# The Biological Fate of Reserpine*

ROBERT E. STITZEL

Department of Pharmacology, West Virginia University, Morgantown, West Virginia

* Authors have often used several terms to refer essentially to either the same type of binding (e.g., "irreversible," "persistent," "tightly," "covalent") or the same subcellular fraction (e.g., "granules," "intracellular vesicles," "intracellular storage organelles," "high-speed particulate fraction"). Since these terms are widely used, they have been retained in the present review. However, the reader should be aware that they are used interchangeably and are intended to describe the same phenomenon or structure.

## I. Introduction

IT has been several years since the pharmacology of reserpine has been summarized (8, 83, 148). Many of these earlier treatments dealt primarily with the botany and chemistry of the *Rauwolfia* alkaloids with only limited emphasis on the emerging pharmacology of the principle alkaloid, reserpine. In the present review, the discussion of chemically related compounds will be minimal and any comments on the amine depleting action of the drug will be severely limited. Proper treatment of the latter topic, although of crucial importance to our developing understanding of the function of the sympathetic nervous system, would necessitate a publication many times the space available here. This review, therefore, is devoted almost entirely to the study of the biological fate of reserpine, *i.e.*, its absorption, distribution, metabolism, and excretion.

## II. Historical Review

More than 100 different species of the genus *Rauwolfia* have been tentatively identified botanically. Approximately 90% of the genus *Rauwolfia* can be found growing indigenously in nearly all of the tropical lands of the world with the principal exception of Australia where it has been artificially introduced. Rain forests and tropical savannas are particularly well suited for this genus.

The members of this group of plants are of major importance to pharmacologists and medicinal chemists particularly because of the complex chemical nature and biological properties of the alkaloids which have been isolated from them. Of all the species comprising this genus, *Rauwolfia serpentina* clearly has become the most

important in both basic research and clinical medicine. *R. serpentina* is widely distributed throughout India, Sri Lanka, Burma, Siam, and Java, and preparations containing its alkaloids have been used for centuries in India for the treatment of a variety of clinical disorders.

The earliest reference in Western literature to the *Rauwolfia* plants is contained in a Portuguese manuscript published in Goa in 1563 (116). This manuscript described the therapeutic properties attributed to the plant and discussed the use of the roots in popular medicine by native doctors. The name *Rauwolfia* was coined by the French botanist Plumier (148) in honor of Leonhart Rauwolf, a 16th-century German or Austrian botanist who in 1582 upon his return from India published a book of his observations on this genus (148).

Systematic investigation of the pharmacological activity of the various *Rauwolfia* species was begun about 1930 with most of the studies concentrating especially on *R. serpentina* from India. In 1931, Siddiqui and Siddiqui (126) isolated and characterized a number of alkaloids from the roots of this species. Although several of these compounds (*e.g.*, serpentine, serpentinine) possessed interesting pharmacological activity, in a series of papers in the early 1940s Chopra and his colleagues (28–30) came to the conclusion that some additional active substance other than the Siddiqui alkaloids must be present in the whole root which accounted for the marked hypotensive effects of the total extracts from *R. serpentina*.

Of all the crude extracts examined by Chopra (17), an oleoresin fraction showed particular promise, especially in regard to the peculiar type of sedation produced in

animals given an alcoholic extract of the oleoresinous material (53).

It was not until 1952, however, that *R. serpentina* became more than a minor drug plant of India. The widespread clinical use of preparations derived from this plant began only after the isolation by Müller *et al.* (105) of the most clinically useful of the *Rauwolfia* alkaloids, reserpine. Many, if not all, of the pharmacological actions described in the early Indian literature as typical of *Rauwolfia* are possessed by this active alkaloid. Although a great deal of research has been devoted to the structure-activity relationships of the *Rauwolfia* alkaloids and although many alkaloids have subsequently been isolated from a variety of species, reserpine still seems to be the most effective of all the compounds thus far isolated and characterized.

### III. Chemistry

#### A. *General Structural Properties of the Alkaloids*

With the exception of thebaine and papaverine, all of the *Rauwolfia* alkaloids are indole bases. Their chemistry is quite complex, which accounts for the relatively

slow progress made in understanding the pharmacology of these polycyclic compounds. A relatively complete listing of the major alkaloids which have been isolated from *R. serpentina* is available (148).

From a clinical standpoint the *Rauwolfia* alkaloids with the most interesting biological activity are those possessing a tertiary indole nucleus with a carboxylic ring E (fig. 1). These alkaloids give the *Rauwolfia* root its distinctive pharmacological characteristics.

At least three alkaloids have been isolated which have a carboxylic ring E.
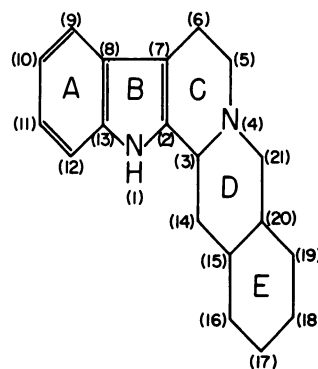


Fig. 1. Chemical skeleton of a tertiary indole alkaloid with a carboxylic ring E.



Deserpidine

Reserpine
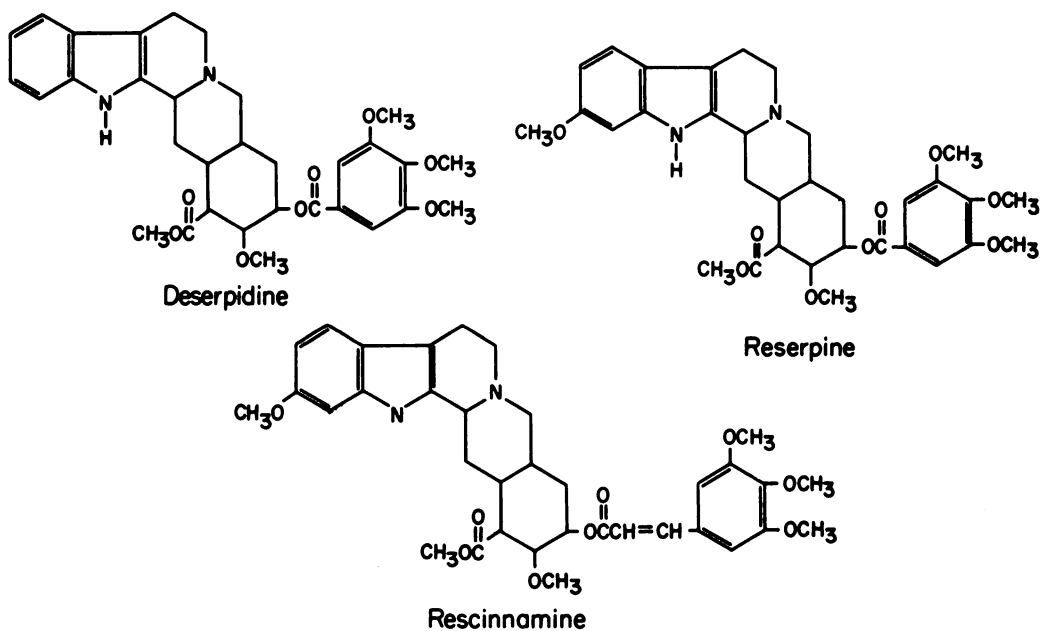
Rescinnamine

Fig. 2. Reserpine and reserpine-like alkaloids isolated from *Rauwolfia serpentina*.

Syrosingopine

Methoserpidine
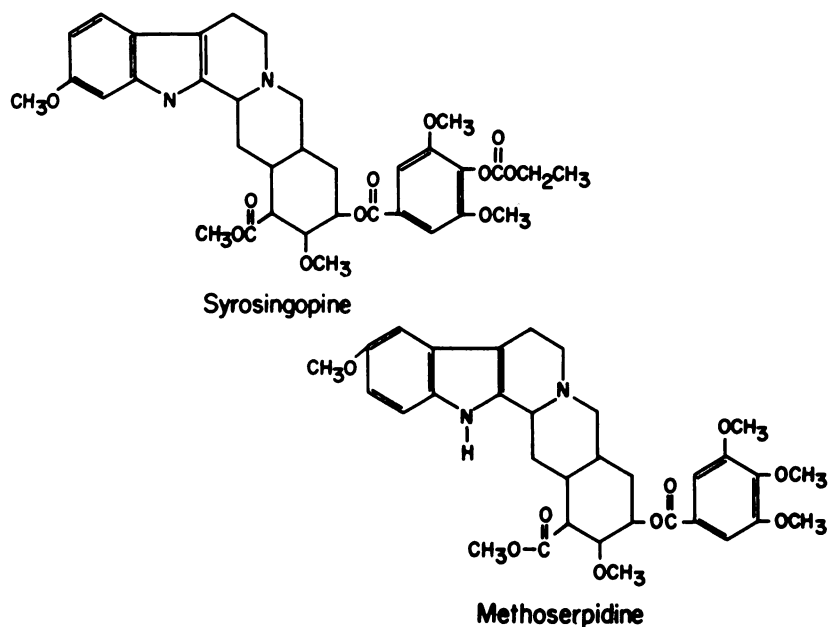
Fig. 3. Semisynthetic derivatives of reserpine.

Prenylamine

Benzquinamide

Tetrabehazine
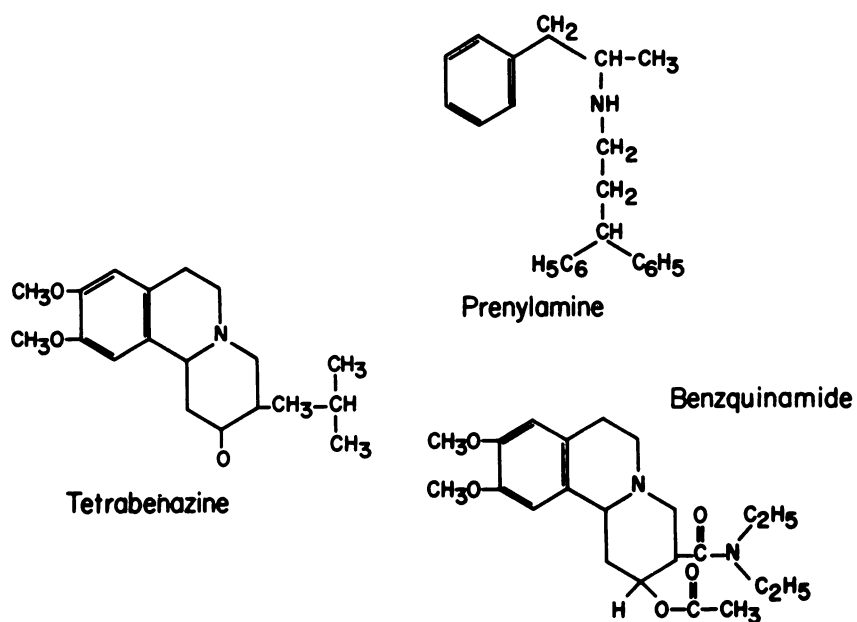
Fig. 4. Synthetic agents possessing reserpine-like activity.

These are reserpine, rescinnamine, and deserpidine, all of which have had their structure and stereochemistry quite well defined (fig. 2). In addition, several semisynthetic (fig. 3) (syrosingopine, methoserpidine) and synthetic (fig. 4) (tetrabenazine, benzquinamide, prenylamine) agents have been produced which have activity spectra somewhat similar to reserpine itself. The latter compounds have been increasingly studied in recent years, and several reports comparing their activity to that of reserpine are available (3, 20, 21, 31, 51, 69, 84, 86, 114).

## B. Physical Properties

Reserpine is a colorless, crystalline powder which is almost insoluble in water at neutral pH, but soluble in solutions of organic acids, such as acetic, ascorbic or citric. Reserpine can also be dissolved in solvent mixtures such as that used commercially: citric acid, benzyl alcohol, polyethylene glycol and water. Reserpine phosphate has also proved to be useful for pharmacological experimentation, especially when reserpine is to be placed in a perfusion medium (19). This material is readily soluble in aqueous solutions up to a concentration of 0.5% reserpine. The phosphate salt should be lyophilized since it is somewhat unstable under atmospheric conditions. Solvents containing mineral salts tend to precipitate reserpine immediately.

## C. Chemical Properties

Reserpine is a diester compound containing six methoxy groups, one basic nitrogen atom which can be quaternized, and a second nitrogen which is weakly basic and is present as an NH group. On alkaline hydrolysis (see fig. 5) reserpine yields reserpic acid, 3,4,5-trimethoxybenzoic acid (TMBA), and methanol. Spectral examination of reserpine confirms the presence of the two ester linkages and also demonstrates a methoxyindole and a second, unsaturated chromophoric system. Derivatives in which the hydrogen atom of the indole nitrogen is replaced by methyl or allyl groupings, or in which the other nitrogen has been quaternized result in compounds which are devoid of all reserpine-like activity (62). Esterification with acids other than TMBA at position 18 in ring E of the reserpine molecule also generally tends to diminish activity (75). However, minor changes in the TMBA ester itself yields compounds with activity almost equivalent to reserpine. For instance, rescinnamine and syrosingopine are somewhat less potent while deserpidine has qualitative and quantitative actions which are almost identical to reserpine. Interestingly, a displacement of a methoxy group on ring A from position 11 to 10 has been claimed to result in a compound which has a more selective peripheral rather than central site of action (52).

## D. Methods of Analysis

In the 1950s, the principal difficulty facing pharmacologists wishing to study the biological fate of reserpine was the unavailability of sufficiently sensitive and specific methods of analysis of this highly lipophilic compound. Some of the earliest methods of analysis were based on the ul-
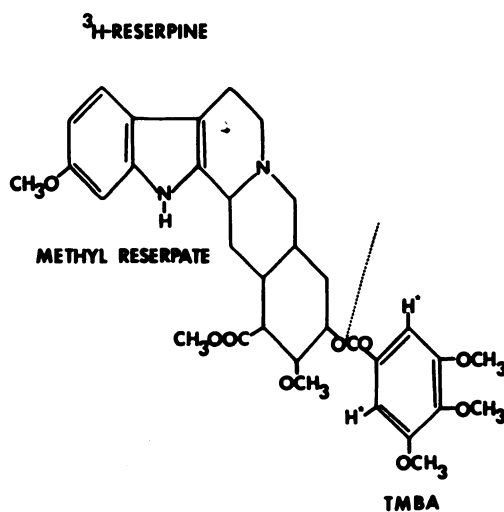


FIG. 5. Hydrolytic cleavage of reserpine.

traviolet absorbing properties of reserpine (12). An isolation step was invariably employed in these procedures in order to separate reserpine from metabolites, decomposition products and/or other chemically related compounds. Banes (6) and Bartelt and Hamlow (7) used chromatographic separation, while Sakal and Merrill (118) combined paper ionophoresis with ultraviolet absorption. McMullen et al. (95) removed decomposition products of reserpine by extracting samples with dilute acid and base. In addition to ultraviolet procedures, a large number of colorimetric methods also were developed (6, 11, 55, 56, 68).

Virtually all of the above analytical methods for the measurement of reserpine involved time-consuming separation and extraction steps before final analysis. In addition the degree of specificity and level of sensitivity was often not adequate, especially when reserpine was to be measured from mixtures containing either metabolites of reserpine or other *Rauwolfia* alkaloids. For these reasons, other means of identification have been sought. The most widely used procedures, at least until radioactive reserpine became available, were those based on fluorescence analysis.

Reserpine, like the biogenic amines which it depletes, does have sufficient native fluorescence to allow direct analysis, provided that it is present in sufficient concentration (55). Generally, however, reserpine must be oxidized in order to convert it to a more highly fluorescent material which can be excited at about 365 nm and fluorescence measured at 420 nm. Several other excitation-emission wavelength combinations have also been suggested (134). Udenfriend (135) points out that the native fluorescence of reserpine (excitation, 300 nm; fluorescence, 375 nm) would probably overlap with that of endogenous indoles (excitation 285 nm; fluorescence, 350 nm), hence the need for oxidation procedures to eliminate such interferences.

Solutions of reserpine will develop a fluorescence when exposed to a variety of conditions, including light (149), acids (56,

73, 137) or oxidizing reagents (37). The fluorescing species thus generated is thought to be 3,4-dehydroreserpine (149) although the presence of other compounds cannot be ruled out since the reaction is quite complex.

One of the first relatively sensitive fluorometric procedures was that of Jakovijevic et al. (68) which could measure reserpine in a concentration as low as 5 ng/ml. This method, while free of such complicating features as interference from closely related compounds such as deserpidine and rescinnamine, was primarily of value when quantifying reserpine in tablets and other nonaqueous materials. Isolation from biological material was not discussed. More recent studies, which are primarily devoted to separation and quantitation of reserpine in the presence of other pharmacologically active compounds, are listed in the reference section (7, 61, 74, 120, 136).

It is not too surprising that the limits of sensitivity of the above assay procedures vary widely. In all likelihood this is a reflection of the efficiency of each procedure in forming the fluorophor and stabilizing it against decomposition. The report of Honigsberg et al. (61) is of interest since it is the first paper reporting on the use of high pressure liquid chromatography to isolate reserpine, a procedure which seems to circumvent some of the difficulties encountered in other methods. This is a two-step analysis with an octadecyl column and a 55:45 mixture of absolute methanol and 0.5% NH$_4$Cl. Unfortunately, as in several previous studies, no isolation from biological material is discussed.

A sensitive fluorometric analysis of reserpine contained in human plasma has been published recently by Tripp et al. (134). This procedure is based on silica gel G thin-layer chromatography, followed by a fluorimetric scanning of the plate with a densitometer. Recovery from human serum averaged about 96%, and values were linear in the range of 0.05–4 ng/ml.

The most sensitive analytical proce-

dures for the measurement of reserpine in biological samples are those which use radiolabeled reserpine. Because reserpine virtually disappears from the body shortly after its administration (57), the need for maximum sensitivity and high specificity is perhaps even more important in measuring reserpine levels than it is for measuring many other active pharmacological agents. It was only through the use of isotopic methods that investigators (90, 122) could measure central nervous system levels of reserpine throughout most of the time-course of its action. The few studies which have involved the disappearance of reserpine from human plasma have also utilized radioactive drug (87, 111, 134, 152). Reasonably detailed studies of the identification and estimation of tritium-labeled reserpine from tissues are those of Manara (89) and Mueller and Shideman (101). A modification of this procedure especially useful for determining ³H-reserpine in subcellular fractions has also been published (139, 140).

## IV. Absorption

Relatively few detailed studies have been published which were concerned with a close analysis of factors influencing the rate of reserpine absorption. Since most of the recent work has dealt with the parenteral administration of ³H-reserpine, factors affecting reserpine absorption become superfluous. In one of the few studies dealing with absorption, Mueller and Shideman (101) demonstrated that the route of administration significantly affected reserpine brain levels achieved both 20 min and 2 hr after injection. In contrast to results often encountered with other drugs, the concentration of ³H-reserpine in both infant and adult rat brains was significantly greater after s.c. injection than it was at similar times after i.p. administration. This observation could be easily explained if reserpine were rapidly metabolized by hepatic enzymes after i.p. administration (i.e., first-pass effect). Since virtually all the drug would initially pass

through the liver after an i.p. injection, rapid metabolic inactivation would result in relatively low blood and tissue levels of the parent compound. The rapid breakdown of the drug would thus more than compensate for the faster absorption usually seen from i.p. rather than from s.c. sites.

Absorption of reserpine was obviously quite rapid from the s.c. site, since the brain levels achieved at 20 min were higher than those found at 2 hr. Although the rates of disappearance of ³H-reserpine from this site were identical in young and mature animals, the concentration in the plasma of the infant rat was about seven times that in adult plasma. The most likely explanation for this finding would be a slower rate of metabolism in the younger animals. Kinetic measurements of hepatic reserpine metabolism seem to support such a hypothesis since the $V_{max}$ values in adults are approximately twice those seen in the infant rats (101).

Some data are also available from studies on ³H-reserpine absorption in man. Maximal blood levels were achieved approximately 2 hr after the oral administration of ³H-reserpine to six normal male volunteers (87, 134), or to six schizophrenic patients (111) with some tendency for peak levels to be reached slightly earlier in the normal subjects. In both of these studies most of the plasma radioactivity was recovered as the trimethoxybenzoic acid metabolite, indicating a rapid rate of hydrolysis after oral absorption. Studies in rats (110) have also indicated a relatively rapid absorption after oral administration, since the overall patterns of blood levels after oral and intravenous reserpine were quite similar. In these studies as well, essentially all the urinary radioactivity was found as TMBA.

Reserpine thus seems to be rapidly absorbed after either p.o., s.c. or i.p. administration. Its subsequent distribution between plasma and blood cells favors plasma while that between plasma and tissues favors the tissue (122). The high

lipid solubility of the compound probably facilitates the passage of reserpine across lipid cell membranes. Reserpine apparently can also cross the placenta and enter the fetal circulation since infants whose mothers received the drug before delivery have shown a number of symptoms associated with reserpine administration (117).

## V. Distribution

The discovery that reserpine can depress both catecholamine and 5-hydroxytryptamine (5-HT) levels led to extensive research into the site and mechanism of action of reserpine. It soon became obvious that tissues responded differently to the amine-depleting action of reserpine. Thus, single doses which have little effect on platelet or intestinal 5-HT levels could deplete the brain of its 5-HT content (113). Similarly, small doses of reserpine deplete the heart of norepinephrine (NE), while higher doses are necessary to deplete brain NE, and still higher doses are required to deplete the adrenal gland of its catecholamine content (24).

### A. Hit and Run Hypothesis

Studies such as those quoted above suggest the importance of understanding the tissue distribution of reserpine as a possible way of gaining insights into the observed differential tissue sensitivity to the depleting activity of reserpine. Shore and his co-workers (125) were among the first to confront this problem directly. They noted a discrepancy between the concentration of reserpine present in the rabbit brain and the ability of reserpine to deplete central 5-HT levels. With methods available at that time, they were unable to detect reserpine 2 hr after its administration, although tissue levels of 5-HT remained depleted for 24 to 48 hr. Consequently, a "hit and run" hypothesis explaining the action of reserpine was postulated; it was believed that the action of reserpine continued after the drug was no longer in the body.

Another important observation made in these early studies was the finding that reserpine-induced sedation did not coincide with its peak concentration in the brain, either as a whole or in its separate parts (122). There also seemed to be little correlation between the levels of known reserpine metabolites in brain and the duration of its pharmacological effects. Although these findings seemed to support the notion of a "hit and run" drug, that is a central action which persists beyond the physical presence of the drug, a number of investigators could not accept this verdict as the ultimate mechanism of action. It was soon appreciated that more sensitive analytical procedures would be required in order to verify such a hypothesis. Both ultraviolet and fluorescence methods of analysis were clearly unsuited to provide the level of sensitivity needed. The availability of $^{14}C$-reserpine and later $^3H$-reserpine possessing fairly high specific activity resulted in fresh interest in reserpine distribution. These studies have now invalidated the "hit and run" theory, since reserpine, although in trace amounts, can be demonstrated in tissues at least as long as a pharmacological effect can be measured.

### B. Tissue Distribution

After intravenous injection, reserpine levels in the blood drop rapidly. For example, 2 min after injection less than 1% of the dose remains in the blood (101, 122), the rest being concentrated in various tissues, especially those with high lipid content. Labeled reserpine administered to rats was found to have its greatest concentration in the lung (46, 47, 122). Of the other tissues examined, high concentrations of radioactivity were found in the kidney, spleen, and liver; intermediate levels were observed in the heart and skeletal muscle, and the lowest levels were found in the blood serum, brain, and body fat. Since neutral fat receives a relatively low blood flow, it is not surprising that this tissue does not have peak reserpine con-

centrations until 4 to 6 hr after drug administration.

Several things should be pointed out concerning these early studies: 1) brain levels are much lower than those observed more recently by other workers; 2) methodological difficulties may have obscured some of the data, e.g., counting methods which employed thin-window Geiger-Müller tubes are known to be far less sensitive than liquid scintillation spectrometry; and 3) most of the radioactivity found in the various tissues more than likely had an appreciable contribution from metabolites.

Sheppard et al. (123) and later Maggiolo and Haley (88) re-investigated the problem of low tissue levels of reserpine. The principal innovation which these studies offered was the recognition that the use of reserpine labeled with $^{14}C$ in the TMBA portion of the ring, as done in previous work, could not sufficiently clarify the reserpine retention problem. This was true both because of the low specific activity of the $^{14}C$-reserpine and because of the rapid hydrolysis of the ester linkage. The above authors, with tritium labeled drug, were able to demonstrate $^3H$-reserpine in the brains of experimental animals 3 to 5 days after its administration. In addition, Maass et al. (87) confirmed the slow clearance of $^3H$-reserpine by finding that fecal samples obtained on the 12th day after drug administration still contained approximately 0.15% of the administered tritium.

These studies finally laid to rest the "hit and run" theory of reserpine action by virtue of their detection of very small amounts of reserpine throughout the duration of its induced clinical symptomatology. Thus the persistence in the body of reserpine and/or its metabolites was now inextricably linked with its long duration of pharmacological activity. Several questions still remained unanswered, however. Neither report (88, 123) satisfactorily accounted for the discrepancy between the small amount of reserpine found and the relatively large depletion of amines effected. Furthermore, the exact site and nature of reserpine binding to tissue components was not examined.

## VI. Binding in Adrenergic Tissue

### A. Tetrabenazine Studies

There is abundant evidence that tetrabenazine, a benzoquinolizine derivative, and reserpine have similar sites of action. Quinn et al. (144) demonstrated that the two drugs compete for the same sites in vivo while Carlsson et al. (22, 23) indicated that both compounds inhibit the $Mg^{++}$-adenosine triphosphate dependent catecholamine uptake mechanism present in adrenergic granules. In 1966, Carlsson and Lindqvist (20) showed that brain dopamine and norepinephrine levels could be protected from the action of reserpine by pretreatment with tetrabenazine. Based on the observation that tetrabenazine administration produced similar pharmacological effects as reserpine, but that its effects were rapidly reversible and therefore much shorter acting, Manara and Garattini (90) studied the time course of $^3H$-reserpine levels in the brains of tetrabenazine-pretreated rats. At 6 and 12 hr after $^3H$-reserpine administration, brain levels of $^3H$-reserpine were indeed lower than in non-pretreated animals. Other groups soon verified these findings (2, 140). These studies were among the first to suggest a relatively specific binding of reserpine to tissues, since tetrabenazine and reserpine seemed to compete at a common and relatively specific cellular site. This site was presumably not at the level of the adrenergic neuron since imipramine and desipramine, membrane pump inhibitors, did not modify the early accumulation of reserpine in tissues (50, 91).

It was noteworthy that in early experiments with tetrabenazine pretreatment, when brain levels were measured only 1 hr after $^3H$-reserpine administration, pretreatment failed to reduce reserpine accumulation (90). This observation seemed to

suggest that there was a large degree of nonspecific binding of reserpine shortly after its injection. This supposition was confirmed by Wagner and Stitzel (139) who showed that the subcellular pattern of ³H-reserpine distribution both 15 and 60 min after its administration paralleled almost exacty the lipid content of each subcellular fraction. Thus the initial binding of reserpine seems to be primarily determined by its high lipid solubility. These authors as well as Alpers and Shore (2) cautioned, however, that the large amount of reserpine bound to lipid at these early times could be masking a smaller more specific binding.

It was reasoned that since most of the ³H-reserpine was probably nonspecifically bound to lipid, especially at early time periods after its administration, one might find a specific tissue binding by waiting until most of the ³H-reserpine was lost from its nonspecific binding sites. This hypothesis was examined with radioactive reserpine with a higher specific activity than that previously available.

After i.v. administration, ³H-reserpine levels in tissues such as heart and spleen decreased relatively rapidly during the first 6 hr and then more slowly over the next 24 hr (2). The half-life of reserpine in these tissues was calculated to be approximately 18 hr. Pretreatment of the animals with either unlabeled reserpine or tetrabenazine reduced the amount of ³H-reserpine recoverable in the heart and spleen 18 hr after its injection, a time when presumably much of the nonspecifically bound drug was gone. Neither tetrabenazine nor unlabeled reserpine had any effect on the binding if they were given after the injection of ³H-reserpine. These observations suggest that a small proportion of the ³H-reserpine was rapidly and irreversibly bound to structures within sympathetically innervated tissue. Since a correlation could be established (2) between the degree of catecholamine depletion produced in the heart and the amount of ³H-reserpine which remained, it was considered quite likely that the prolonged depletion of

norepinephrine produced by reserpine was both produced and maintained by the presence of these small amounts of very highly bound reserpine molecules.

## B. Subcellular Binding Studies

Since the depletion of biogenic amines induced by reserpine in vivo is in all probability due to a blockade of amine uptake into storage sites (9, 130), it might also be expected that the granules are a subcellular binding site of reserpine. This hypothesis is supported by the observation that after reserpine treatment, i.e., at a time when fresh uninhibited granules could be expected to reach the periphery from cell bodies (32, 42, 54), the return of sympathetic nerve function correlates closely with the reappearance of granular uptake (85).

1. Neural tissue. Several investigators (2, 139) have pointed out the difficulty inherent in studying the subcellular distribution of highly lipid soluble compounds such as reserpine. The similarity of distribution of ³H-reserpine given intravenously and that added to tissue homogenates suggested to Alpers and Shore (2) that information drawn from fractionation studies may be largely artifactual since drug redistribution might occur during the preparative separation procedures. In contrast to this suggestion, Wagner and Stitzel (140) found that the microsomal fraction, i.e., that fraction containing a large amount of catecholamine granules, had three times the ³H-reserpine content after administration in vivo then did the same fraction after addition in vitro. If the retention in vivo of ³H-reserpine was expressed as amount retained per gram of protein in each fraction, then the microsomal fraction contained much more reserpine than any other subcellular fraction. Studies in vivo, therefore, may reveal more than addition in vitro of ³H-reserpine.

Two additional approaches to investigating this problem were tried in an attempt to demonstrate a small amount of more persistently bound ³H-reserpine in

the presence of larger quantities of reversibly bound drug. By both reducing the dose of reserpine administered and by waiting 18 hr or longer after ³H-reserpine injection, several laboratories (43, 92, 140) found evidence for the occurrence of a persistent binding *in vivo* to the microsomal fraction. However, if measurements were made only 1 hr after ³H-reserpine administration no unique binding to any single fraction could be shown.

Pretreatment with unlabeled reserpine or tetrabenazine reduced the ³H-reserpine content in the microsomal fraction more than in any other subcellular compartment (140). This decrease after drug pretreatment seems to provide direct evidence of a small, but significant, amount of specific binding in this fraction. This conclusion is supported by brain subcellular distribution studies (92) which showed that the microsomal (*i.e.*, granular) fraction accounted for about 50% of the total reserpine present in the brain 24 hr after its administration and thus is likely to be the major site for persistent binding *in vivo*.

If ³H-reserpine is indeed bound in a specific manner to some component of monoamine storage organelles, then one should see higher concentrations of the depleting agent in those tissues having the highest amine content. Studies attempting to relate the density of adrenergic innervation to the binding capacity of the tissue for reserpine have been published (40, 107, 140). There seems to be a fairly direct relationship between tissue norepinephrine content in peripheral tissue and the amount of ³H-reserpine retained by that tissue. For instance, adrenal glands have been shown to contain the highest concentration of ³H-reserpine, followed by almost equal amounts in heart and spleen, and lower levels in the small intestine (107).

A comparison of the abilities of cardiac and skeletal muscle to accumulate both ³H-NE and ³H-reserpine showed that cardiac muscle retained 15 times more of both drugs than did skeletal tissue (140). Thus, in the periphery, the data are compatible with at least a portion of administered ³H-

reserpine being associated specifically with adrenergic neurons. The binding of ³H-reserpine in these nerves is apparently quite firm since conditions which augment adrenergic nerve activity fail to decrease the amount of bound reserpine (42, 108).

The correlation between reserpine distribution and monoamine content is roughly similar in the central nervous system (40, 58), where areas rich in monoamines such as the striatum, do have a higher ³H-reserpine content while areas relatively deficient in adrenergic innervation have the lowest content. The lack of an ideal correlation, especially in other brain areas, is probably the result of ³H-reserpine binding to several types of monoamine storage vesicles in a given brain area. In general, no single biogenic amine is found exclusively in a given brain region and thus it is not at all surprising that there is no absolute correlation between the regional binding of reserpine and the regional distribution of any single endogenous monoamine. In the central nervous system, it is probable that one may find a better correlation between the reserpine-binding capacity of a particular brain area and the total number of transmitter-containing vesicles found in that area. The possibility of ³H-reserpine being bound to non-monoaminergic storage vesicles should also be considered.

*2. Adrenal granules.* Evidence derived from experiments with several different types of isolated monoaminergic storage granules have all pointed to the granular membrane as the most likely site of irreversible reserpine binding. Adrenal medullary granules incubated with reserpine and then sedimented, washed, dialyzed overnight, and incubated with ¹⁴C-NE showed a 50% inhibition of amine uptake (76). Clearly, exposure of the granules to reserpine resulted in an irreversible binding of this uptake blocker since even prolonged dialysis could not reverse the inhibition.

Kirshner (77) and later Giachetti *et al.* (43, 44) examined the location of the granular binding site directly. After exposure

of isolated adrenal granules of 1 mμmole of $^{14}$C-reserpine, followed by granular lysis, about 30% of the added radioactivity was found associated with the insoluble residue. Incubation with higher concentrations of reserpine resulted in 50 to 80% of the label being bound irreversibly to the residue. Subsequent chloroform extraction and thin layer chromatography showed that the reserpine bound to the lysed granules remained as unchanged reserpine. The reserpine must have reacted irreversibly with the granular membrane rather than with any of the soluble intragranular constituents since those substances were lost after granular lysis. This has also been shown *in vivo* when it was found that bound reserpine was not released during sympathetic nerve stimulation (108) as are the soluble components (*e.g.*, chromogranins, dopamine-β-hydroxylase, *etc.*) of adrenergic granules (138).

Since reserpine does not block dopamine uptake and conversion to NE by the insoluble granular residue but does block uptake into intact granules (77), it is likely that the binding site of reserpine is restricted to the external portion rather than to the internal membrane surface. This finding suggests both that dopamine-β-hydroxylase is located on the interior surface of the intact granule membrane and that this enzyme is unaffected by reserpine directly. Presumably, then, the ability of reserpine to prevent amine uptake by intact granules is due strictly to an effect on the function of some component of the exterior membrane of adrenergic storage vesicles.

## VII. Binding to 5-HT Containing Cells

### A. Blood Platelets

*1. Reserpine binding.* Reserpine, in a manner similar to its action on the accumulation of NE, also inhibited the uptake of 5-HT into platelets (16, 63). The endogenous 5-HT of the blood platelets is localized primarily in special intracellular vesicles (96, 97) which have been isolated in essentially pure form by density gradient centrifugation and are the site of monoamine uptake *in vitro* (4, 33-35, 133). Incubation of platelets in plasma containing 0.2 μg/ml of reserpine results in a depletion of 5-HT (25, 97). Subcellular centrifugation of platelet homogenates showed that the granule-rich layer was the subfraction most sensitive to the depleting action of reserpine.

Studies on the intracellular localization of reserpine in these platelets (34, 36, 97, 127) established that the distribution of the $^3$H-reserpine accumulated by platelets was similar to that of 5-HT, namely a concentration within the 5-HT storing vesicles. A steady state distribution of drug was reached within 5 min after exposure (127). Approximately 80% of the reserpine found in the platelets was associated with a low density membrane fraction, with 50% of this reserpine being tightly bound to membrane fragments (97). This strongly supports the hypothesis that, in the platelets, reserpine exerts its action at the level of the intracellular storage organelle.

As mentioned previously, the proposed primary site of action of reserpine in blocking 5-HT uptake by platelets is the granular storage particles. This is an almost instantaneous phenomenon and therefore implies a rapid interaction with some biochemical component of these vesicles. Several workers (41, 98) examined the distribution of reserpine bound to subcellular fractions prepared from whole platelets. In addition, these groups further subfractionated lysed 5-HT storage vesicles into soluble and particulate components. An appreciable proportion of the total reserpine present in the platelets was always found to be tightly bound to the particulate subfraction. The degree of particulate binding seemed to be directly proportional to the amount of reserpine present in the incubation medium. These results, therefore, also point to the membrane of the storage vesicle having an important and irreversible binding affinity for reserpine.

It is interesting that platelets incubated in plasma accumulated much less reser-

pine than did those incubated in Tris-buffered saline. Platelets incubated in buffer containing albumin showed an intermediate reserpine uptake (127). Apparently, protein molecules can also serve as binding sites for reserpine with plasma proteins able to bind up to 80% of the reserpine incubated with it. Much of the binding of reserpine to proteins seems to be reversible since almost 70% of that bound to albumin was extractable (93) in contrast to the relative resistance to extraction of the reserpine bound to amine storage granules.

2. Binding of other depleting agents. It is important to point out that the distribution of tyramine and reserpine differ considerably within these organelles (36). The majority of $^{14}$C-tyramine is located in the granule supernatant while almost all of the $^{3}$H-reserpine remains irreversibly bound to the membrane components. These results support a hypothesis previously put forth that tyramine and reserpine reduce monoamine stores by different mechanisms (17-19, 79). Tyramine stoichiometrically displaces the monoamines bound to adenosine triphosphate and $Mg^{++}$ within the interior of the granules, while reserpine probably acts by altering the membrane characteristics of the organelles.

It is also of interest that guanethidine, an agent which has several pharmacological effects in common with reserpine, also can be accumulated in platelets (13) and sympathetically innervated tissue (14, 112, 119). Furthermore, reserpine has been demonstrated to interfere with guanethidine uptake and binding in rat heart in vivo (26). However, the subcellular binding sites of these two drugs may not be identical since studies with mast cells have not shown a mutual competition for uptake and have shown a temperature dependency for the accumulation for guanethidine, but not for reserpine (49).

### B. Mast Cells

Reserpine is known to decrease the endogenous content of 5-HT and to depress 5-HT uptake into mast cells (69-71). When these cells were exposed to 5 × $10^{-6}$ M reserpine (49), the drug was quickly taken up and retained. Intracellularly bound reserpine was concentrated approximately 75-fold over that present in the incubation medium (50) with incorporation being maximal at a pH of 7.5 to 7.7. The binding of reserpine increased as its medium concentration was increased and showed no tendency toward saturation, at least in the range employed. Steady state conditions seemed to be achieved in less than 40 min. The rate of incorporation was both independent of temperature (since the amount of reserpine accumulated was the same at 0° and 37°C) and independent of metabolic energy (since metabolic inhibitors and uncouplers of oxidative phosphorylation did not impair its incorporation).

Reserpine seems to be largely located in the amine storage granules found within the mast cells (49, 50, 72). The close correlation between the subcellular distribution of 5-HT and the subcellular binding of reserpine also suggests the mast cell granules as the site of binding of reserpine. Differential centrifugation of a disrupted mast cell population preloaded with reserpine yielded a nearly pure granular fraction. About 25% of the total reserpine present in the granules was firmly bound; another 40% was found loosely bound in the nuclear sediment. These results suggest both a specific, irreversible binding to the mast cell granules and a nonspecific, reversible binding to other mast cell components. The latter binding of $^{3}$H-reserpine probably is accounted for largely by the lipid solubility of reserpine.

### VIII. Reserpine Action and Amine Deficit

It is quite apparent that the depleting action of reserpine is not the result of a one-for-one displacement of reserpine for bound amine. Reserpine exerts its depleting effects through an interaction with a component of the amine granular membrane involved in actively taking up the biogenic amine. In this way reserpine-

treatment results in a granular deficit of amines in excess of that which could occur *via* stoichiometric displacement.

A number of research groups have estimated the approximate amine deficit produced by each mole of reserpine tightly bound to tissue. Reserpine-inhibited noradrenergic granules obtained from rat heart were calculated to have an impairment of the binding of about 500 moles of NE per mole of reserpine (2). This would mean that there were approximately 20 molecules of reserpine bound to each affected granule. DaPrada and Pletscher (34), working with platelet 5-HT, found that at the time of maximal 5-HT depletion, there was a deficit of 30,000 moles of 5-HT for each mole of reserpine bound. Granules obtained from peritoneal mast cells were much more resistant to the depleting action of reserpine since one molecule of the alkaloid was able to inhibit the uptake of only 6 molecules of 5-HT (50). Although the values obtained may differ because of the varying ways in which they are calculated, it may also be that different amine storage organelles possess different numbers of binding sites and/or affinities for reserpine.

## IX. Chemical Nature of Reserpine Binding

### A. Theories of Reserpine Accumulation

Several possibilities have been examined in an attempt to understand the primary factor or factors which influence the initial accumulation of reserpine within tissues. Simple diffusion, diffusion with subsequent ionic trapping and carrier-mediated uptake have all been considered.

Based on the knowledge that the internal pH of the blood platelet is acid compared to its surrounding medium (150), it was thought that weak organic bases such as reserpine might accumulate within the cell. If no appreciable cellular binding were to occur, then reserpine accumulation should be strictly a function of the external-internal hydrogen ion gradient. An examination of reserpine accumulation

*in vitro* at various external hydrogen ion concentrations clearly showed that the very large accumulation of reserpine was not markedly influenced by the hydrogen ion gradient (50, 127). Therefore, ionic trapping cannot play a major role in determining cellular accumulation of reserpine.

The existence of an active transport of reserpine across cellular membranes has also been studied. In these experiments reserpine was accumulated over a wide range of extracellular concentrations both in mast cells (50) and platelets (127) *in vitro*. A steady state was generally reached within 5 min. The observation that this uptake of reserpine increased proportionally with its medium concentration suggests that a carrier-mediated transport process is probably not involved in reserpine accumulation.

Cell penetration is most markedly affected by the lipid solubility of a drug, although the degree of ionization of the compound may also play a role. Reserpine is a very lipid-soluble compound, as evidenced by its solubility in chloroform and, thus, it is not surprising that it reaches a steady state tissue distribution very rapidly. The lipophilicity of reserpine seems to be the single most important factor in determining its cellular distribution. The accumulation of reserpine within tissues is primarily the result of a rapid permeation across lipid cell membranes, due to its lipophilic properties, followed by an extensive degree of binding to components within the interior of the cell. The reserpine found within the tissues exists initially as both reversibly and irreversibly bound drug. Presumably, it is the persistently bound fraction that is of functional significance in understanding the site and mechanism of action of reserpine.

### B. Strength and Permanence of Reserpine Binding

It is now generally accepted that the rapid initial decline in tissue reserpine levels represents the nonspecifically and reversibly bound portion of the drug. From

18 to 30 hr after drug administration, however, the amount of ³H-reserpine remaining in the tissues although small is irreversibly bound and is largely confined to the membranes of the monoamine storage granules (42). The permanence of the reserpine binding at this time is compatible with it being a covalent process. This is supported by the finding that both lipophilic extraction (e.g., peanut oil) (93) and prolonged dialysis (44) could not remove the alkaloid from its binding sites 24 or 60 hr after drug administration.

Lysis of the amine storage granules shows that the permanently bound reserpine is associated exclusively with granular membranes and not with the intragranular constituents (e.g., amines, adenosine triphosphate, soluble proteins) (36, 44). The irreversible nature of this binding is emphasized by the finding that incubation of prelabeled membranes with fresh unlabeled amine granules does not result in any exchange of ³H-reserpine (44).

It should be mentioned that a specific and persistent binding to granule membranes in vitro is not readily demonstrated. The reason for this is not as yet clear. Perhaps the formation of the covalent bonds is a relatively slow process which has not been sufficiently completed to be detectable during the usual 5- to 60-min incubation period. Alternatively, the incorporation of reserpine into the granular membranes may require a factor or active process which is present in vivo, but not in vitro.

### C. Binding to Membrane Lipids

Balzar et al. (5) have found that reserpine combines in vitro with vesicle membranes obtained from rabbit sarcoplasmic reticulum. The binding isotherms at 20 to 25°C extend from $10^{-7}$ to $10^{-4}$ M. The authors felt that the combination of high binding capacity and somewhat uniquely shaped binding isotherm reflected a special type of interaction between reserpine and the membrane constituents. When similar binding studies were conducted with lipids isolated from the vesicles it was clear that the binding capacity for reserpine resided exclusively in the membrane lipids.

Isolated vesicle lipids have been treated with phospholipases in an attempt to identify the lipid fraction most involved in reserpine binding. Particular attention was placed on the phospholipid fractions since they predominate in most metabolically active membranes (80). Drug binding was not impaired by removal of phosphatidylcholine by phospholipase C or by cleavage of the β-ester linkage by phospholipase A (5).

Of the remaining constituents only the unsaturated fatty acids proved to be capable of drug binding. This was supported by the observation that hydrogenation of the isolated phospholipids as well as of the membrane bound lipids completely abolished drug binding. Although unsaturated fatty acids were found in all lipid fractions except the lysolecithin fraction, there did seem to be some specificity of binding since the lecithin and cephalin fractions contained most of the drug. At saturating concentrations there were about four lipid molecules for each drug molecule.

Although the studies cited above all deal with reserpine binding to skeletal muscle sarcoplasmic vesicles, it is quite probable that the lipids in monoamine storage vesicles also are the site of reserpine binding.

The insoluble matrix obtained from both adrenal medullary vesicles (10) and adrenergic nerve vesicles (81, 82) are quite similar in their lipid composition. The nerve vesicles do have somewhat more phospholipid and cholesterol, but the cholesterol/phospholipid ratio is of the same magnitude (ca. 0.5). Lechithin and phosphatidylethanolamine are the major phospholipids found in both types of vesicles while lysolecithin is present in much larger amounts in adrenal granules.

It would seem reasonable to propose that, just as in sarcoplasmic vesicles, a primary site of reserpine binding would be to the lecithin portion of the granular membrane. Lysolethicin would seem to be

ruled out as a binding site since it is present only in relatively small amounts in adrenal adrenergic granules (10).

All of the sarcoplasmic reticulum studies quoted above were performed *in vitro* and therefore reserpine binding was readily reversible and temperature dependent (5). This is in accord with the already mentioned difficulty in demonstrating irreversible binding *in vitro* of reserpine to adrenergic and serotonergic vesicles. Since covalent bonds are obviously not formed during incubation *in vitro* the reversible interaction that does occur between reserpine and phospholipids may be primarily an electrostatic binding of the cationic portion of the drug with fatty acid sites on the phospholipids. Electrostatic binding to phospholipids *in vivo* may also account for the initial widespread distribution of reserpine in a variety of tissues. However, once this reversible binding is established in monoaminergic tissue other more permanent bonds may then slowly be formed.

## X. Metabolism

The duration and intensity of drug action are dependent upon the mechanisms by which animals can rid themselves of foreign substances. Through evolution and natural selection, organisms have developed specialized enzyme systems by which metabolism or detoxification of these foreign compounds can occur. Despite the large numbers of such foreign substances, most are biotransformed along a relatively few chemical pathways including oxidation, reduction, hydrolysis, and conjugation. These reactions are catalyzed by both specific and nonspecific enzymes which have been localized in greatest concentration in blood plasma, cell cytoplasm, mitochondria, and endoplasmic reticulum. Some of the pathways involved in the metabolism of reserpine are discussed below.

The rapid disappearance of reserpine from the plasma is due both to tissue uptake and metabolism. The major portion of intravenously administered reserpine is so rapidly removed from the blood that less

than 1% remains in the blood 2 min after injection. Observations reported by many research groups (38, 47, 57, 110, 122) indicate that this rapid disappearance primarily reflects degradation of the compound rather than extensive tissue binding. Since the most widely examined reactions involved in the biotransformation of reserpine have been the hydrolysis of its ester linkage and the oxidative demethylation of the TMBA portion of the molecule, these will be discussed in most detail. The principle reserpine metabolites which have been reported to occur either *in vitro* or *in vivo* are methyl reserpate, TMBA, reserpine acid, syringomethyl reserpate (SMR), syringic acid (SA), and carbon dioxide.

### A. Esteratic Hydrolysis

Hydrolytic reactions constitute one of the major ways in which drugs are broken down. Drug metabolism by hydrolysis is limited to those compounds having either an ester or amide grouping (48). Although both amides and esters undergo hydrolysis their end products are slightly different. De-esterification by an esterase results in the formation of an acid and an alcohol while deamidation by an amidase yields an acid plus an amine.

Methyl reserpate and TMBA (fig. 5) are the two principle metabolites which result from the biotransformation of reserpine by hydrolysis. This reaction involves the hydrolytic cleavage (see fig. 5) of the ester through the action of an as yet unidentified esterase(s) to an acid (TMBA) and an alcohol (methyl reserpate). Although a variety of both specific and nonspecific esterases are found in plasma, there are no studies available which have exclusively examined the participation of these enzymes in the metabolism of reserpine. Only hepatic esterase activity has been examined in any detail. It should be kept in mind, however, that since enzymes which hydrolyze drugs are found both in plasma and in the liver, the question of the relative importance of each to metabolize reserpine *in vivo* is still an open question.

There is no doubt, however, that both *in vitro* and *in vivo* hydrolysis of reserpine does take place, with TMBA and methyl reserpate being the breakdown products formed.

The route of administration seems to be an important factor in determining the relative rate of hydrolysis. For example, on oral administration to rats, 22% of the administered reserpine appears in the urine as methyl reserpate while the same dose given parenterally results in a urinary methyl reserpate level of less than 1% of the administered drug (47). Intestinal mucosa of the rat has been shown to hydrolyze reserpine (47, 124) although controversy still exists relating to the ability of human intestine to metabolize the drug (87, 94).

Several laboratories (121, 124, 128) have examined the *in vitro* metabolism of reserpine in liver slices as well as in subcellular fractions. Reserpine hydrolysis seems to be enzymatically mediated (128, 131) since the rate of hydrolysis *in vitro* is markedly affected by: 1) prior heating to 60°C, 2) substitution of albumin for hepatic tissue; 3) lower temperatures; and 4) increased amounts of enzyme protein.

Subcellular studies of hepatic reserpine-metabolizing activity *in vitro* indicate that the highest amount of hydrolytic activity is found in the microsomal fraction (121, 128). The hydrolysis of reserpine found to occur in other subfractions may be the result either of microsomal enzymes trapped in these fractions or of the presence of soluble esterases. Since the rate of reserpine hydrolysis in the supernatant fraction is relatively low (128, 131) it does not seem likely that reserpine serves as a substrate for the several soluble esterases that have been shown to be present in hepatic cytosol (39).

## B. Oxidation

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and $O_2$ were required for the microsomal metabolism of reserpine (131). This was an unexpected finding. It was suggested that an intermediate metabolic step may precede hydrolytic clevage since it would be highly unusual for an esterase to require NADPH as a cofactor (78). It was further proposed that this intermediate step involved participation by the microsomal mixed-function oxidase system. Precedence for such a hypothesis is found in the work of Hollunger (59, 60) who has shown that oxygen and NADPH are necessary for the microsomal enzymes which oxidize lidocaine to ethylglycinexylidide but are not required for the subsequent hydrolysis reaction. The finding that NADH could not replace NADPH as a cofactor in the microsomal metabolism of reserpine (128, 131) is in accord with previous observations (15, 45) dealing with microsomal drug oxidations.

The inhibition of reserpine metabolism by carbon monoxide (128, 131) and SKF 525-A (128) further supports the idea that the biotransformation of reserpine involves enzymes which work through an electron transport chain containing cytochrome P-450.

The intermediate oxidative step preceding reserpine hydrolysis probably involves the demethylation of the 4-methoxy group on the TMBA moiety (122). This has been suggested by the work of Sheppard and Tsien (121) who showed that when demethylation of the 4-methoxy group was inhibited, the rate of reserpine hydrolysis was much reduced. Rats given injections of $^{14}C$-reserpine, which has been labeled in the 4-methyl group of the TMBA portion, readily oxidize the methoxy-$^{14}C$ to $^{14}CO_2$ (122). Although one would presume that this carbon would go through a formaldehyde intermediate before being converted to $CO_2$, no evidence of such a product has as yet been demonstrated (128).

Two O-demethylated metabolites of reserpine have been isolated from urine and feces of experimental animals: syringoyl methyl reserpate (SMR) and syringic acid (SA). SMR is the ester form of SA and would be the product which resulted from the oxidative demethylation of the 4-meth-

oxy group of the TMBA portion of reserpine. The existence of SMR as a reserpine metabolite was established (124) with an elegant experimental design which employed both reserpine-(TMBA-methoxy)-$^{14}$C and reserpine-(TMBA-carboxy)-$^{14}$C. When the isotope was located in the carboxy position, it was not lost to $CO_2$ as it was when located in the 4-methoxy portion. Paper chromatography showed that $^{14}$C-SMR contributed to about half of the recovered radioactivity. The rat did not seem to hydrolyse SMR readily since free SA could not be detected in these experiments nor could free TMBA.

The possible appearance of metabolites of reserpine other than those mentioned above have been examined. For instance, the further metabolism of the reserpine hydrolysis product, TMBA, was investigated. When $^{14}$C-TMBA alone was given orally to mice (110), essentially all the radioactivity was recovered from the urine as unchanged TMBA in only 4 hr. Clearly much of this highly water soluble compound could be eliminated via renal excretion without the need of further metabolism. Although no evidence of ring cleavage or demethylation of TMBA was found in these studies, recently, measurable quantities of the glucuronide and sulfate conjugates of TMBA have been found in urine (152). Other compounds which have been ruled out as potential metabolites of reserpine are isoreserpine, reserpine N-oxide, didehydro-reserpine and tetradehydro-reserpine (123).

Attempts to impair the rate of reserpine metabolism in vivo have not met with notable success. SKF 525-A, an agent which has been shown to inhibit numerous microsomal reactions, had no effect on the rate of disappearance of $^{3}$H-reserpine from either the liver, heart, or whole body of the rat (100). This is not surprising, however, since reserpine could serve as a substrate for nonmicrosomal esterases which may be unaffected by SKF 525-A. Since enzymes which hydrolyze drugs are found in plasma, liver, and other organs, it is diffi-

cult to determine the relative importance of each with an in vivo system. A more direct approach was that of Stitzel et al. (131) who examined the in vitro inhibitory action of SKF 525-A. In those experiments SKF 525-A added directly to the incubation flasks did impair reserpine metabolism, and did so in a concentration-dependent manner.

The apparent contradiction between the negative in vivo and positive in vitro results may be resolved in the following manner. Reserpine administered in vivo is probably metabolized primarily by a number of nonhepatic esterases. Many of these enzymes would be unaffected by SKF 525-A (78) and therefore this inhibiting agent would have little effect on the overall hydrolysis of reserpine. However, since reserpine also can be metabolised by hepatic microsomal enzymes, studies in vitro should then reveal any direct inhibitory action of SKF 525-A on reserpine microsomal metabolism. Although SKF 525-A does inhibit reserpine metabolism in vitro, it is not completely clear at what point the inhibition takes place. Either SKF 525-A inhibits a hepatic esterase as it does procaine esterase (144), or it inhibits reserpine metabolism at a point before hydrolytic cleavage. Support for the latter hypothesis has already been suggested by the finding that inhibition of demethylation of the 4-methoxy group reduces the rate of subsequent hydrolysis.

## C. Factors Affecting in Vitro Microsomal Metabolism

1. Incubation conditions. Reserpine metabolism by both mouse (131) and rat (128) liver microsomes was shown to be dependent on NADPH and oxygen. Metabolism did not occur if these cofactors were excluded from the incubation medium. Frozen microsomes showed decreased hydrolytic activity only after 28 days of storage at $-20°C$. Reserpine metabolism was found to be both temperature and pH sensitive with the greatest rate of biotransformation occurring at 37°C and at a pH of 7.

*2. Sex differences.* Male and female rats have been shown to metabolize some foreign compounds at different rates. However, when the influence of sex on the hydrolysis of reserpine was examined no differences were observed (128).

*3. Inducibility.* The ability of reserpine metabolism to be increased by pretreatment with either phenobarbital or 3-methylcholanthrene, two different types of enzyme-inducing agents, was also studied (128). At first glance at the data it seems that neither agent increased the rate of reserpine hydrolysis. The ability of phenobarbital to induce reserpine metabolism should not be completely ruled out, however. If the oxidative demethylation of reserpine to SMR, but not its subsequent hydrolysis to TMBA, was specifically increased then we should expect to see a decrease in the amount of TMBA formed after phenobarbital with either no change or a slight decrease in reserpine levels. This is exactly what has been observed (128). The final proof for such a speculation would lie in the measurement of SMR formation both before and after phenobarbital pretreatment. Unfortunately, SMR concentrations were not examined in that study.

*4. Species differences.* Sheppard and Tsien (121) studied the metabolism *in vitro* of reserpine with liver slices from the dog, pigeon, rabbit, mouse, rat, and guinea pig. The rat had the greatest ability to oxidize the 4-methoxy carbon of TMBA to $CO_2$, while the pigeon and rabbit were devoid of such activity. However, rat, dog, and pigeon liver slices did not readily hydrolyze reserpine to form free TMBA. On the other hand, guinea pig liver was very active in this regard, hydrolyzing about 80% of the reserpine. Glazko *et al.* (47) found high levels of methyl reserpate in rat tissues, but practically none in the dog. Rabbits also hydrolyze reserpine extensively *in vivo* (57).

In a 1974 study (128) in which the comparative rates of ³H-reserpine microsomal metabolism among a number of common laboratory species were examined, the mouse and rat showed the greatest ability to hydrolyze reserpine. The dog and cat hydrolyzed reserpine at somewhat slower rates while the guinea pig had the lowest activity. The latter study differs in the relative order described by previous groups (47, 121). The differences may be due in part to the way in which the results were expressed. The former studies did not attempt to correlate the individual species either with the specific activity of the reserpine hydrolyzing protein or with the total amount of reserpine metabolized, but reported metabolism as percentages of recovery of a given dose of reserpine. The expression of the data in terms of per g of liver or per mg of microsomal protein may provide a more quantitative assessment of the comparative rates of hepatic metabolism among the various species.

Studies with human beings (87, 94, 152) all confirm that there is extensive metabolism of reserpine *in vivo*. Most individuals showed virtually no free reserpine in the urine after drug administration although some was detectable in feces. The major metabolites were methylreserpate and TMBA and their glucuronide or sulfate conjugates. It should be remembered, however, that all of the data pertaining to human beings thus far available are obtained from urinary and fecal sampling. No information exists which is solely derived from hepatic metabolism.

All of the above studies relating to species differences in reserpine metabolism seem to allow certain speculations to be put forward. Firstly, it is quite likely that all species are capable of hydrolyzing reserpine to form methyl reserpate and TMBA *in vivo*. Presumably several of the variety of plasma and tissue esterases present in the intact animal participate in the enzymatic hydrolysis of reserpine. Secondly, the liver and intestine are probably the organs responsible for the largest amount of reserpine breakdown. Thirdly, reserpine is in part metabolized by the same hepatic microsomal enzymes which

participate in many drug oxidative reactions. This idea is supported by the observation that other drug substrates, particularly ester compounds, can compete for the microsomal metabolism of reserpine (128).

### D. Effects of Reserpine on Selected Enzymes

*1. Monoamine oxidase.* In addition to being acted upon by degradative enzymes, reserpine has been shown to alter levels of endogenous enzymes involved in a variety of metabolic processes. Several papers (64, 66, 67) have been published dealing with the stimulatory effect of reserpine on tissue monoamine oxidase activity (MAO). Both brain and heart MAO activity can be increased after a single dose of reserpine. Heart slices and mitochondria taken from guinea pigs 2.5 hr after reserpine showed much higher MAO activity than did control tissue. This stimulation of heart mitochondrial MAO by reserpine was observable as early as 1 hr after drug treatment, reached maximum activity after 2 to 5 hr and declined thereafter, although some increased MAO stimulation was still detectable after 24 hr. Reserpine did not increase mitochondrial MAO when added *in vitro,* however, nor did it increase deamination of substrates other than NE.

One possible mechanism by which reserpine stimulates heart mitochondrial MAO would be through reserpine-induced damage to the mitochondrial membranes (65). This could lead to an alteration in activity, since MAO activity is related to the structural state of the mitochondria (1, 109). Reserpine, particularly at higher doses, seems to have an appreciable effect on mitochondrial integrity. These ultrastructural changes include swelling, fusion, alterations in the cristae and the appearance of myelin-like structures in the mitochondria (65, 132, 142, 143). The work ofd Iwayama *et al.* (65) have made it quite apparent that the changes induced by large doses of reserpine are not the consequence of transmitter loss, but rather are due to a direct toxic effect on cells. Other studies

demonstrating a toxic action of large doses of reserpine on tissues have been reported previously (106, 146, 147).

*2. Tyrosine hydroxylase and dopamine-β-hydroxylase.* When sympathetic neuronal activity is increased, there is a rapid acceleration of NE formation (141). The enhanced rate of catecholamine synthesis seems to be regulated by a feedback control of tyrosine hydroxylase (TH) activity. In addition to this rapid regulation of TH activity, the total amount of enzymatically active TH present in the adrenergically innervated tissue can also be altered. When reserpine interferes with the function of the sympathetic nervous system, there is an increase in the amount of TH (103, 104, 115, 151) and dopamine-β-hydroxylase (99) in the adrenal medulla. Because protein synthesis inhibitors prevent the elevation of activity (102), reserpine administration is thought to trigger induction or derepression of TH.

*3. Microsomal enzymes.* Short-term reserpine administration leads to an induction of at least one microsomal enzyme, *i.e.,* aniline hydroxylase (129). The increased microsomal metabolism produced by reserpine more closely resembled that caused by polycyclic hydrocarbons than it did that of the phenobarbital-type of inducing agent. Both polycyclic hydrocarbons and reserpine, but not phenobarbital, increased microsomal enzyme activity after a single injection.

It was postulated that the increased aniline metabolism might be due more to a reserpine-induced starvation than to a direct effect of reserpine. Both reserpine administration and starvation result in similar histological changes in hepatocytes (145), including prominent increases in smooth-surfaced endoplasmic reticulum. This structure has been shown to be the subcellular component most directly involved in microsomal drug oxidation.

### XI. Excretion

Relatively little intact reserpine is eliminated *via* the various excretory routes. In

the intact animal much of the administered reserpine is hydrolyzed to methyl reserpate and TMBA, and it is these metabolites which have been most extensively examined in studies of drug elimination.

## A. Renal Elimination

In the rat about 8% of the injected dose of reserpine (4-methoxy-^{14}C), as measured by total ^{14}C, appeared in the urine within 6 hr, with the amount at 24 hr being about 14% (122–124). In the guinea pig, approximately 20% of the injected dose was excreted in the first 24 hr with some ^{14}C still being eliminated for up to 96 hr (121, 124). Of all species examined, mice showed the highest rate of drug elimination, with approximately 50% of the administered radioactivity appearing in the urine within 24 hr (110), mostly as TMBA. Once TMBA is formed it apparently does not undergo further metabolic breakdown, but is rapidly cleared by the kidneys, with essentially all of the ^{14}C-TMBA appearing in the urine within 4 hr after its oral administration (110).

Studies with human being shows that the average excretion of methyl reserpate per hr was 1.43 $\mu$g, with the 24-hr rate of urinary excretion amounting to about 6.4% of the daily dose of reserpine (94). Although some ^{3}H-reserpine was detectable, most of the radioactivity in the urine was identified as metabolites (87, 152). The initial rapid excretion of approximately 0.6% of the administered dose/hr ($T_{1/2}$ = 4.5 hr) was followed by a much slower phase of tritium elimination ($T_{1/2}$ = 46 hr). Some tritium was still found in the urine on the 11th day after ^{3}H-reserpine administration. There is little doubt that this activity was derived from ^{3}H-reserpine since there was no evidence of [3]H-$H_2$O being present (87). These long drug retention times are in agreement with the data of Numerof et al. (111), who found radioactivity in the blood of schizophrenic patients 6 weeks after a single dose of ^{14}C-reserpine.

Changes in urinary pH, specific gravity, or the presence of proteinuria did not seem to result in major alterations in reserpine excretion (152). Although patient sample size was small, there was also no evidence that diabetes, age, sex, decreased renal function, or the concomitant administration of other drugs, had any significant effect on the urinary excretion of reserpine (152). Obesity, however, may play a role in the rate of elimination of this highly lipid-soluble drug since the plasma half-life of radioactivity was increased and the urinary excretion decreased in one obese patient.

## B. Fecal Elimination

A considerable degree of variability is found in the amount of reserpine and its breakdown products that have been found in feces. Contributing to this variability is the effect that the route of administration has in determining the amount of drug eliminated. However, even when reserpine is administered intravenously rather than orally, appreciable amounts of reserpine and its metabolites are found in the feces (121, 122). This points to the possibility of an enterohepatic shunt of reserpine from the liver into the small intestine with its subsequent elimination.

The studies of Numerof et al. (110) show that 24 hr after an oral dose of ^{14}C-reserpine about 15% of the administered radioactivity was found in the gastrointestinal (GI) tract. Most of this activity was present in the fecal matter with only small amounts of activity being associated with the stomach, small intestine, and large intestine. When ^{14}C-reserpine was given intravenously, nearly 30% of the administered dose was associated with the feces. Again only traces of radioactivity were found in the tissues of the GI tract. Furthermore, there was a higher percentage of the total fecal radioactivity present in the form of ^{14}C-reserpine than as metabolites. Fecal excretion of unchanged reserpine was about 8% after an oral dose and 35% after intravenous administration.

This finding would seem to suggest reserpine itself as the molecular species undergoing biliary transport (124).

Oral doses of reserpine administered to rats were largely hydrolyzed in the GI tract (46, 47) and it was shown that the intestinal mucosa contributed to this hydrolytic cleavage. No significant degree of hydrolysis was found when the intestinal mucosa of the dog and monkey were used. It seems that in the rat, at least, a considerable fraction of orally administered reserpine is hydrolyzed in the intestinal tract before absorption.

More than 60% of the tritium-labeled reserpine given orally to human beings (87) was recovered in 96 hr from the feces, with most of this appearing as intact ^{3}H-reserpine. Even on the 12th day after administration fecal samples still contained about 0.15% of the tritium administered. ^{3}H-reserpine given intravenously (152) to human patients also appeared in the feces, with about 30% of the intravenously injected drug appearing in the feces during the first 96 hr. The radioactivity present in a 24-hr fecal sample was about one-half intact ^{3}H-reserpine. Samples obtained on the 2nd and 3rd days after drug administration showed only 0.1 of the radioactivity present as intact ^{3}H-reserpine.

The above results indicate that human beings also can transport intact reserpine *via* the biliary system from the liver to the small intestine for elimination. It is not clear, however, whether or not reserpine undergoes extensive biliary recirculation before final excretion. The prolonged blood levels seen after ^{3}H-reserpine administration (134) may, in part, reflect such a continued intestinal reabsorptive process, although extensive tissue storage probably is the most dominant factor involved.

Labeled TMBA, when given orally in equimolar doses as reserpine, appeared only in minute amounts in the feces (110). Over 90% of the ^{14}C-TMBA was found in the urine as the unchanged material. Clearly, TMBA is absorbed almost completely from the GI tract where it is subse-

quently cleared almost quantitatively by the kidneys. Alkaline hydrolysates of guinea pig fecal material after intravenously administered reserpine contained only TMBA, whereas those from rats contained levels of SA amounting to almost 4% of the injected dose.

## C. Pulmonary Excretion

Very little data are available relative to the pulmonary excretion of $CO_2$ derived from the metabolic breakdown of reserpine. The principle study is that of Sheppard *et al.* (122) who examined the cumulative excretion of [14]$CO_2$ as a function of time after an intravenous injection of reserpine (TMBA-methoxy-^{14}C). These authors found that almost 25% of the injected radioactivity was given off as [14]$CO_2$ in the first 6 hr. Although this seems to represent a large amount of oxidation of the 4-methoxy group of TMBA, it is in line with the observation that almost 20% of the radioactivity from reserpine (TMBA-methoxy-^{14}C) metabolized by liver slices *in vitro* appears as [14]$CO_2$. When the ^{14}C was located in the carboxy carbon of TMBA, the label was not lost to $CO_2$ but appeared in the feces as part of the oxidation product, presumably as SMR. The guinea pig also possesses some ability to oxidize the 4-methoxy carbon to yield $CO_2$ (124).

## XII. Summary

Orally administered reserpine is readily absorbed from the GI tract. During this process at least a portion of the drug is metabolized by the intestinal mucosa and then presumably is acted upon by serum esterases. Methylreserpate and trimethoxybenzoic acid are the primary metabolites which result from the hydrolytic cleavage of reserpine. Since most of the blood leaving the GI tract passes through the liver *via* the portal vein, hepatic metabolism would also be expected to reduce reserpine levels in the blood. The relative contributions of serum esterases *versus* hepatic metabolism in the biotransformation of reserpine *in vivo* are not known. However,

very little unmetabolized reserpine is eventually eliminated in the urine.

In the liver, it is quite likely that both microsomal oxidative and hydrolytic enzymes contribute to the metabolism of reserpine. It seems that microsomal oxidation (such as the demethylation of the 4-methoxy group on the TMBA moiety) must precede hydrolysis since inhibition of demethylation markedly reduces the rate of hydrolysis. In addition to oxidation and hydrolysis, conjugative reactions also must occur in liver or extrahepatic tissues since both glucuronide and sulfate conjugates of TMBA have been identified.

Some reserpine molecules do seem to escape metabolism, however, since significant amounts of intact reserpine have been found in fecal samples taken from both experimental animals and human beings after either oral or parenteral drug administration. Presumably reserpine is transported from the blood via the biliary tree into the small intestine where it is either reabsorbed or eliminated in the feces. Pulmonary elimination of $CO_2$ produced after complete oxidation of the 4-methoxy group of TMBA has also been shown to occur both in vivo and in vitro.

The following may serve as a model for the relationship between the subcellular distribution of reserpine and its site of action. After a single intravenous injection most of the reserpine, probably loosely bound to plasma albumin, is distributed to tissues on the basis of their blood flow. Because of its lipophilic properties, reserpine would easily penetrate cell membranes and then bind possibly electrostatically to intracellular membrane components, particularly those rich in phospholipids. Much of the circulating reserpine would then either be metabolized or be taken up by the lipid depots of the body, leading to a rapid redistribution of the reversibly bound reserpine from the tissues. During this time a relatively small fraction of the total reserpine administered by injection would become associated with monoaminergic granular membranes in a

more specific and irreversible manner. This would result in a persistent, nonstoichiometric inhibition of monoamine uptake. Such a small specific binding would not be detectable for at least 18 hr after reserpine administration, i.e., until most of the reversibly bound alkaloid had been metabolized and/or excreted. In such a model, inhibition of amine uptake would continue until sufficient quantities of newly synthesized uninhibited granules arrived in the nerve terminals to replace those permanently impaired by reserpine.

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