20 minutes. DA content, representing the free and conjugated DA in the urine, was determined as described before.

Measurement of 3,4-dihydroxyphenylacetic acid and homovanillic acid

3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined simultaneously on the JXR column at a temperature of 125°C and flow rate of 40 ml/min by the procedure previously described (Watson et al., 1974). These samples were also injected on a 3% XE60 column at a temperature of 150°C and a flow rate of 13 ml/min to confirm the HVA values.

Measurement of renal plasma flow in rats

Female Sprague-Dawley rats weighing 250 to 325 g were anesthetized with ethyl ether. The femoral vein was cannulated with a PE-50 catheter filled with normal saline and the femoral artery was cannulated with a PE-50 catheter filled with a heparin-saline solution. The bladder was exposed and catheterized with a No. 8 polyethylene catheter. The urethra was ligated. Renal plasma flow was measured by determining the clearance of [^{14}C]-p-aminohippurate (Schlegel et al., 1962). An infusing solution of 0.45% saline-p-aminohippurate was prepared by diluting normal saline with distilled water and adding 0.025 μCi of glycyl-[1-^{14}C]-p-aminohippurate per ml of infusing solution (specific activity 43 mCi/mmole). Drug effects were determined by dissolving the drug in the infusing solution. The solution was infused into the femoral vein at a rate of 0.17 ml/min using a Sage model 355 syringe pump. After a 1-hour equilibration period urine was collected continuously over 30-minute intervals. At the midpoint of each collection period 0.2 ml of blood was withdrawn from the femoral artery. A total of five collections were made. The blood samples were deproteinized by addition of 0.5 ml of 10% trichloroacetic acid and centrifuged. Aliquots of the supernatant and

urine samples were counted in a Nuclear-Chicago iso-cap scintillation counter. Renal plasma flow was calculated assuming a constant hematocrit of 50% by the general formula:

$$\text{Renal plasma flow} = \frac{\text{urine(cpm/ml)} \times \text{urine flow(ml/min)}}{\text{plasma(cpm/ml)}}$$

Renal plasma flow after oral administration of drugs was determined using the procedure described above with minor modifications. Thus after two 20 min control periods, DA or one of its prodrugs dissolved in 2 ml of water was given via a stomach tube. The tube was rinsed with 1 ml of water to wash out the remaining drug in the dead space. As a control, seven animals were given an equal amount of water.

Renal plasma flow after intravenous injection of a drug was determined following the procedure described before. In this case, however, the infusing solution was made up of 5% mannitol, 0.45% saline and glycyl-[1-^{14}C]-p-aminohippurate (0.033 μCi/ml). After a 45 min equilibration period, two successive 20 min control urine collections were performed. L- γ-Glutamyl-DOPA (dissolved in 0.2 ml of 0.9% saline) was then injected intravenously and four 20 min experimental urine collections each lasting 20 min were performed.

Measurement of glomerular filtration rate in rats

Animals were prepared as described in 'measurement of renal plasma flow in rats'. The general procedure used in measuring renal plasma flow was followed except in this case the infusing solution contained inulin-[^{14}C]-carboxylic acid (0.025 μCi/ml). Glomerular filtration rate

was calculated from the clearance of radioactive inulin.

Measurement of Na^+ excretion in rats

Female rats were anesthetized with sodium pentobarbital (50 mg/kg). The femoral vein and bladder were cannulated and the urethra was ligated. After 30 min of 5% dextrose infusion at a rate of 0.09 ml/min, two 30 min control urine collection periods were started. At the end of this time, the initial infusing solution was changed to that containing a drug. This was followed by five 30 min experimental collection periods. Urinary concentration of sodium was measured by flame photometry.

Measurement of blood pressure in rats

Female Sprague-Dawley rats weighing 275 to 325 g were anesthetized with sodium pentobarbital (50 mg/kg i.p.). In some cases tracheotomy was performed. The femoral vein was cannulated with a PE-50 catheter filled with normal saline and the femoral artery was cannulated with a PE-50 catheter filled with a heparin-saline solution. The arterial catheter was connected to a blood pressure transducer and the blood pressure was recorded on a Grass model 7 polygraph upon stabilization of arterial blood pressure. The effect of drugs on blood pressure was evaluated by dissolving the drug in normal saline and infusing it into the femoral vein at a constant rate of 0.17 ml/min.

Measurement of plasma glucose levels after administration of DA and its precursors in rats

Female rats weighing 250 to 275 g were starved overnight; free access to water was allowed. Following the induction of anesthesia with sodium

pentobarbital (30mg/kg), a tracheal tube was introduced. The right femoral artery and vein were cannulated. Each drug was dissolved in normal saline before intravenous infusion through the femoral venous catheter. A total of four 0.3 ml arterial blood collections were made. The first blood sample was obtained immediately before the drug infusion. At time 0, a constant infusion at a rate of 0.067 ml/min for 30 min was started. After 15 and 30 min, two additional collections were made. The last blood sample was taken 30 min after the cessation of the drug infusion. Preliminary experiments showed that normal saline infusion had no effect on plasma glucose levels.

Each blood sample was placed in a heparinized centrifuge tube and centrifuged at 4°C for 10 min at 2000 rpm. The plasma was separated and immediately assayed for glucose using the glucose oxidase method according to the manufacturers' recommendation.

Measurement of renal clearances and urine output after L-γ-glutamyl-DOPA administration in dogs

Female mongrel dogs weighing between 16 to 25 kg were anesthetized with sodium pentobarbital (50 mg/kg). A juglar vein and femoral artery were cannulated for the introduction of the infusing solution and blood collection respectively

In the first group of dogs, L-γ-glutamyl-DOPA was infused intravenously. After the priming dose of p-aminohippurate (PAH; 5 mg/kg) and creatinine (70 mg/kg), sustaining solution made up in 5% mannitol-Ringer's solution, was infused at a rate approximately equal to the rate of urine flow. The sustaining solution contained PAH (10 mg/kg/hr) and creatinine (50 mg/kg/hr) in amounts required to maintain plasma PAH and

creatinine concentrations of about 1 mg/dl and 10 mg/dl respectively. After three or four control periods, the infusion of L-γ -glutamyl-DOPA was started. This was followed by six 20 min experimental periods. Urine was collected through a Foley catheter and blood samples were obtained at the midpoint of each collection period.

In the second group of dogs, the right renal artery was exposed retroperitoneally. L- γ-Glutamyl-DOPA, dissolved in 0.9% saline, was infused via a small needle placed into the renal artery. The rate of the infusion ranged from 0.5 to 2.0 ml/min. Both ureters were cannulated and urine from each kidney was collected separately. The general procedure for the clearance study described above was followed. For these experiments the sustaining solution was made up in isotonic Ringer's solution.

Measurement of p-aminohippurate concentration

PAH concentrations in urine and plasma were measured as described by Smith et al. (1940).

Measurement of creatinine concentration

Creatinine concentrations in urine and plasma were determined using the method described by Bonsnes and Taussky (1945).

Direct measurement of renal blood flow in dogs

Dogs were prepared as described previously in 'measurement of renal clearances and urine output after L- γ -glutamyl-DOPA administration in dogs'. The mean renal blood flow was measured by placing an electro-

magnetic probe around the right renal artey; the flow rate was read on a CME Cliniflow flowmeter (Carolina Medical Electronics).

Measurement of arterial blood pressure in dogs

Mean arterial blood pressure was measured from the femoral artery using a Baumanometer (W A Baum Co., 300 model).

Measurement of cardiac output

Cardiac output was measured by the dye dilution method of Hamilton (1962).

Measurement of rate of sulfamethoxazole release in vitro

Enzymatic release of sulfamethoxazole (SM) from glutamyl and N-acyl-L-γ-glutamyl derivatives of SM by tissue homogenates was measured in an incubation mixture (final volume 0.25 ml) containing substrate (0.5 μmole), homogenate (0.05 ml) and Tris-HCl buffer (0.1M; pH 8.0). Homogenates were made in cold 0.9% saline. Reactions were started by addition of homogenate and incubations were carried out at 37°C. Reactions were terminated by adding 0.25 ml of 25% trichloroacetic acid. After removal of protein by centrifugation, free SM was determined in the supernatants by the diazotization procedure of Bratton and Marshall (1939) as modified by Goldbarg and Rutenburg (1958). Controls in which either the enzyme or substrate were omitted were also carried through the procedure.

Measurement of rate of deacylation from N-acyl-L- γ-glutamyl derivatives of SM in vitro

The release of SM from N-acyl-L- γ-glutamyl derivatives of SM was measured after addition of excess sheep kidney γ-glutamyl transpeptidase (Zelazo and Orlowski, 1976). Incubation mixtures (final volume 0.5 ml) contained homogenate (0.05 ml), substrate (1 μmole), glycylglycine (7.5 μmole; pH 8.0) and γ-glutamyl transpeptidase (1 unit). Glycylglycine in the incubation mixture functions as an acceptor for the γ-glutamyl moiety of γ-glutamyl-SM generated during the acylase reaction and thus ensures its complete cleavage. Control experiments in which either the substrate or homogenate were omitted, were included in all determinations.

Measurement of tissue levels of SM after administration of SM and its precursors

SM or one of its derivatives were injected intraperitoneally to mice, rats and guinea pigs as a solution in 0.9% saline after the pH had been adjusted to 7.6. The volume of the injected solutions was 0.1 ml/g. The animals were killed and the tissues were immediately excised, weighed and homogenized in ice-cold 0.9% saline using a Potter-Elvehjem glass homogenizer equipped with a motor driven teflon pestle. Blood samples were collected in heparinized tubes and centrifuged to obtain plasma. Organ homogenates and plasma were deproteinized with 25% trichloroacetic acid and centrifuged. SM concentration in the supernatant was measured by the diazotization procedure as described.

Measurement of urinary excretion of SM after administration of SM and its precursors

Following the intraperitoneal injection of SM and its prodrugs (0.25 μmole/g) to rats and guinea pigs, the animals were placed in metabolic cages, one per cage. Food was withheld with free access to water throughout the experimental period. Two 24 hour urinary collections were made. Urine was acidified with 25% trichloroacetic acid and free SM was determined as described.

Determination of protein concentration

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

RESULTS

A. Prodrugs of dopamine

Tissue distribution of DA after DA prodrugs

The tissue distribution of DA after intraperitoneal administration of L- γ-glutamyl-DOPA and L-DOPA (0.5 μmole/g) has been studied previously (Wilk et al., 1978). Administration of L-γ -glutamyl-DOPA led to a marked accumulation of DA in the kidney. The level of DA in the kidney was considerably higher than that in all the other tissues examined. By contrast, an equimolar amount of L-DOPA produced a much more uniform tissue distribution of DA. An even more uniform distribution has been reported after infusion of free DA (Halushka and Hoffman, 1968). Since the tissue distribution of DA after administration of L- γ-glutamyl-DA had not yet been studied, a study was carried out to determine if administration of this DA prodrug induces a selective accumulation of DA in the kidney. The results obtained after injection of L- γ-glutamyl-DA, L- γ-glutamyl-DOPA and L-DOPA (0.5 μmole/g) are shown in Figure 5. Like L-γ -glutamyl-DOPA, the administration of L-γ-glutamyl-DA results in a selective accumulation of DA in the kidney. The kidney concentration of DA, however, was higher after administration of L- γ-glutamyl-DOPA than after L-γ -glutamyl-DA.

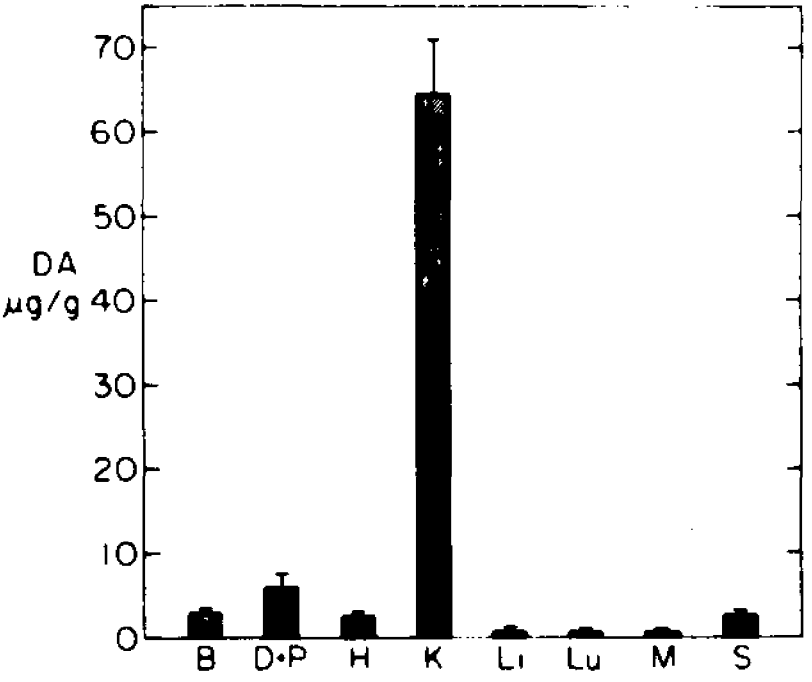
Time course of DA accumulation in the kidney after DA precursors

It is likely that γ-glutamyl derivatives of DOPA and DA act as a store of DA from which this catecholamine is released over a prolonged period of time. These derivatives protect DA from metabolic inactivation to which free DA is highly susceptible. It was therefore of interest to examine if the γ-glutamyl derivatives of DOPA and DA are capable of

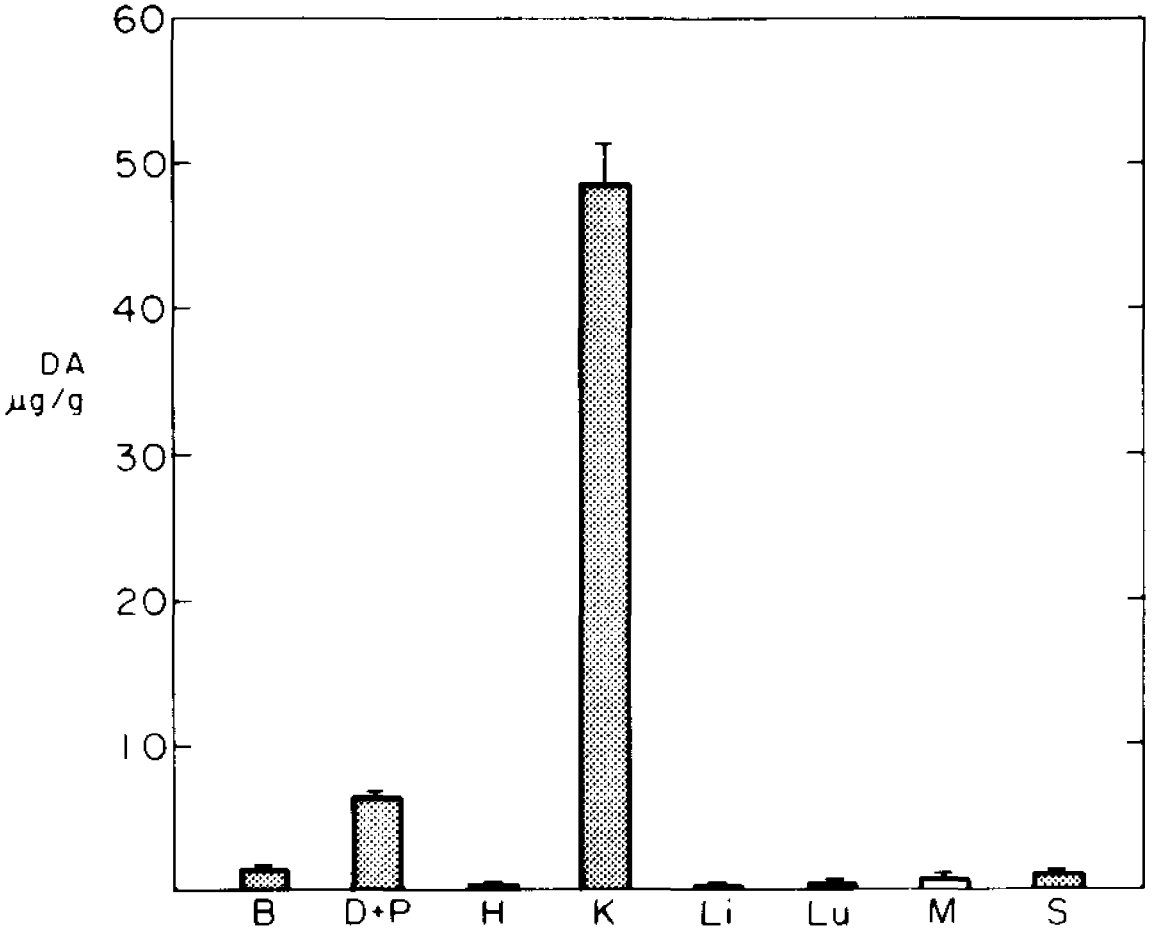
Figure 5

Tissue distribution of dopamine after dopamine precursors

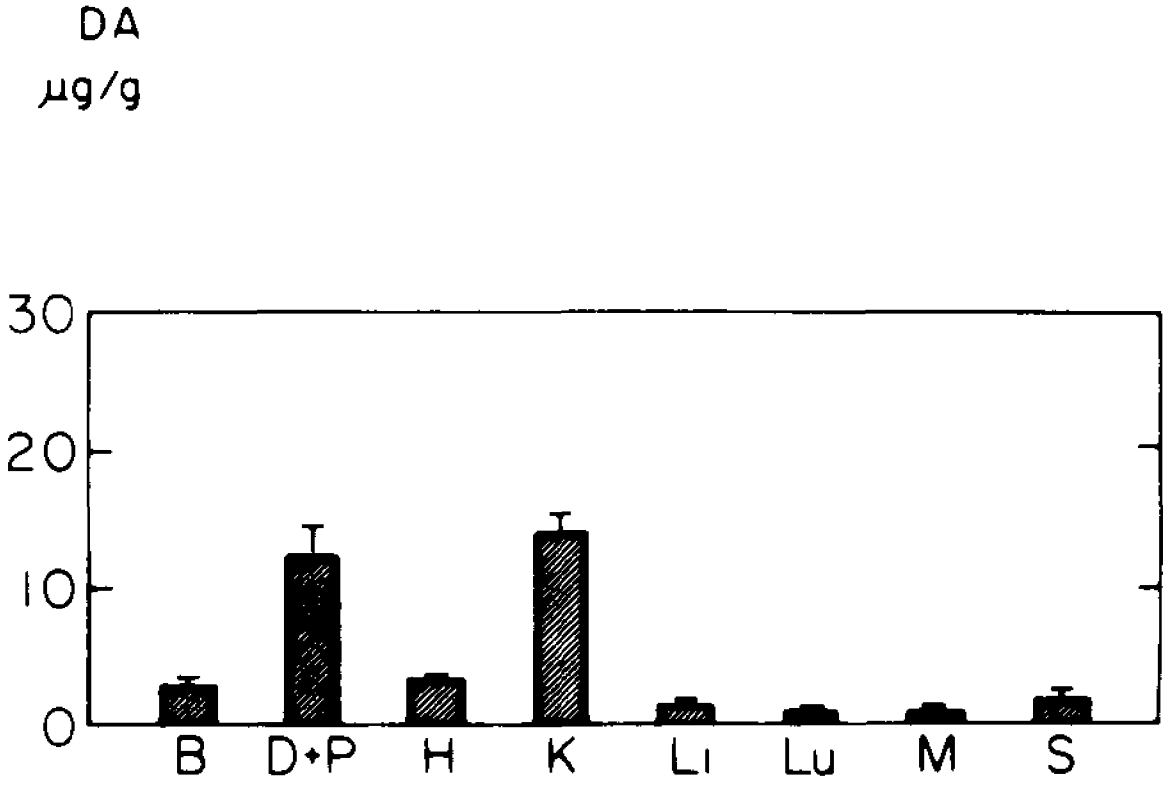
Concentration of dopamine in various tissues 20 minutes after administration of a) γ -glutamyl-DOPA (0.5 µmole/g i.p.)
b) γ -glutamyl-dopamine (0.5 µmole/g i.p.)
c) L-DOPA (0.5 µmole/g i.p.).
B, brain; D+P, duodenum+pancreas; H, heart; K, kidney; Li, liver; Lu, lung; M, muscle; S, spleen.



a) γ-Glutamyl-DOPA



b) γ-Glutamyl-dopamine



c) L-DOPA

maintaining high kidney concentrations of DA for prolonged periods of of time. The time course of DA accumulation in the kidney after the prodrugs was therefore studied. Table 1 compares the time course of DA accumulation after administration of L- γ-glutamyl-DOPA, L-γ -glutamyl-DA and L-DOPA (0.5 μmole/g i.p.). A peak level of DA was reached 10 min after the drug administration in all the cases. A high level of DA was more persistently maintained by L- γ-glutamyl derivatives of DOPA and DA than by L-DOPA.

Renal effects of DA and its prodrugs in rats

The kidney-selective accumulation of DA after administration of L-γ -glutamyl-DOPA and L- γ-glutamyl-DA prompted studies on the effect of the locally generated DA on renal plasma flow in rats. Since DA has been reported to increase glomerular filtration rate, Na^+ excretion and urine flow, the effects of the DA precursors on these renal functions were studied.

1. Effect on renal plasma flow

The effects of L- γ-glutamyl-DOPA and L-γ -glutamyl-DA on renal plasma flow (RPF) were compared to those of L-DOPA and DA (Table 2). In control rats, RPF amounted to 2.58 ml/min/100g, a value in close agreement with values reported by others [2.6 ml/min/100g (Friedman, 1947), 2.85 ml/min/100g (Hsu et al., 1975)]. L- γ-Glutamyl-DOPA at a dose of 10 nmole/g/30min significantly elevated the RPF by 60%. By contrast an equimolar dose of L-DOPA was without effect on the RPF. When the dose of L-DOPA was doubled, a smaller but significant increase in the RPF (by 29%) was observed. L- γ-glutamyl-DA also increased the RPF (by 50%), but the dose required was 50 nmole/g/30min, five times

Table 1

Time course of DA accumulation in kidney after DA precursors

Time [min]	Dopamine concentration [µg/g] after L- γ - Glutamyl-DOPA[a]	L- γ - Glutamyl-DA	L-DOPA[a]
10	66.9 ± 14.9 [4]	48.7 ± 3.8 [5]	23.2 ± 1.9 [4]
20	64.5 ± 7.4 [4]	48.1 ± 2.6 [10]	13.9 ± 1.6 [4]
40	---	16.1 ± 0.4 [5]	---
60	9.9 ± 2.3 [4]	3.2 ± 0.8 [4]	2.6 ± 0.6 [4]

[a]The values are obtained from Wilk et al., 1978 for comparative purposes. Dose of 0.5 µmole/g was given. The number of animals is indicated in brackets.

Table 2

Effects of DA and its precursors on renal plasma flow (RPF), glomerular filtration rate (GFR) and urine flow

Treatment	Dose [nmole/g/30min]	RPF [ml/min/100g]	GFR [ml/min/100g]	Urine flow [ml/30min/100g]
Saline	---	2.58 ± 0.14 [8]	0.76 ± 0.07 [8]	0.70 ± 0.12 [11]
DA	1	4.10 ± 0.43[c] [5]	0.99 ± 0.10 [7]	1.11 ± 0.12[a] [11]
L-γ -Glutamyl-DOPA	10	4.17 ± 0.45[c] [7]	0.89 ± 0.04 [12]	0.97 ± 0.10 [17]
L-γ -Glutamyl-DA	50	3.86 ± 0.33[c] [5]	0.97 ± 0.06[a] [7]	1.05 ± 0.10[a] [14]
L-DOPA	10	2.58 ± 0.29 [6]	---	---
L-DOPA	20	3.33 ± 0.23[b] [7]	---	---

Values are expressed as mean ± S.E. The number of animals is given in brackets. Student's t-test was performed: [a] $p < 0.05$, [b] $p < 0.025$, [c] $p < 0.005$.

greater than that of L- γ-glutamyl-DOPA. DA at a dose of 1 nmole/g/30min elevated the RPF by 60%.

2. Effect on glomerular filtration rate

In control rats, values of glomerular filtration rate (GFR) averaged 0.76 ml/min/100g, a value in the range of 0.7 - 0.8 ml/min/100g reported by others (Friedman et al., 1947; Shikita, 1962). The doses of L-γ - glutamyl-DOPA, L- γ-glutamyl-DA and DA which elevated the RPF also increased the GFR (Table 2). However the increase was statistically significant only after L- γ-glutamyl-DA.

3. Effect on urine flow

Considerable variation in urine flow was noted among different animals. DA and L- γ-glutamyl derivatives of DOPA and DA increased the average urine output in anesthetized and volume expanded rats. The increase in urine flow after L- γ-glutamyl-DA and DA but not that after L- γ -glutamyl-DOPA was statistically significant (Table 2).

4. Effect on Na^+ excretion

The effects of DA and its precursors on Na^+ excretion were studied in rats receiving a constant infusion of 5% dextrose. Initial studies indicated that there was no change in Na^+ excretion in animals infused with 5% dextrose for the duration of experiments (240 min). L- γ - Glutamyl-DOPA, L- γ -glutamyl-DA and DA all induced natriuresis (Table 3). The lowest effective doses needed to increase Na^+ excretion were 5, 50 and 100 nmole/g/30min for DA, L- γ-glutamyl-DOPA and L- γ-glutamyl-DA respectively.

Table 3

Effects of DA and its precursors on Na^+ excretion

Treatment	Dose [nmole/g/30min]	Control [µeq/min]	Drug infusion [µeq/min]
DA [6]	5	0.06 ± 0.01	0.33 ± 0.11[a]
L- γ -Glutamyl-DOPA [8]	50	0.06 ± 0.02	0.13 ± 0.02[a]
L- γ -Glutamyl-DA [8]	100	0.11 ± 0.04	0.49 ± 0.17[a]

Values are expressed as mean ± S.E. The number of animals is given in brackets. Paired t-test was performed: [a] $p < 0.05$.

Effect of DA and its precursors on blood pressure in rats

Our studies have shown that DA, L-DOPA and their γ-glutamyl derivatives cause an increase in RPF in rats. This action could have resulted either from a direct effect of the drugs on kidney circulation or from a general systemic effect. It was therefore of interest to evaluate whether the increase in RPF was caused by a selective effect of the prodrugs on the kidney. Experiments were designed using blood pressure elevation as an indicator of extrarenal action since DA is known to elevate blood pressure by activating peripheral α-adrenergic receptors. If γ-glutamyl derivatives of DA and L-DOPA possess kidney-specific action, such organ specificity should be reflected in a greater separation of doses required to elevate blood pressure as compared to doses required to increase RPF. Studies were therefore carried out to determine the minimum effective doses of DA and its precursors required to elevate arterial blood pressure in rats. The results are shown in Table 4. The lowest effective dose causing an elevation of arterial blood pressure after L- γ-glutamyl-DA was one hundred times greater than after free DA whereas the lowest effective dose for L- γ-glutamyl-DOPA and L-DOPA was twenty times greater than after free DA. The nature of the pressor response elicited by L- γ-glutamyl-DOPA and by L-DOPA differed from that caused by L- γ-glutamyl-DA and DA in that the time to peak response was more gradual for the former two compounds. Compared to other compounds, L-γ -glutamyl-DOPA produced a smaller increase in diastolic pressure. The ratio of dose required to elevate arterial blood pressure to that required to elevate renal plasma flow was taken as an index of renal specificity. This ratio was 10 for DA and L-DOPA and 20 for L- γ-glutamyl-DA and L-γ -glutamyl-DOPA.

Table 4

Effects of DA and its precursors on blood pressure

Treatment	Dose [nmole/g/30min]	Change in systolic pressure [mm Hg]	Change in diastolic pressure [mm Hg]	Time to peak response [min]
Saline [5]	---	- 2	- 6	---
DA [4]	10	+ 23	+ 20	1 - 3
L- γ -Glutamyl-DOPA [6]	200	+ 17	+ 5	5 - 17
L- γ -Glutamyl-DA [6]	1000	+ 21	+ 18	2 - 6
L-DOPA [12]	200	+ 25	+ 15	4 - 20

Values are expressed as average of several determinations. The number of animals is given in brackets.

Effects of DA and its precursors on plasma glucose levels

In addition to affecting blood pressure, DA has been reported to elevate plasma glucose levels (Horwitz et al., 1962; Mueller and Horwitz, 1962; Hakanson et al., 1967). Hyperglycemia as a possible side effect of action of the γ-glutamyl derivatives of DOPA and DA therefore required exploration, especially since we have previously shown that after administration of L- γ-glutamyl-DOPA and L-γ -glutamyl-DA, the levels of DA in the pancreas were higher than in any other organ except the kidney (Figure 5). Since catecholamines have been shown both to inhibit insulin release and to stimulate glucagon release (Woods and Porte, 1974), DA released from its precursor may have altered the release of the pancreatic hormones and subsequently plasma glucose levels. Because hyperglycemia can be an undesirable side effect in certain patients requiring treatment with precursors of DA, we compared the effect of L- γ-glutamyl-DOPA and L- γ-glutamyl-DA to that of L-DOPA and DA on plasma glucose in rats.

Doses of 1 nmole/g/30min DA and 50 nmole/g/30min L- γ-glutamyl-DA were chosen since a previous study showed that these are the minimum effective doses for increasing renal plasma flow in rats. L- γ-Glutamyl-DOPA and L-DOPA, 50 nmole/g/30min, were used in order to compare the hyperglycemic effect of these agents with that of L- γ-glutamyl-DA. Table 5 summarizes the results. The hyperglycemia produced by DA (1 nmole/g/30min) and L-DOPA (50 nmole/g/30min) was similar in magnitude and time course. Both agents produced an increase in plasma glucose in the range of 15 - 31 mg/dl. Plasma glucose returned to the basal level 30 min after cessation of the infusion. On the other hand, L- γ-glutamyl-DA (50 nmole/g/30min) raised plasma glucose by 44 and 56 mg/dl at times

Table 5

Effects of DA and its precursors on plasma glucose

Treatment	Dose [nmole/ g/30min]	Plasma glucose concentration[mg/dl] Pre-infusion	15 min	30 min	Post-infusion
DA [6]	1	125±5.0	140± 5.3[b]	142± 4.9[b]	113±26.5
L-DOPA [5]	50	117±9.8	138±11.7[c]	132±12.5[a]	117± 7.0
L-γ-Glutamyl-DA [6]	50	119±7.7	163± 3.8[d]	175±11.4[d]	157±10.2[b]
L-γ-Glutamyl-DOPA [7]	50	111±1.7	114± 2.6	117± 3.3	116± 7.3

Data are mean values ± S.E. The number of animals is given in brackets. The plasma glucose levels measured at 15 and 30 min, and post-infusion were compared to the pre-infusion value with Student's t-test for paired values: [a] $p < 0.05$, [b] $p < 0.025$, [c] $p < 0.01$, [d] $p < 0.005$.

15 and 30 min respectively; moreover even after 30 min following the cessation of drug infusion, plasma glucose remained significantly elevated by 38 mg/dl. In contrast, L-γ -glutamyl-DOPA, 50 nmole/g/30min had no effect on plasma glucose at any time. This dose of L-γ -glutamyl-DOPA was five times higher than the lowest effective dose which increased renal plasma flow. The administration of the lowest effective dose of this compound similarly failed to affect plasma glucose levels.

Concentrations of DA, DOPAC and HVA in pancreas after DA and its precursors

In order to examine whether the hyperglycemic effect of DA and its precursors could be related to the pancreatic content of DA or its metabolites, the levels of DA, DOPAC and HVA in the pancreas were determined by gas chromatography, 30 min after drug infusion. The results summarized in Table 6 show that the concentration of DA or its metabolites in the pancreas was not directly related to the ability of the drug to induce hyperglycemia. Thus the greatest accumulation of DA was produced by L- γ-glutamyl-DOPA, a compound which failed to elevate plasma glucose.

Effect of intravenously injected L- γ-glutamyl-DOPA on renal plasma flow in rats

The findings that L- γ-glutamyl derivatives of DOPA and DA were able to maintain a high level of DA in the kidney suggested that these γ-glutamyl compounds may act as long-acting DA prodrugs. The duration of renal plasma flow elevation was therefore determined after a single intravenous injection of L- γ-glutamyl-DOPA. This compound was chosen

Table 6

Concentrations of DA, DOPAC, HVA in pancreas after the infusion of DA and its precursors

Treatment	Dose [nmole/g/30min]	Concentration[ng/mg tissue] DA	DOPAC	HVA
Control[a] [5]	---	0.1	0.11±0.10	0.09±0.08
DA [4]	1	0.1	0.22±0.16	0.17±0.06
L-DOPA [4]	50	1.34±0.26	12.00±0.95	2.20±0.14
L-γ-Glutamyl-DA [4]	50	0.61±0.04	2.80±0.53	1.11±0.20
L-γ-Glutamyl-DOPA [4]	50	3.07±0.60	14.35±0.87	2.07±0.22

[a]Control animals were infused with normal saline.
Data are mean values ± S.E. The number of experiments is given in brackets.

for the present study because it induced the most selective accumulation of DA in the kidney. It was also capable of increasing RPF with doses that had no effect on either arterial blood pressure or plasma glucose levels. The effect on RPF after a single intravenous injection of L-γ -glutamyl-DOPA (1 mg/kg) is shown in Table 7. Within 20 min after the injection, an increase of 20% in RPF was observed. Although RPF remained elevated for the next 60 min, only the increase during the first collection period was statistically significant.

Oral efficacy of DA prodrugs in rats

The possibility that L-γ -glutamyl-DOPA may be effective as a renal vasodilator after oral administration was studied. Efficacy after oral administration could be expected if the presence of a γ-glutamyl moiety in the DA prodrugs protects the drug from metabolic inactivation in the gastrointestinal tract and during the first pass through the liver. The effects on RPF of orally administered L-γ -glutamyl-DOPA and L-γ -glutamyl-DA were therefore studied and compared to those of DA (Table 8). A significant increase in RPF by these DA precursors was demonstrable only after the dose was increased to 50 mg/kg. At this dose, however, DA itself was capable of elevating RPF when given orally. A possible explanation for the lack of oral efficacy of L-γ -glutamyl derivatives of DOPA and DA is that these γ-glutamyl derivatives are hydrolyzed by γ-glutamyl transpeptidase present in the gastrointestinal tract. In order to retard the rate of such cleavage the D-isomers of these DA precursors were synthesized. It was expected that a decrease in the rate of hydrolysis of the γ-glutamyl derivatives may lead to the entry of a greater portion of the orally administered DA prodrugs into the general

Table 7

Effect of an intravenous bolus injection of L- γ -glutamyl-DOPA on renal plasma flow

Treatment[1 mg/kg]	Renal plasma flow[ml/min/100g]				
	Before drug	0-20 min	20-40 min	40-60 min	60-80 min
L- γ -Glutamyl-DOPA [6]	2.64±0.20	3.16±0.23[a]	2.93±0.22	2.89±0.23	2.98±0.19

Data are mean values ± S.E. The number of animals is given in brackets. Renal plasma flow measured at each of four experimental periods was compared to that before drug with Student's t-test for paired values: [a] $p < 0.025$.

Table 8

Effect of an oral administration of DA and its precursors on renal plasma flow

Period [min][1]	Treatment[50mg/kg]				
	D-γ-Glutamyl-DOPA	D-γ-Glutamyl-DA	L-γ-Glutamyl-DOPA	L-γ-Glutamyl-DA	DA
Control	2.59+0.25 [5]	2.24+0.27 [6]	2.59+0.13 [13]	2.52+0.24 [11]	2.68+0.21 [6]
0- 20	2.60+0.28 [5]	2.20+0.25 [6]	2.49+0.11 [7]	2.21+0.21 [6]	2.54+0.27 [6]
20- 40	2.62+0.14 [5]	2.42+0.32 [6]	2.40+0.15 [7]	2.37+0.27 [6]	2.74+0.18 [6]
40- 60	2.75+0.19 [5]	2.80+0.38[b] [6]	2.71+0.12 [7]	2.30+0.30 [6]	2.98+0.25[a] [6]
60- 80	3.06+0.18[b] [5]	2.86+0.39[a] [6]	2.79+0.19 [7]	2.44+0.21 [6]	3.17+0.36 [6]
80-100	---	---	2.91+0.26[a] [6]	3.20+0.57[a] [5]	2.96+0.30 [6]
100-120	---	---	3.14+0.28[a] [6]	3.19+0.48[a] [5]	3.43+0.30[b] [6]

[1]At zero time, a compound was given to animals by a stomach tube. Data are mean values [expressed in ml/min/100g] ± S.E. The number of animals is given in brackets. Significant difference from control value was calculated using paired t-test: [a]$p<0.05$, [b]$p<0.025$.

circulation. However an increase in RPF was observed only after oral administration of 50 mg/kg of the D- γ-glutamyl derivatives of DA and DOPA. This dose is the same as that required for oral efficacy of the L-isomers. In our rat model, therefore, none of the γ-glutamyl derivatives of DOPA or DA was orally effective at doses lower than free DA.

Renal and cardiovascular effects of L- γ-glutamyl-DOPA in dogs

The studies in rats demonstrated that L- γ-glutamyl-DOPA is capable of increasing RPF in doses that do not affect blood pressure or plasma glucose levels. Despite the minimal extrarenal effects associated with L- γ-glutamyl-DOPA, the possibility that the increased RPF observed after administering this prodrug is due, at least partially, to increased cardiac output could not be completely ruled out. This possibility was examined in the following studies utilizing dogs.

First, the effects on cardiac output and several renal parameters were studied during the intravenous infusion of L- γ-glutamyl-DOPA. The infusion of the prodrug in doses up to 1.5 μmole/kg/min produced no significant increase in cardiac output. The clearance of PAH (C_{PAH}) was increased by 14 to 27% during the prodrug infusion while no significant change in the clearance of creatinine (C_{Cr}) or urine flow was observed. A small decrease in mean arterial blood pressure was seen during the prodrug infusion. In these dogs, therefore, the observed increase in C_{PAH} could not be a secondary effect related to an increase in cardiac output.

In an attempt to further dissociate the direct action on the kidney from possible systemic actions, L- γ-glutamyl-DOPA was infused directly

into a renal artery. Since the kidney from the drug infused side and that from the contralateral side would be affected equally by systemic change, any difference in the responses of these two kidneys must be due to the direct action of the drug on the kidney.

L-γ-Glutamyl-DOPA in doses ranging from 0.08 to 1 μmole/kg/min was infused into the right renal artery. The administration of the prodrug led to a prompt increase in urine flow on the side of its infusion (Table 9). At no time during the prodrug infusion did the urine flow from the infused kidney become less than the uninfused kidney. The maximal difference in urine flow between the two kidneys occurred at about 60 min after initiation of the prodrug infusion. The difference then declined; this was probably due to the systemic recirculation of the prodrug. There was also a moderate increase in Na^+ excretion on the infused side as compared to the contralateral side. However, no consistent difference in either C_{PAH} or C_{Cr} between the infused and uninfused kidneys was observed during L-γ-glutamyl-DOPA infusion.

The increase in RPF in response to the intravenous infusion of L-γ-glutamyl-DOPA in dogs was less than that found in rats. Furthermore, there was no increase in RPF after its infusion directly into a renal artery. One possible explanation for the apparent difference in response to this prodrug in these two species is the method utilized in measuring RPF. For determination of C_{PAH} only a tracer amount of PAH was administered to rats. In dogs, on the other hand, a plasma PAH concentration of approximately 1 mg/dl was maintained. Therefore if the tubular secretion of PAH is inhibited during the L-γ-glutamyl-DOPA infusion, an increase in RPF as measured by C_{PAH} will be obscured.

Table 9

Effect of L- γ -glutamyl-DOPA on urine flow after infusion into the right renal artery in dogs

Urine flow [ml/min]								
Dog#1			Dog#2			Dog#3		
Right	Left	%increase in Right	Right	Left	%increase in Right	Right	Left	%increase in Right
control			control			control		
3.10	3.17	-2	2.00	2.15	-7	2.90	2.80	-4
2.47	2.47	0	3.18	3.58	-11	2.75	2.70	2
1.87	1.87	0	3.60	3.80	-5	2.60	2.55	2
						2.37	2.17	9
0.25 μmole/kg/min			0.25 μmole/kg/min			0.08 μmole/kg/min		
1.75	1.70	3	4.20	3.90	8	2.33	1.93	21
2.00	1.75	14	4.05	3.70	9	2.33	1.93	21
2.58	1.98	30				2.20	1.97	12
0.5 μmole/kg/min			0.5 μmole/kg/min			0.16 μmole/kg/min		
2.55	1.98	29	3.70	3.30	12	2.50	1.90	32
2.13	1.63	31	3.25	3.00	8	2.50	2.13	17
			3.50	3.25	8	2.40	2.17	11
1.0 μmole/kg/min			1.0 μmole/kg/min			0.25 μmole/kg/min		
1.83	1.53	20	3.75	3.40	10	2.33	2.17	7
1.70	1.48	15	3.35	3.25	3	2.40	2.17	11
						2.10	2.10	0

Both control and experimental urine collection periods lasted from 10 to 20 min. Following three or four control urine collection periods, L- γ -glutamyl-DOPA [0.25 or 0.08 μmole/kg/min] was infused into the right renal artery. After two or three collection periods, the dose was increased as indicated. 'Right' and 'Left' refer to the urine flow from the right and left kidney respectively. '% increase in Right' is defined as $\frac{UF_{right} - UF_{left}}{UF_{left}}$ times 100% where UF is urine flow.

There are several reports which suggest that the administration of L- γ-glutamyl-DOPA may indeed interfere with the PAH secretory mechanism. First, it has been reported that the infusion of DA caused a reduction in the renal extraction of PAH in dogs (Meyer et al., 1967). Since L- γ-glutamyl-DOPA generates large amounts of DA in the kidney, such generation might lead to a reduction in the PAH extraction ratio. In addition, L-DOPA, DOPAC and HVA have all been shown to be capable of inhibiting renal tubular transport of PAH (Bierer et al., 1979). L-DOPA is released from L- γ-glutamyl-DOPA by a reaction catalyzed by γ-glutamyl transpeptidase. DOPAC and HVA are major metabolites of DA. Thus the formation of these compounds from this DA prodrug may also contribute to the inhibition of the PAH secretion. Furthermore L- γ-glutamyl-DOPA is an organic acid so that the compound itself may interfere with the PAH secretion by the organic acid transport system. Finally sodium pentobarbital, used to anesthetize the dogs, is known to depress the transport maximum of PAH ($T_{m\ PAH}$) by its direct effect on the renal tubular transport mechanism (Harvey, 1975). In view of these possible interfering factors, the use of the PAH clearance method may not provide an accurate account of changes in the renal blood flow (RBF) induced by L- γ-glutamyl-DOPA. Therefore it became desirable to measure RBF directly by means of electromagnetic flow probes. In addition, the direct measurement allows recording of the instantaneous change in RBF. This could not be done with the clearance method since this method measures the average effective RPF over a period of at least 10 min.

Dogs were prepared as described in 'The Methods'. The responsiveness of our dog preparations was tested by injecting DA (15 μ g/kg i.v.). The results are shown in Table 10. Immediately after the DA injection,

Table 10

Effects of an intravenous injection of DA (15 µg/kg) on renal blood flow and mean arterial blood pressure in dogs

Renal blood flow			Mean arterial blood pressure	Source
Maximal intensity [%change]	Time to peak [min]	Duration [min]	[%change]	
35	2	18	-15	This study (n=2)
31±10	---	8	-16±5	Kyncl et al., 1975

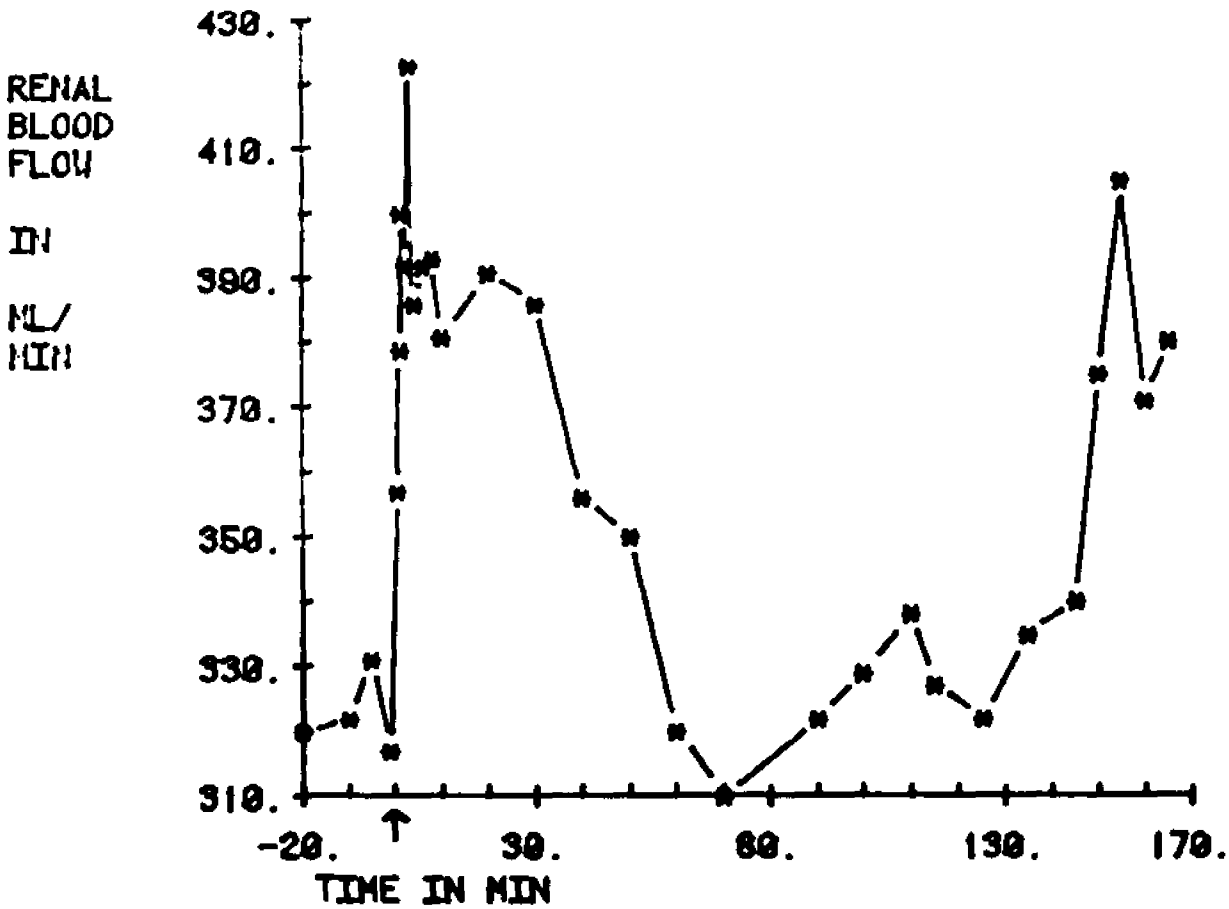
RBF increased by 35%, lasting for 18 min. The increase was accompanied by a decrease in the mean arterial blood pressure by 15%. These findings are similar to those reported by Kyncl et al (1975) (Table 10).

The renal and cardiovascular effects of L- γ-glutamyl-DOPA (2.5 mg/kg) administered as an intravenous bolus injection were studied in three dogs. In each case there was an immediate increase in RBF by 13 to 29% which lasted about 40 min. After this time, RBF returned to the base value except in one dog in which the RBF remained elevated. At about 150 min after the prodrug injection, the RBF began to increase for the second time. In this second phase of RBF elevation, up to a 68% increase in RBF was recorded. The recording of RBF from one animal is shown as an example of the response to the intravenous injection of L- γ-glutamyl-DOPA (Figure 6). No increase in mean arterial blood pressure or heart rate was recorded at any time during these experiments.

If the release of DA from L- γ-glutamyl-DOPA in the kidney is responsible for the observed increase in RBF, then the presence of DA in urine might be expected during such an increase. The urine samples obtained at various times during the experiments were therefore analyzed for DA. The excretion of free and total (free + conjugated) DA before and after the injection of L- γ-glutamyl-DOPA (2.5 mg/kg) is shown in Figure 7. The results presented in Figure 7 and Figure 6 were obtained from the same dog. As shown, there was a surge of DA excretion which peaked at about 40 min following the prodrug injection. After this time, the DA excretion declined but it nevertheless remained above 100 ng/min for the rest of the experimental period. No detectable amount of free DA was measured prior to the injection of L- γ-glutamyl-DOPA. The results also demonstrate that a considerable portion of DA is excreted

Figure 6

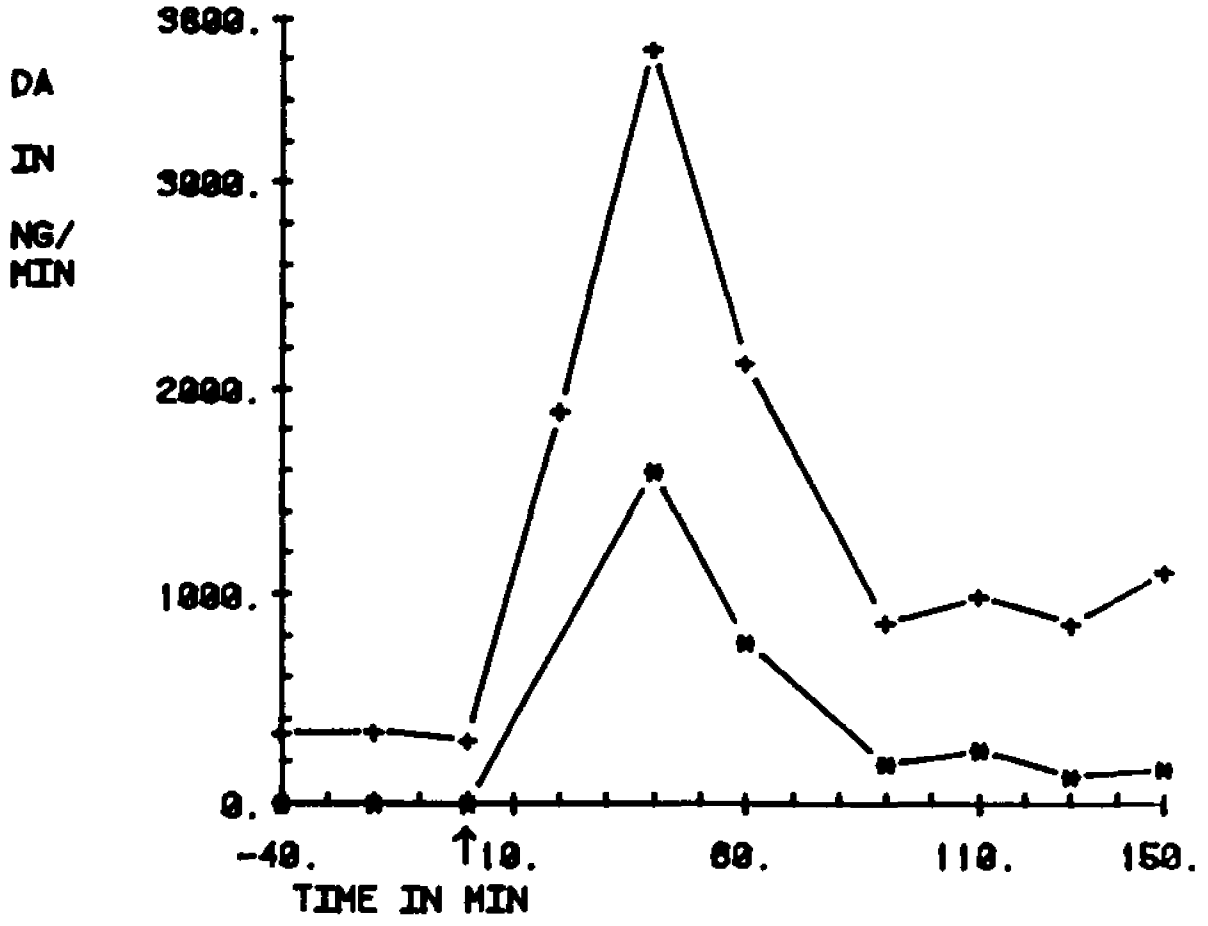
Effect of L- γ-glutamyl-DOPA on renal blood flow in the dog



-*- Renal blood flow
↑ indicates the injection of L-gamma-glutamyl DOPA[2.5mg/kg]

Figure 7

Urinary excretion of free and total dopamine in the dog



as its conjugated metabolite in urine. As a comparison, free DA excreted after the injection of DA (15 μg/kg) was also determined. The maximum excretion of free DA was approximately 300 ng/min and occurred at about 5 min after the injection. The DA excretion then declined rapidly.

B. Prodrugs of sulfamethoxazole

Rate of release of SM from its glutamyl derivatives in vitro

The release of SM from its γ-glutamyl derivatives is catalyzed by γ-glutamyl transpeptidase and that from L-α -glutamyl-SM by aminopeptidase A. Therefore the results of the measurement of SM release from γ-glutamyl and α-glutamyl derivatives of SM provide estimates for the γ -glutamyl transpeptidase and aminopeptidase A activities respectively.

The rate of SM release from γ- and α-glutamyl derivatives of SM by mouse tissue homogenates is summarized in Table 11. Among the tissues examined the kidney exhibited the fastest release of SM. There were, however, great differences in rates among the various derivatives. L- γ-Glutamyl-SM was cleaved most rapidly reflecting the high activity of γ-glutamyl transpeptidase in the kidney. The rate of release from D- γ-glutamyl-SM was several times slower, a finding consistent with earlier observations, which showed that D- γ-glutamyl derivatives are much poorer substrates of γ-glutamyl transpeptidase than the respective L-isomers (Orlowski and Meister, 1965). Among other tissues only the pancreas and small intestine show appreciable release of SM from the γ-glutamyl derivatives; the release in other tissues was slower by a factor of 300 to 1000 than in the kidney. The hydrolysis of L- α-glutamyl-SM although slower than that of the L- γ-glutamyl derivative proceeded nevertheless at a relatively rapid rate in the kidney. Aminopeptidase A, the enzyme responsible for this reaction is also most active in the kidney, however this enzyme seems to be much less kidney-specific than γ-glutamyl transpeptidase. With the exception of the kidney and pancreas its activity in all other tissues was much higher than the

Table 11

Rate of release of SM from its glutamyl derivatives by several organ homogenates

	L- γ -Glutamyl-SM		D- γ -Glutamyl-SM		L- α -Glutamyl-SM	
Kidney	71.5	[100]	11.9	[100]	32.5	[100]
Pancreas	10.4	[15]	2.49	[21]	1.04	[3]
Small Intestine	1.25	[2]	0.23	[2]	3.86	[12]
Liver	0.02	[<0.1]	0.002	[<0.1]	3.51	[11]
Spleen	0.126	[0.2]	0.008	[<0.1]	1.74	[5]
Lung	0.094	[0.1]	0.026	[0.2]	16	[49]
Heart	0.005	[<0.1]	0.003	[<0.1]	1.45	[4]
Brain	0.25	[0.3]	0.05	[0.4]	1.25	[4]

Results are expressed as nmoles of SM released per mg protein per min. Data are mean values of 2-4 determinations. Numbers in brackets represent relative rates, with the activity in kidney homogenates arbitrarily set as 100.

activity of γ-glutamyl transpeptidase.

Tissue concentration of SM after administration of its glutamyl derivatives in vivo

The kidney selective release of SM from its glutamyl derivatives in vitro prompted studies on the tissue distribution of SM after administration of these compounds in vivo. The concentration of SM in various tissues were determined in mice after SM and its glutamyl derivatives (0.5 μmole/g i.p.). The animals were killed 20 min after the drug injection since the peak concentration of SM in the kidney was attained at this time. The results are summarized in Table 12. The concentration in the kidney was higher than that in any of the tissues studied, nevertheless with the exception of the brain considerable accumulation of SM was found in all tissues. In absolute terms the concentration of SM in the kidney after L- γ-glutamyl-SM was similar to that obtained after free SM. The concentrations after the other two glutamyl derivatives were significantly lower than that after free SM. In relative terms, there was only a small preferential accumulation of SM in the kidney with respect to other tissues after the γ-glutamyl prodrugs compared with that after free SM. Although this kidney-specific accumulation of SM was greater after D- γ-glutamyl-SM than after the L-isomer, it was accompanied by a considerable reduction of the absolute concentration of SM in the kidney. No kidney-specific accumulation of SM was observed after L- α-glutamyl-SM. Indeed the distribution of SM among different tissues after this derivative was even more uniform than after free SM.

Table 12

Concentrations of SM in several tissues after intraperitoneal administration of SM and its glutamyl derivatives

	SM	L-γ-Glutamyl-SM	D-γ-Glutamyl-SM	L-α-Glutamyl-SM
Kidney	108±9 [100]	97±8 [100]	72±4[b] [100]	66±5[c] [100]
Pancreas	51±4 [47]	66±4 [69]	43±7 [60]	46±8 [69]
Small Intestine	54±2 [50]	35±2[e] [36]	18±1[e] [25]	45±3[a] [69]
Liver	78±4 [73]	55±4[c] [57]	34±2[e] [48]	55±2[d] [85]
Spleen	51±3 [47]	32±4[c] [33]	18±1[e] [25]	43±3 [67]
Lung	58±3 [55]	47±3[a] [50]	24±1[e] [33]	42±2[d] [64]
Heart	57±3 [54]	47±4 [49]	24±2[e] [33]	38±1[d] [59]
Brain	11±1 [10]	5±1 [5]	3±0.3[d] [4]	4±1[c] [7]

The dose of each drug was 0.5 μmole/g. Data are mean concentrations (expressed in μg/g tissue) ± S.E. obtained in 4 to 5 experiments. Values in brackets represent relative concentrations, with those in the kidney arbitrarily set as 100. Statistical significance of concentration differences after glutamyl derivatives of SM are compared with those after administration of SM using the t-test: [a] $p<0.05$, [b] $p<0.025$, [c] $p<0.01$, [d] $p<0.005$, [e] $p<0.001$.

Rate of release of SM from its N-acyl-L-γ-glutamyl derivatives in vitro

Since only a small preferential accumulation of SM was found in the kidney after administration of γ-glutamyl derivatives of SM, a chemical modification of the prodrugs became desirable so as to obtain more kidney-specific derivatives. Acylation of L-γ-glutamyl-SM was thus carried out in order to make the release of SM dependent on the actions of both acylase and γ-glutamyl transpeptidase, two kidney-specific enzymes. Several N-acyl derivatives of γ-glutamyl-SM including N-acetyl-L-γ-glutamyl-SM, N-chloroacetyl-L-γ-glutamyl-SM and N-butyryl-L-γ-glutamyl-SM were synthesized. Glycyl-L-γ-glutamyl-SM was also synthesized by ammonolysis of N-chloroacetyl-L-γ-glutamyl-SM.

Enzymatic release of SM from the N-acyl-L-γ-glutamyl derivatives was studied as described before with glutamyl derivatives of SM. The release of SM from the N-acyl-γ-glutamyl derivatives proceeds in a two step reaction catalyzed in sequence by acylase and γ-glutamyl transpeptidase. With N-acetyl-L-γ-glutamyl-SM, the reaction proceeds as follows (reaction 8 and 9).

N-Acetyl-L- γ-glutamyl-SM

$$\xrightarrow{\text{acylase}}$$

acetate + L-γ-glutamyl-SM (8)

L- γ-Glutamyl-SM + H_2O

$$\xrightarrow{\gamma\text{-glutamyl transpeptidase}}$$

L-glutamate + SM (9)

Enzymatic hydrolysis of glycyl-L- γ-glutamyl-SM also requires two steps, the first of which is catalyzed by one or more aminopeptidases and the second by γ-glutamyl transpeptidase. Thus the rates of SM release from N-acyl- γ-glutamyl-SM and glycyl- γ-glutamyl-SM measure the release caused by the presence of deacylating enzyme and γ-glutamyl transpeptidase in a given tissue homogenate.

The rate of release of SM from its N-acyl-L- γ-glutamyl derivatives by several organ homogenates is summarized in Table 13. Aside from glycyl-L- γ-glutamyl-SM which was relatively rapidly hydrolyzed in the kidney by the combined action of several aminopeptidases and γ-glutamyl transpeptidase, other N-acyl- γ-glutamyl derivatives of SM were hydrolyzed at a small fraction of the rate observed with either the γ- or α-glutamyl derivatives of SM (Table 11). The hydrolysis of these N-acyl-L- γ-glutamyl derivatives shows high kidney specificity, proceeding only extremely slowly in other tissues. The N-acetyl derivative of γ-glutamyl-SM was hydrolyzed almost exclusively in the kidney. The rates of hydrolysis of the N-butyryl and N-chloroacetyl derivatives were several times higher than that of the N-acetyl derivatives, nevertheless these derivatives were hydrolyzed in almost all tissues at a much slower rate than the glutamyl derivatives of SM.

Rate of deacylation of N-acyl-L- γ-glutamyl-SM in vitro

Under the assay conditions used previously to measure the release of SM from its N-acyl-L- γ-glutamyl derivatives, either deacylating enzyme or γ-glutamyl transpeptidase could have been rate-limiting. In the present study, the release of SM from these compounds was measured in the presence of excess sheep kidney γ-glutamyl transpeptidase.

Table 13

Rate of release of SM from its N-acyl-L-γ-glutamyl derivatives by several organ homogenates

	N-acetyl-L-γ-glutamyl-SM	N-butyryl-L-γ-glutamyl-SM	N-chloroacetyl-L-γ-glutamyl-SM	Glycyl L-γ-glutamyl-SM
Kidney	0.46 [100]	1.76 [100]	2.79 [100]	42.0 [100]
Pancreas	0.001 [<0.2]	0.19 [11]	0.35 [13]	3.8 [9]
Small Intestine	0.001 [<0.2]	0.11 [6]	0.17 [6]	0.77 [2]
Liver	0.002 [0.4]	0.014 [0.8]	0.051 [2]	0.21 [0.5]
Spleen	0.001 [<0.2]	0.024 [1]	0.065 [2]	0.39 [0.9]
Lung	0.001 [<0.2]	0.016 [0.9]	0.034 [1]	0.41 [1]
Heart	0.001 [<0.2]	0.003 [0.2]	0.005 [0.2]	0.055 [0.1]
Brain	0.001 [<0.2]	0.067 [4]	0.055 [2]	0.27 [0.6]

Results are expressed as nmole of SM released per mg protein per min. Data are mean values of 2-4 determinations. Numbers in brackets represent relative rates, with the activity in kidney homogenates arbitrarily set as 100.

Under these conditions, the reaction catalyzed by deacylating enzymes becomes rate-limiting therefore the rate of release of SM measures the activity of deacylating enzymes.

The results summarized in Table 14 show that the most rapidly hydrolyzed derivative is glycyl-L- γ-glutamyl-SM. The hydrolysis of this compound although most rapid in the kidney nevertheless proceeded at a high rate in all other tissues. This finding suggests that amino-peptidases capable of hydrolysing the glycyl- γ-glutamyl bond are widely distributed, and exhibit high activity in all tissues. By contrast deacylation of the other N-acyl-L- γ-glutamyl derivatives of SM proceeded by two orders of magnitude slower than that of the glycyl derivative. Among the three compounds tested the release of γ-glutamyl-SM was slowest from its N-acetyl derivative, however, the deacylation of this derivative showed the highest kidney-specificity since its deacylation in other tissues was extremely slow. Higher deacylation rates were obtained with the N-butyryl and N-chloroacetyl derivatives, with the kidney again showing the highest rate. The deacylation rate of N-acyl- γ-glutamyl derivatives of SM in various tissues reflects the activity of deacylating enzymes. The distribution of activity of these enzymes seems to be more uniform than that of γ-glutamyl transpeptidase, nevertheless a considerable kidney specificity is clearly noticeable.

Tissue concentrations of SM after administration of its N-acyl-L-γ -glutamyl derivatives in vivo

Studies of the SM release from N-acyl-L- γ-glutamyl-SM in vitro have shown that acylation of γ-glutamyl derivatives of SM significantly slows the rate of release of the SM moiety from these derivatives. In

Table 14

Enzymatic release of γ-glutamyl-SM from its N-acyl derivatives

	N-acetyl-L-γ-glutamyl-SM	N-butyryl-L-γ-glutamyl-SM	N-chloroacetyl-L-γ-glutamyl-SM	Glycyl-L-γ-glutamyl-SM
Kidney	0.74 [100]	1.85 [100]	3.14 [100]	239 [100]
Pancreas	0.031 [4]	0.24 [13]	0.24 [8]	24.2 [10]
Small Intestine	0.002 [0.3]	0.28 [15]	0.20 [6]	73.5 [31]
Liver	0.045 [6]	0.40 [22]	1.70 [54]	69.7 [29]
Spleen	<0.001 [<0.1]	0.38 [21]	0.36 [11]	54.6 [23]
Lung	<0.001 [<0.1]	0.24 [13]	0.22 [7]	38.4 [16]
Heart	<0.001 [<0.1]	0.049 [3]	0.042 [1]	19.7 [8]
Brain	<0.001 [<0.1]	0.13 [7]	0.11 [4]	40.5 [17]

Activity was measured in a coupled enzyme assay in the presence of excess γ-glutamyl transpeptidase. Data are expressed in nmole/mg protein/min and represent mean values of 2-4 determinations. Numbers in brackets represent relative activities with those in the kidney arbitrarily set as 100.

addition it makes the reaction dependent on the action of deacylating enzymes and γ-glutamyl transpeptidase, both having significant kidney-specificity. These findings encouraged us to examine the tissue distribution of SM after N-acyl-L- γ-glutamyl derivatives of SM in vivo.

In contrast to the observations made earlier with the glutamyl derivatives of SM, administration of the N-acetyl, N-butyryl and N-chloroacetyl derivatives of γ-glutamyl-SM (0.25 μ mole/g i.p.) resulted in a highly specific accumulation of SM in the kidney (Table 15). Among the three N-acyl derivatives the results with N-chloroacetyl-L- γ-glutamyl-SM were the most striking (Figure 8). A dose of this prodrug equimolar with SM resulted in 2.2 times higher concentration of SM in the mouse kidney. At the same time the concentration of SM in other tissues was only a small fraction of that observed after free SM. As a result the concentration of SM in these tissues was only about 2% of that in the kidney with somewhat higher concentrations occurring in the liver. Administration of N-chloroacetyl-L-γ -glutamyl-SM (0.25 μmole/g i.p.) to rats and guinea pigs also led to a highly kidney selective accumulation of SM (Table 16). The kidney concentration of SM after the prodrug was three times higher in guinea pigs and almost two times higher in rats than that after an equimolar dose of SM. As in mice, only small amounts of SM were found in the liver and pancreas after this prodrug.

Similar results were obtained after N-acetyl-L-γ -glutamyl-SM in mice (Table 15, Figure 8). The concentration of SM in the kidney after this prodrug was about 50% higher than that after an equimolar concentration of free SM, with only small concentrations of SM present in other tissues studied. A kidney-specific accumulation of SM was also observed

Table 15

Concentrations of SM after intraperitoneal administration of SM and its N-acyl-γ-glutamyl derivatives

	SM	N-Acetyl-L-γ-glutamyl-SM	N-Chloroacetyl-L-γ-glutamyl-SM	N-Butyryl-L-γ-glutamyl-SM	Glycyl-L-γ-glutamyl-SM
Kidney	62+4 [100]	91+9[a] [100]	137+11[e] [100]	33+6 [100]	37+5[c] [100]
Pancreas	29+3 [47]	5+1[e] [5]	3+1[e] [2]	1+0.1[e] [4]	20+4 [54]
Small Intestine	19+2 [31]	4+1[e] [4]	3+0.3[e] [2]	1+0.1[e] [4]	12+1[b] [32]
Liver	43+5 [69]	11+1[e] [12]	9+0.4[e] [7]	7+0.1[e] [23]	19+1[d] [51]
Spleen	23+6 [37]	4+1[c] [4]	3+0.2[b] [2]	1+0.3[e] [2]	11+1 [30]
Lung	30+1 [48]	4+1[e] [4]	4+1[e] [3]	1+1[e] [4]	14+1[e] [38]
Heart	37+3 [60]	5+1[e] [5]	3+0.4[e] [2]	0.4+0.3[e] [1]	14+2[e] [38]
Brain	5+2 [8]	2+1 [2]	0.3+0.1[b] [0.2]	0	2+0.1 [5]

The dose of each drug was 0.25 μmole/g with the exception of N-butyryl-L-γ-glutamyl-SM which was given at a dose of 0.5 μmole/g. Statistical differences after this derivative were compared with SM levels given in Table 12. Other explanations are the same as in legend to Table 12.

Figure 8

Tissue distribution of sulfamethoxazole after N-chloroacetyl-L-γ-glutamyl-sulfamethoxazole, N-acetyl-L-γ-glutamyl-sulfamethoxazole and sulfamethoxazole(0.25 μmole/g i.p.)

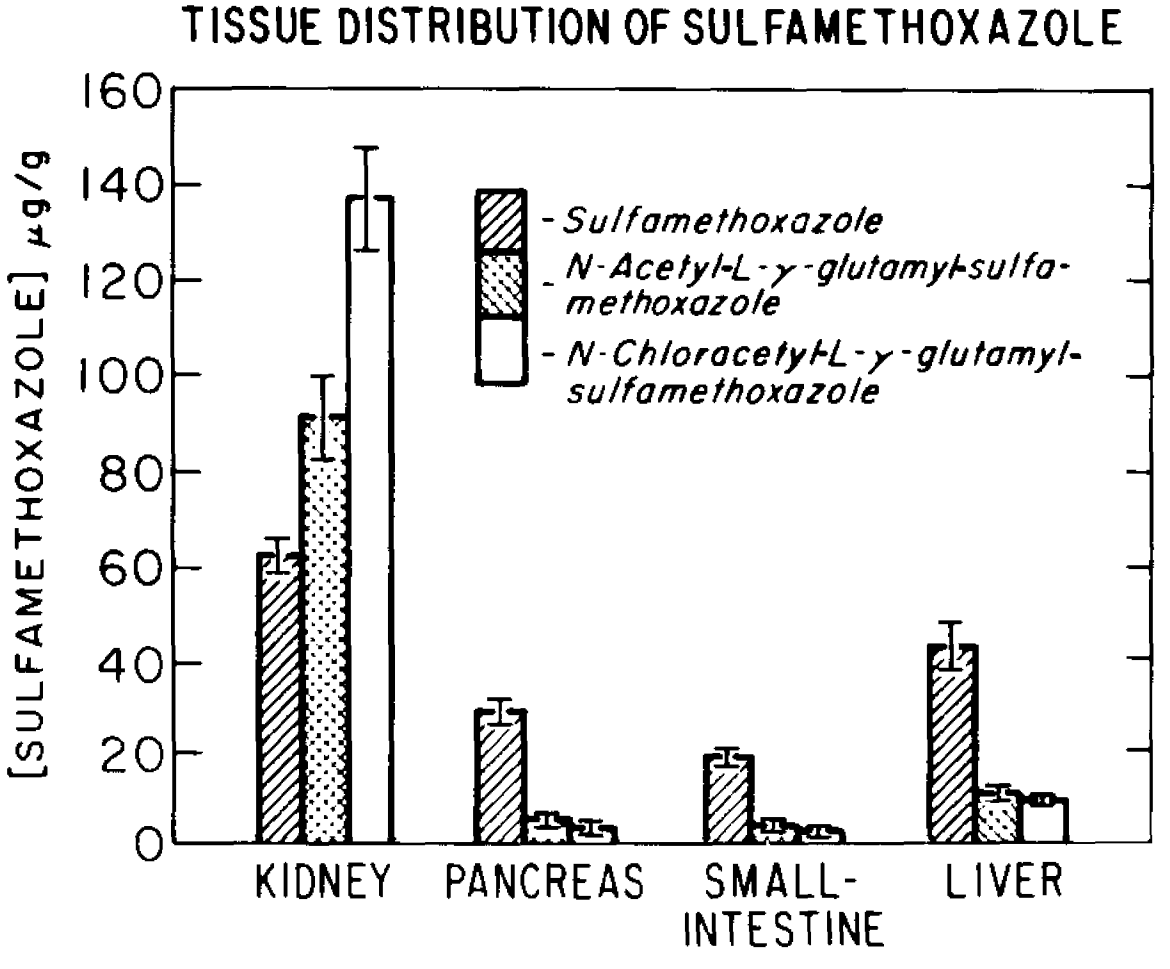

TISSUE DISTRIBUTION OF SULFAMETHOXAZOLE
[SULFAMETHOXAZOLE] µg/g
160
140
120
100
80
60
40
20
0
- Sulfamethoxazole
- N-Acetyl-L-γ-glutamyl-sulfamethoxazole
- N-Chloracetyl-L-γ-glutamyl-sulfamethoxazole
KIDNEY
PANCREAS
SMALL-INTESTINE
LIVER

Table 16

Concentrations of SM in tissues after SM and N-chloroacetyl-L-γ -glutamyl-SM in rats and guinea pigs

	Guinea pig		Rat	
	SM	N-Chloroacetyl-L- γ -glutamyl-SM	SM	N-Chloroacetyl-L- γ -glutamyl-SM
Kidney	40±4	122±18[b]	42±2	78±7[b]
Pancreas	88±18	5±1[b]	54±6	11±1[b]
Liver	45±5	10±1[a]	35±2	10±1[b]

The dose of each drug was 0.25 μmole/g. Animals were killed 20 min after the drug injection. Data are mean values (expressed in μg/g tissue) ± S.E. obtained from 5 to 7 animals. Statistical significance of concentration differences after N-chloroacetyl-L- γ -glutamyl-SM are compared to those after SM using the t-test: [a]$p<0.005$, [b]$p<0.001$.

after administration of N-butyryl-L-γ-glutamyl-SM, however, for as yet unknown reasons the absolute concentration of SM in the kidney after this prodrug was only 50% of that after SM given at a dose only half of that of the prodrug. No kidney-specific accumulation was observed after administration of glycyl-L-γ-glutamyl-SM.

Plasma concentration of SM after N-chloroacetyl-L-γ-glutamyl-SM

After demonstration of the high organ-selective accumulation of SM in the kidney following administration of N-chloroacetyl-L-γ-glutamyl-SM, it was of interest to determine the plasma concentration of free SM after this prodrug. The plasma levels of SM 20 min after intraperitoneal injection of N-chloroacetyl-L-γ-glutamyl-SM (0.25 μmole/g) were measured in rats and guinea pigs. Control animals were injected with an equivalent dose of free SM (Table 17). After the administration of the prodrug, only a small amount of free SM was detected in plasma of these animals. The plasma levels of SM after the prodrug were 8 and 12% of those found after SM in guinea pigs and rats respectively.

Time course of tissue accumulation of SM after N-chloroacetyl and N-acetyl derivatives of γ-glutamyl-SM

Since the administration of the N-chloroacetyl and N-acetyl derivatives of γ-glutamyl-SM resulted in a highly selective accumulation of SM in the kidney, it was important to determine the duration of this selectivity. Thus the tissue distribution of SM was investigated as a function of time after intraperitoneal administration of N-acetyl-L-γ-glutamyl-SM (0.25 μmole/g) and N-chloroacetyl-L-γ-glutamyl-SM (0.25 μmole/g) into mice (Table 18). The kidney specific accumulation of SM

Table 17

Plasma concentrations of SM after SM and N-chloroacetyl-L-γ -glutamyl-SM in rats and guinea pigs

Treatment	SM concentration	
	Guinea pig	Rat
N-Chloroacetyl-L- γ-glutamyl-SM	8 ± 1[a]	15 ± 1[a]
SM	104 ± 10	129 ± 6

The dose of each drug was 0.25 μ mole/g. Data are mean values (μg/ml of plasma) ± S.E. obtained from 5 to 7 animals. Statistical significance of concentration differences after N-chloroacetyl-L- γ -glutamyl-SM are compared to those after SM using the t-test: [a] $p<0.001$.

Table 18

Concentrations of SM one and two hours after administration of SM and its N-acyl-L- γ -glutamyl derivatives

	after 1 hour					
	SM		N-Acetyl-L- γ-glutamyl-SM		N-Chloroacetyl-L- γ-glutamyl-SM	
Kidney	51±5	[100]	65±7	[100]	54±6	[100]
Pancreas	22±2	[43]	7±1	[11]	6±1	[11]
Small Intestine	18±2	[35]	7±1	[11]	5±1	[9]
Liver	33±2	[65]	14±1	[22]	12±1	[22]
Spleen	20±2	[39]	7±1	[11]	5±1	[9]
Lung	28±2	[55]	7±1	[11]	6±0.3	[11]
Heart	25±2	[49]	8±2	[12]	7±1	[13]
Brain	5±1	[10]	2±1	[3]	1±0.2	[2]

The dose of each drug was 0.25 μmole/g. Other explanations are the same as given in legend to Table 12.

after 2 hours					
SM		N-Acetyl-L-γ-glutamyl-SM		N-Chloroacetyl-L-γ-glutamyl-SM	
25±4	[100]	14±1	[100]	22±2	[100]
11±2	[44]	7±1	[50]	7±1	[32]
10±2	[40]	5±1	[36]	7±2	[32]
23±4	[92]	12±2	[86]	12±1	[55]
11±2	[44]	6±1	[43]	6±1	[27]
14±3	[56]	7±1	[50]	8±1	[36]
18±3	[72]	7±1	[50]	8±1	[36]
4±1	[16]	2±0.3	[14]	3±1	[14]

was maintained 1 hour after administration of the prodrugs. Absolute concentrations of SM in the kidney, however, declined considerably by this time, so that the degree of accumulation was not significantly different from that after free SM. A further decline in SM concentrations in the kidney was observed after 2 hours. The concentrations of SM in other tissues after the two prodrugs remained at about the same levels after 1 and 2 hours. Because, however, of the marked decline in kidney levels of SM after 2 hours, there was a decline in the relative kidney-specific accumulation of SM at that time. It is, however, of interest to note that even 2 hours after administration of the prodrugs all tissue concentrations of SM other than those in the kidney remained distinctly lower than those after free SM.

Possible relationship between the rate of SM release from N-chloroacetyl-L-γ-glutamyl-SM by kidney homogenates and the kidney concentration of SM after N-chloroacetyl-L-γ-glutamyl-SM

Previous studies showed that there are differences in the extent of SM accumulation in the kidney among different species after administration of N-chloroacetyl-L-γ-glutamyl-SM. It was found that while the concentrations of SM in mouse and guinea pig kidney after the prodrug were similar, considerably less SM was found in the rat kidney. The kidney concentration of SM in rats was only 57% of that found in mice. A possible explanation for these differences is that the rates of SM release from the prodrug in the kidney differ among different species. In Table 19, the rates of SM release by kidney homogenates of mouse, rat and guinea pig are listed together with the kidney concentrations of SM after N-chloroacetyl-L-γ-glutamyl-SM (0.25 μ mole/g i.p.) in these

Table 19

Rate of SM release from N-chloroacetyl-L- γ -glutamyl-SM by kidney homogenates and kidney concentrations of SM after N-chloroacetyl-L-γ - glutamyl-SM in mice, guinea pigs and rats

	Rate of SM release from N-chloroacetyl-L- γ - glutamyl-SM by kidney homogenate [nmole/min/mg protein]	Concentration of SM in the kidney after N-chloroacetyl-L- γ - glutamyl-SM[a] [μ g/g]
Mice	2.79[c]	137 ± 11[b]
Guinea pigs	3.83	122 ± 18
Rats	0.85	78 ± 7

[a] N-Chloroacetyl-L- γ -glutamyl-SM (0.25 μmole/g i.p.) was administered.
[b] Data are mean values ± S.E. obtained from 5 to 7 animals.
[c] Data are mean values obtained from 3 animals.

animals. The slowest rate of SM release from the prodrug was found in rats. Compared to the rat kidney homogenate, the rates of SM release by guinea pig and mouse kidney homogenates were more than 3 to 4 times faster. It therefore seems that the slow rate of SM release in the rat kidney may at least partially be responsible for the low accumulation of SM in the kidney after administration of N-chloroacetyl-L- γ-glutamyl-SM.

Effect of sodium pentobarbital on kidney concentration of SM after N-acetyl-L- γ-glutamyl-SM

In the course of these studies, it was noted that the concentrations of SM in the kidney of sodium pentobarbital-anesthetized rats after administering N-acyl-L- γ-glutamyl derivatives of SM were considerably lower than in non-anesthetized rats. The possible effect of sodium pentobarbital on the kidney concentration of SM was therefore studied. Rats were pretreated with sodium pentobarbital (50 mg/kg i.p.) in 0.9% saline. After 10 min, N-acetyl-L- γ-glutamyl-SM (0.5 μmole/g i.p.) was injected. In control animals, saline solutions were injected prior to the prodrug administration. Sixty minutes after the injection of N-acetyl-L- γ-glutamyl-SM, the animals were decapitated and the kidneys were removed. As shown in Table 20, there was an average reduction in SM concentration by 48% in the kidneys of rats pretreated with sodium pentobarbital.

Urinary excretion of SM after SM and its N-acyl-L- γ-glutamyl derivatives in guinea pigs and rats

Since the N-acyl-L- γ-glutamyl derivatives of SM were found to promote a kidney selective accumulation of SM, it was also of interest

Table 20

Effect of sodium pentobarbital on kidney concentration of SM after N-acetyl-L- γ -glutamyl-SM

Treatment		SM concentration[μg/g]
N-acetyl-L- γ -glutamyl-SM alone	[8]	29 $\pm$ 3
Sodium pentobarbital pretreatment followed by N-acetyl-L- γ-glutamyl-SM	[9]	15 $\pm$ 3[a]

N-Acetyl-L- γ-glutamyl-SM (0.5 μg/g i.p.) was administered.
Data are mean values $\pm$ S.E. The number of animals is given in brackets.
Statistical significance of concentration difference after administration of N-acetyl-L- γ-glutamyl-SM alone and after the prodrug following the sodium pentobarbital pretreatment was determined using the t-test :
[a] $p<0.005$.

to investigate the urinary excretion of SM after these prodrugs. The urinary excretion of SM after intraperitoneal injection of N-acetyl-L-γ-glutamyl-SM (0.25 μmole/g), N-butyryl-L- γ-glutamyl-SM (0.25 μmole/g) and N-chloroacetyl-L- γ-glutamyl-SM (0.25 μmole/g) in guinea pigs and rats is shown in Table 21. During the first 24 hours, the amount of SM excreted after all the prodrugs was about 60% of that found after an equivalent dose of SM in guinea pigs. In rats, considerably less SM was excreted in the urine after the prodrugs (15 - 20% of the amount after free SM). By the second 24 hours, the urinary excretion of SM was similar regardless of previous drug treatment.

Table 21

Urinary excretion of SM after SM and its N-acyl-L- γ -glutamyl derivatives in rats and guinea pigs

Treatment [0.25 μmole/g]	Guinea pig		Rat	
	0-24 hr	24-48 hr	0-24 hr	24-48 hr
SM	13.8±0.9	5.7±0.8	25.3±1.6	2.8±0.4
N-Acetyl-L- γ -glutamyl-SM	8.5±0.2	6.9±0.9	3.6±0.4	3.0±0.6
N-Butyryl-L- γ -glutamyl-SM	7.5±0.6	5.3±0.6	5.2±1.2	4.3±0.6
N-Chloroacetyl-L- γ -glutamyl-SM	8.3±1.0	4.9±0.2	4.5±0.5	3.3±0.5

Data are mean values obtained from 4 to 5 animals (expressed in μmole/24 hr) ± S.E.

DISCUSSION

One of the goals of a rational therapy is to increase drug specificity by directing drug action toward a desired organ or tissue with the possible exclusion of other sites. The work presented here explores the possibility of utilizing the prodrug approach to promote the organ selective delivery of a drug. As models of organ selective prodrugs, we synthesized γ-glutamyl derivatives of DA, DOPA and SM. The release of the active drug from these prodrugs requires the action of γ-glutamyl transpeptidase, a kidney specific enzyme, either alone or in conjunction with aromatic L-amino acid decarboxylase or acylase. In addition, a prodrug requiring the action of aminopeptidase A was synthesized. Since these enzymes exhibit greater activity in the kidney compared to other tissues, the release of DA and SM from the prodrugs was expected to take place preferentially in the kidney.

The release of DA from γ-glutamyl-DA involves a single enzymatic reaction catalyzed by γ-glutamyl transpeptidase. The sites of DA generation from this prodrug in the kidney are likely to be in the proximal tubules, loops of Henle and/or medullary vascular bundles where the enzyme has been localized (Albert et al., 1961,1964; Glenner et al., 1962_a). By contrast conversion of γ-glutamyl-DOPA to DA requires the action of two enzymes, γ-glutamyl transpeptidase and aromatic L-amino acid decarboxylase. The localization of the decarboxylase and its relationship to γ-glutamyl transpeptidase is thus an additional factor in the organ specificity of γ-glutamyl-DOPA.

Despite the difference in the mechanisms of DA release from γ-glutamyl-DOPA and γ-glutamyl-DA, the administration of these γ-glutamyl derivatives to mice resulted in a similar selective

accumulation of DA in the kidney. Such selective accumulation of DA from its prodrugs is consistent with previous findings, showing that the metabolism of γ-glutamyl derivatives of amino acids and peptides occurs preferentially in the kidney (Orlowski and Wilk, 1976,1978$_b$). Furthermore the results show that both γ-glutamyl transpeptidase and aromatic L-amino acid decarboxylase are functionally active in the kidney. The kidney concentration of DA after injection of L-γ -glutamyl-DOPA and L- γ-glutamyl-DA was five and three times greater, respectively, than that after an equimolar dose of L-DOPA. This finding indicates that the presence of a γ -glutamyl moiety in these prodrugs promotes not only greater organ selectivity but also delivery of a larger amount of DA to the kidney. The observation that a higher kidney concentration of DA was attained after administration of L-γ -glutamyl-DOPA compared to that after L- γ-glutamyl-DA may be related to the difference in the rate of cleavage of the γ -glutamyl bond in these two derivatives. Thus γ-glutamyl transpeptidase is known to cleave a γ-glutamyl bond in γ -glutamyl amino acids (as in γ -glutamyl-DOPA) faster than γ-glutamyl amides in which the amino component represents a primary aliphatic amine (such as the ethylamine moiety of DA). Another possible explanation is that γ -glutamyl-DOPA is more readily taken up by the kidney than γ -glutamyl-DA.

Since DA has been shown to induce renal vasodilation by interacting with DA receptors in renal vessels (Goldberg, 1972), DA released from its precursors may also cause renal vasodilation and an increase in RBF. The effect on RPF by DA and its precursors was therefore determined in rats under ether anesthesia. The lowest effective dose of L-γ -glutamyl-DOPA was 10 mmole/g/30 min. By contrast, an equimolar dose of either

L-DOPA or L- γ-glutamyl-DA was without effect. The observation that of the three DA precursors, L- γ-glutamyl-DOPA required the smallest dose to elevate RPF is consistent with previous findings that the highest DA concentration was attained in the kidney after administration of L- γ-glutamyl-DOPA. The ability of L-γ -glutamyl-DA to elevate RPF confirms the increase in RBF seen after its injection to dogs (Kyncl et al., 1975, 1976). A significant increase in RPF was also observed after administration of L-DOPA. Such an increase in RBF induced by L-DOPA has been reported in man by Finlay et al. (1971). Comparison of ability of L- γ-glutamyl-DA and L-DOPA to increase RPF showed that a larger dose of L- γ-glutamyl-DA was required to produce a significant increase in RPF. This was not expected since administration of L- γ-glutamyl-DA has been shown previously to result in a greater kidney concentration of DA than an equimolar dose of L-DOPA. It is possible that this apparent discrepancy is due to a difference in the intra-renal site of DA release from γ-glutamyl-DA and L-DOPA.

The kidney selective accumulation of DA observed after injection of L- γ-glutamyl derivatives of DOPA and DA prompted us to determine if such selectivity is reflected in their ability to induce specific renal vasodilation without causing systemic side effects. Since DA is known to elevate blood pressure by activating peripheral α-adrenergic receptors, blood pressure elevation produced by DA and its precursors was used as a measure of a systemic side effect. The ratio of dose required to elevate systemic blood pressure to that required to elevate RPF was taken as an index of renal specificity. Using this ratio, L- γ-glutamyl-DOPA and L- γ-glutamyl-DA were shown to have a greater kidney-specific action as compared to L-DOPA or DA. Such a kidney-

specific action of the γ-glutamyl derivatives of DOPA and DA is likely to be the result of a kidney selective generation of DA from these precursors causing renal vasodilation with only minimal systemic effects.

The effect of L- γ-glutamyl-DOPA on RBF and some hemodynamic parameters was also studied in sodium pentobarbital anesthetized dogs. The observation that L- γ-glutamyl-DOPA increased C_{PAH} in rats without causing a significant increase in systemic blood pressure suggests that this prodrug is capable of increasing RBF without affecting the systemic hemodynamics. This conclusion is supported by the results of our experiments in dogs, in which no increase in cardiac output was observed after administration of L- γ-glutamyl-DOPA. A similar dissociation of RBF elevation from a possible stimulatory action on the heart has been shown with L- γ-glutamyl-DA. Thus the injection of this prodrug in dogs increased RBF without accompanying positive inotropic or chronotropic effects (Kyncl et al., 1975).

In order to further exclude the possibility that the elevation of RBF after L- γ-glutamyl-DOPA is secondary to systemic hemodynamic effects, we administered the prodrug directly into the renal artery. Based on the previous studies in rats demonstrating the kidney selective elevation of RPF by L- γ-glutamyl-DOPA, intraarterial infusion of this prodrug was expected to produce an ipsilateral increase in C_{PAH}. The infusion of the prodrugs in doses up to 1 μmole/kg/min, however, failed to produce any significant increase in C_{PAH} on the side of infusion, although a marked increase in urine output was observed. There are several lines of evidence, however, suggesting that L- γ-glutamyl-DOPA and its metabolites may inhibit the tubular secretion of PAH (p. 67). Therefore, the use of the PAH clearance method in the above study might

have given erroneously low values for effective RPF during the infusion of L-γ-glutamyl-DOPA. In order to circumvent such complication, RBF was measured directly using an electromagnetic flow probe. In these experiments, a clear increase in RBF was recorded after intravenous injection of L-γ-glutamyl-DOPA.

While there is considerable evidence that the γ-glutamyl derivatives of DOPA and DA are capable of increasing RBF, the mechanism whereby these compounds promote such an increase is not fully understood. The presence of a high concentration of γ-glutamyl transpeptidase in the brush border membrane of the proximal tubules suggests that these γ-glutamyl compounds may be filtered and then hydrolyzed by the enzyme at this site. The exact fate, however, of DA after its release from the prodrugs is not known. Some of it is apparently excreted into the urine since a considerable excretion of DA follows the injection of L-γ-glutamyl-DOPA. Some DA is probably released into the blood and enters the general circulation. The appearance of free DA in the urine of the contralateral kidney after direct infusion of DA into the renal artery supports this conclusion. DA may also be stored in the intracellular space or within nerve terminals.

Elucidation of how DA released from its prodrugs reaches the DA receptor sites is hampered by a lack of information concerning the localization of the renal DA receptors. Recently Dinerstein et al. (1979) have demonstrated the presence of DA-containing neuronal elements at the glomerular vascular poles. It is possible that these neuronal elements represent the DA nerve terminals. The release of DA from these nerve terminals could then result in activation of DA receptors which causes renal vasodilation. Their findings, however, do not

exclude the possibility that DA receptors exist elsewhere in the kidney. Until more information becomes available on the localization of the DA receptors and the distribution of DA after administration of the γ-glutamyl prodrugs, the mechanism by which DA released from the γ-glutamyl derivatives of DOPA and DA elevate RBF, remains speculative.

To further characterize the actions of the DA prodrugs on renal functions, we examined their effects on GFR, Na^+ excretion and urine flow. The effects of the DA prodrugs on these renal functions were particularly of interest since DA has been shown to increase GFR and induce both natriuresis and diuresis (Goldberg, 1972). If the action of the DA prodrugs is mediated by DA released in the kidney, then these renal functions may also be influenced by administration of these prodrugs. The γ-glutamyl derivatives of DOPA and DA, and DA itself, all induced natriuresis and showed a tendency to increase GFR and urine flow. These findings are, therefore, consistent with the hypothesis that the action of the DA prodrugs is mediated by DA.

The mechanism whereby DA and its prodrugs increase GFR may be related to their effect on RBF. An increase in RBF induced by these compounds causes greater plasma flow into the glomerulus. Consequently the difference between the capillary pressure and the sum of glomerular oncotic pressure and capsular pressure becomes zero at a more distal part of the glomerular capillary. This then results in a larger amount of plasma being filtered at the glomerulus. It is also possible for an increase in the glomerular filtration pressure, produced by the dilation of the afferent arteriole and/or constriction of the efferent arteriole, to result in a greater GFR.

The natriuretic action of DA appears to involve a direct action on the kidney since the intraarterial infusion of DA causes an ipsilateral increase in Na^+ excretion (Meyer et al., 1967). It is also interesting to note that DA has been postulated to act as an intra-renal natriuretic hormone (Ball and Lee, 1977; Kuchel et al., 1978). A Similar direct action on the kidney is suggested for the natriuretic action of L-γ-glutamyl-DOPA. Thus administration of this DA prodrug into a renal artery produces a moderate increase in Na^+ excretion from the infused kidney. It therefore seems likely that the natriuretic action of the DA prodrugs is mediated by DA released from its prodrugs in the kidney.

The mechanism whereby the released DA promotes greater Na^+ excretion has not been established. It is possible that an increase in GFR produced by DA results in a greater filtered load of Na^+, and consequently greater Na^+ excretion. Alternatively, the inhibitory action of DA on proximal tubular Na^+ reabsorption (Seely and Dirks, 1967) may be responsible for its natriuretic effect. Another possible mechanism involves DA-induced redistribution of the intrarenal blood flow. Juxtaglomerular nephrons with long loops of Henle have higher reabsorptive capacities than superficial cortical nephrons. Since the glomeruli of juxtaglomerular nephrons are located in the inner cortex, the redistribution of blood flow away from the inner cortex could conceivably reduce the amount of Na^+ reabsorption. Interference with the action of aldosterone could also increase Na^+ excretion. However it has been reported that L-DOPA potentiates an aldosterone induced decrease in Na^+ excretion. This action of L-DOPA is believed to be mediated by DA since the observed potentiation was inhibited by an aromatic L-amino acid decarboxylase inhibitor and by haloperidol (Adam, 1979). Therefore it

seems unlikely that the interference of aldosterone action is involved in the natriuresis induced by DA.

As in the case with the natriuretic action, the induction of diuresis by the DA prodrugs appears to involve direct action on the kidney. A unilateral increase in urine flow after infusion of L- γ-glutamyl-DOPA into a renal artery, provides evidence for such direct action. Various intrarenal mechanisms are possible for induction of diuresis. Since a small change in GFR can promote a significant increase in urine flow, the observed diuresis may be secondary to the increase in GFR by the DA prodrugs. The diuretic response could also be a consequence of the natriuretic action of the DA prodrugs. The presence of an excess of an osmotically active substance, like sodium, is expected to retard the water reabsorption from the renal tubules. The diuresis may also occur from interference with the action of antidiuretic hormone (ADH), a hormone which increases the permeability of the tubular membrane to water. In this respect, it is of interest that DA has been shown to reverse the antidiuretic reponse to ADH in rats (Deis and Alonso, 1970). The inhibitory effect of DA on the tubular action of ADH was also reported to occur in the toad bladder (Bentley, 1972). Thus, the inhibition of the action of ADH by DA released from its prodrugs may be responsible, at least partially, for the diuretic action of the DA prodrugs.

In studies on the systemic effects of the DA precursors, we directed our attention to the pancreas. The pancreas is the second richest organ in γ-glutamyl transpeptidase with about eight percent of the activity in the kidney (Orlowski and Szewczuk, 1961). Moreover aromatic L-amino acid decarboxylase has also been found in this organ

(West, 1958). It was therefore not surprising to find that after administration of γ-glutamyl derivatives of DOPA and DA, levels of DA in the pancreas were higher than in all other organs except the kidney.

As DA has been shown to induce hyperglycemia by either stimulating glucagon release (Leblanc et al., 1977; George and Bailey, 1978) or inhibiting insulin release (Hakanson et al., 1967), it was important to learn whether the DA precursors also produced hyperglycemia. DA, L-DOPA and L-γ-glutamyl-DA all caused a significant increase in plasma glucose levels. The increase was highest and most persistently maintained after administration of L-γ-glutamyl-DA. In contrast, L-γ-glutamyl-DOPA had no effect on plasma glucose levels.

In order to examine if the ability of the DA precursors to induce hyperglycemia is related to the pancreatic content of DA or its metabolites, their concentrations in the pancreas were determined. The greatest concentration of DA and DOPAC occurred following infusion of L-γ-glutamyl-DOPA, a prodrug which failed to elevate plasma glucose levels. Thus the hyperglycemic effect of the DA precursors was not related to the concentration of DA or its metabolites in the pancreas.

The reason for the difference in the hyperglycemic effect between L-γ-glutamyl-DA and L-γ-glutamyl-DOPA is unclear. The distribution of DA in the pancreas generated by the two prodrugs is not known. The action of γ-glutamyl transpeptidase on γ-glutamyl-DA releases the active principle directly whereas the formation of DA from γ-glutamyl-DOPA requires two enzymatic steps. If γ-glutamyl transpeptidase and aromatic L-amino acid decarboxylase are localized in different parts of the pancreas or in different cell compartments, then DA derived from the two prodrugs may have a differential distribution,

which in turn could account for the difference in the hyperglycemic response. Such a possibility is supported by the finding that the $\frac{DOPAC}{HVA}$ ratios in the pancreas differ markedly after the two prodrugs. Other possibilities, however, should also be considered. Thus the hyperglycemic response might be due not only to changes of insulin and glucagon release but also to stimulation of glycogenolysis in liver.

In addition to promoting greater organ selectivity, the incorporation of a transport group such as a γ -glutamyl moiety to a labile drug may slow down the rate of the drug inactivation. Rapid metabolic inactivation and excretion of free DA is evidenced by the observation that injection of DA in dogs elevates RBF for only 8 minutes (Kyncl et al., 1975). In our dog experiments, the increase in RBF after the same dose of DA persisted longer, but still lasted only 18 minutes. In contrast, the injection of L- γ-glutamyl-DOPA resulted in an immediate RBF elevation lasting for about 40 minutes. Interestingly there was a second rise in RBF starting at about 150 minutes after the prodrug injection. Similarly, a bolus injection of L-γ -glutamyl-DOPA to rats increased RPF over a period of 80 minutes, although only the increase observed during the first urine collection period was statistically significant. These findings indicate that L-γ -glutamyl-DOPA has a longer duration of action and causes a more prolonged increase in RBF than free DA.

The ability of L- γ-glutamyl-DOPA to act as a long-acting renal vasodilator is apparently related to its ability to act as a store of DA. The generation of DA from such a store occurs over a prolonged period of time. Consistent with this interpretation is the observation that the injection of L-γ -glutamyl-DOPA results in excretion of elevated

levels of free and conjugated DA in the urine lasting for at least 150 minutes. In addition, the results of the present studies demonstrate the possibility of utilizing the prodrug approach to prolong the pharmacological action of DA and other labile drugs.

An orally effective renal vasodilator would have a clear advantage over drugs effective only via an intravenous infusion. This consideration prompted us to explore the oral efficacy of γ-glutamyl derivatives of DOPA and DA. The D-isomers of these compounds were synthesized and their efficacy as oral renal vasodilators was compared with those of the L-isomers in ether anesthetized rats. RPF increased significantly 40 to 80 minutes after administration of the D-γ-glutamyl derivatives of both DOPA and DA. During this period the L-isomers had no effect. Some significant increase in RPF after administration of the L-isomers was, however, seen 80 to 120 minutes following their oral administration. The faster onset of renal vasodilation after the D-γ-glutamyl derivatives of DOPA and DA can probably be explained by the fact that these derivatives are much more slowly cleaved by intestinal γ-glutamyl transpeptidase, than their L-counterparts. It may be thus expected that more of the D-isomer is absorbed from the gastrointestinal tract and enters the circulation in an intact form. By contrast, most of the L-isomers probably undergo degradation in the gastrointestinal tract, and more time is apparently required for some of these isomers to reach the systemic circulation. The oral doses of the γ-glutamyl derivatives (50 mg/kg) required for producing renal vasodilation are rather high and in fact at this dose, DA also increased RPF. Similar results were reported by Kyncl et al. (1976). They showed that oral administration of L-γ-glutamyl-DA (25 mg/kg) in dogs increased RBF

but at this dose, free DA was also capable of increasing RBF. These findings indicate that γ -glutamyl-DOPA and γ -glutamyl-DA have only limited oral efficacy. It seems however likely that a further chemical modification of the molecule in a manner which would accelerate its absorption from the gastrointestinal tract and make it less susceptible to metabolic inactivation, would make the oral administration of these prodrugs more practical. Esterification of the carboxyl group of DOPA and acylation of its catechol grouping might achieve this objective.

The ability of DA to increase both RBF and cardiac output while maintaining adequate peripheral resistance is unique among sympathomimetic amines. α-Agonists such as norepinephrine and metaraminol increase the peripheral resistance, thus causing a decrease in RBF. Isoproterenol, a β-agonist, is effective in increasing cardiac output; however, the simultaneous lowering of the peripheral resistance by this drug tends to shunt blood away from the kidney.

The observation that DA induces renal vasodilation and stimulates cardiac performance has led to the use of this drug in treatment of refractory congestive heart failure (McDonald et al., 1964; Rosenblum et al., 1972), acute heart failure during and after cardiac surgery (Rosenblum and Frieden, 1972; Holzer et al., 1973), and septic shock (Loeb et al., 1971; Thompson et al., 1975). The unusual renal vasodilating action of DA may also be useful in treatment of other clinical conditions characterized by reduced RBF. However, because DA is capable of activating α -receptors, it may cause hypertensive crisis as well as gangrene as a result of excessive peripheral vasoconstriction (Alexander et al., 1975; Stetson and Reading, 1977). While the cardiostimulatory effect of DA is an important pharmacological action of this drug, this

action may also induce cardiac arrhythmia and possibly precipitate angina pectoris (McDonald et al., 1964; Talley et al., 1969). Thus agents such as the γ-glutamyl derivatives of DOPA and DA which are capable of increasing RBF with minimal systemic effect associated with DA, are of considerable therapeutic interest. It is possible that such agents could play a role in the treatment of hypertension, acute renal failure, phenobarbital intoxication and other conditions where greater renal perfusion is desired.

The possibility of utilizing the prodrug approach to targeting drug action was investigated further using γ -glutamyl derivatives of antibacterial agents as kidney directed prodrugs. From previous studies involving the γ -glutamyl derivatives of DOPA and DA, γ -glutamyl derivatives of antibacterial agents were expected to be converted selectively to the active drugs in the kidney. This was expected to result in a high concentration of the drugs in the kidney. A kidney-selective accumulation of antibacterial agents would be of considerable therapeutic interest in the treatment of kidney and urinary tract infection.

As models of kidney directed antibacterial agents, γ - and α - glutamyl derivatives of SM were synthesized initially. Consistent with previous findings showing a high kidney specificity of localization of γ-glutamyl transpeptidase (Albert et al., 1961; Orlowski and Szewczuk, 1961), we found that both D- and L- γ-glutamyl derivatives of SM are cleaved in the kidney to SM at a rate of one to three orders of magnitude greater than in any other tissue studied. However, there was only a small preferential accumulation of SM in the kidney, with respect to other tissues, after intraperitoneal administration of these γ -glutamyl

compounds as compared with that after administering free SM. It is of interest that D-γ-glutamyl-SM, which was cleaved by mouse kidney homogenates at a rate about 6 times slower than the L-isomer, showed a more selective kidney accumulation than the L-isomer. Since γ-glutamyl-SM is an excellent substrate of γ-glutamyl transpeptidase, its rapid hydrolysis even in those tissues having a low enzyme activity may have contributed to the observed loss in kidney selectivity. No kidney-specific accumulation of SM was observed after administration of L-α-glutamyl-SM. This finding is consistent with the more uniform tissue distribution of aminopeptidase A activity compared to γ-glutamyl transpeptidase activity.

In order to increase the kidney-selectivity of γ-glutamyl-SM, a further chemical modification of this prodrug became desirable so as to decrease the rate of its cleavage, and make it dependent on the action of a second kidney specific enzyme. The kidney is known to have high levels of enzymes capable of hydrolyzing N-acylamino acids and several deacylating enzymes, differing in substrate specificity, have been described (Birnbaum et al., 1952; Fones and Lee, 1953; Endo, $1978_{a,b}$). Our attention was therefore directed toward N-acyl-γ-glutamyl derivatives of SM, and several of these derivatives were synthesized. As expected N-acetyl, N-chloroacetyl and N-butyryl derivatives of L-γ-glutamyl-SM were hydrolyzed at greatly reduced rates compared to the glutamyl derivatives of SM. Application of a coupled enzyme assay made possible the determination of the rate of deacylation of N-acyl-γ-glutamyl derivatives. This assay showed that the deacylation of these derivatives proceeds with the highest rate in the kidney.

Administration of all three N-acyl-L- γ-glutamyl derivatives resulted in a remarkable kidney-specific accumulation of SM in mice. With both N-acetyl and N-chloroacetyl derivatives of γ-glutamyl-SM, SM concentrations in the kidney greatly exceeded those observed after an equimolar dose of SM. Apparently by making the release of the active component from a prodrug dependent on two organ-specific enzymes that are localized in the same target organ, it is possible to achieve a very high concentration of a drug in that organ. The high concentration of the sulfonamide in the kidney was accompanied by a great decrease of the drug concentration in other tissues. Thus the levels of SM after N-acyl- γ-glutamyl-SM, in tissues other than the kidney, were greatly reduced compared with those seen after administration of free SM. This high kidney-selective accumulation of SM was seen after all the N-acyl- γ -glutamyl derivatives of SM. When the distribution of free SM after N-chloroacetyl-L- γ -glutamyl-SM was measured in rats and guinea pigs in addition to mice, a similar kidney-selective accumulation of SM was found in all these species. Moreover plasma concentrations of SM in these animals were also very low after administration of the prodrug. These findings, therefore, support the hypothesis that a prodrug circulates in an intact form and its conversion to the active drug occurs in the target organ where the enzymes required for such conversion are present.

The observed kidney specificity of N-acyl- γ -glutamyl derivatives of SM is likely to be due to at least two factors. The first is the slow release of SM from the N-acyl- γ -glutamyl derivatives which effectively prevents SM release in the tissues having low activities of the enzymes required for the release. The second factor is dependence

of SM release on the activity of the two kidney specific enzymes. This point is of considerable importance particularly in designing new organ-selective prodrugs. Thus, an increase in organ selectivity of a prodrug can be expected if its conversion to the active drug is made dependent on the action of two organ-specific enzymes localized in the target organ.

It was of interest to determine the time period during which the organ-selectivity of the prodrug is maintained. We therefore studied the concentration of SM in the kidney after various time intervals following administration of N-acyl-L- γ-glutamyl derivatives of SM. The finding that the kidney-selective accumulation of SM was maintained for 1 hour after the prodrug administration indicates that the observed organ-selectivity is not a transient phenomenon. Even at 2 hours following injection of the prodrugs, the tissue concentration of SM, in tissues other than kidney, remained distinctly lower than those after injection of free SM. It seems that only a minimal amount of SM ever enters organs other than the kidney after administration of the prodrugs.

In the course of studies on the concentration of SM in the kidney after N-acyl-L- γ-glutamyl derivatives, it was noted that of the three species studied (rat, guinea pig and mouse) the lowest accumulation of free SM was seen in the rat. It was then found that the deacylation of N-chloroacetyl-L- γ-glutamyl-SM proceeded in the rat kidney at a considerably lower rate than in the other species. Thus the rate of prodrug hydrolysis and the kidney concentration of the active component appear to be related. Such a relationship is of interest since it offers a possibility of utilizing the <u>in vitro</u> assay to predict the

effectiveness of a given prodrug as a precursor for the targeted delivery of the active drug.

In addition to the rate of prodrug hydrolysis, the rate of uptake of the prodrug by kidney cells may affect the kidney accumulation of the active component. In this respect, it is of interest that the kidney concentration of SM after administration of N-acetyl-L- γ-glutamyl-SM was significantly lower in rats pretreated with sodium pentobarbital as compared to untreated rats. A likely explanation for this result is based on a postulate that N-acetyl-L- γ-glutamyl-SM is taken up into the kidney cells, at least partially, by the renal organic acid transport system. Since barbiturates are known to inhibit the renal organic acid transport (Harvey, 1975), such inhibition could reduce the uptake of the prodrug. As a consequence of reduced uptake, the kidney concentration of the active component is lowered. Other factors which can influence the accumulation of the active component after administration of prodrugs include access to the site of enzyme localization, plasma protein binding, and the rates of elimination into urine.

The finding of a high kidney concentration of SM after administration of its N-chloroacetyl-L-γ-glutamyl and N-acetyl-L-γ-glutamyl derivatives, induced us to examine the urinary excretion of SM after these derivatives. This concentration of SM in the kidney could have resulted from its accumulation either in the cellular elements of the tubules or in the urinary spaces. If the high kidney concentration of SM is due to SM localized in the urinary spaces, then correspondingly high urinary excretion of SM could have been expected. Compared to SM, administration of its N-chloroacetyl-L-γ-glutamyl and N-acetyl-L-γ-glutamyl derivatives resulted in a lower urinary excretion of free SM.

This occurred even though the concentration of SM in kidney homogenates was higher after these derivatives than after free SM. This finding suggests that the concentration of SM is not evenly distributed between the cellular elements and urinary spaces and that the sulfonamide is probably more concentrated in the cellular spaces of the kidney than in the urine.

Although sulfonamides with an activity limited to the kidney and urinary tract would be of considerable therapeutic interest, evaluation of the usefulness of the N-acyl- γ-glutamyl derivatives of sulfonamides in this respect would require further studies. Factors such as toxicity, rate of intestinal absorption, intrarenal distribution of a sulfonamide, duration of action, and rates of elimination and metabolism would have to be determined in such evaluation. A large number of sulfonamides with a wide spectrum of properties and relatively limited toxicity are currently available. Replacement of these drugs with derivatives showing activity limited to a single organ may therefore be considered of therapeutic use only in special situations. N-acyl- γ-glutamyl derivatives of a variety of drugs may however be of general interest when limitation of action to the kidney is a desired objective.

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