TOXICOLOGICAL AND BIOCHEMICAL INVESTIGATIONS OF ALPHA-CHACONINE IN POTATO (SOLANUM TUBEROSUM L.) TUBERS: PHYSIOLOGIC DISPOSITION AND TISSUE BINDING, AND INHIBITION OF TISSUE CHOLINESTERASES AND ISOENZYMES

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Toxicology

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Sydney Obodoechina Alozie
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ABSTRACT

Toxicological and Biochemical Investigations of Alpha-Chaconine in Potato (Solanum tuberosum L.) Tubers: Physiologic Disposition and Tissue Binding, and Inhibition of Tissue Cholinesterases and Isoenzymes

by

Sydney O. Alozie, Doctor of Philosophy

Utah State University, 1977

The distribution, absorption, metabolism and tissue binding of radioactivity were studied in hamsters after oral and intraperitoneal administration of alpha-chaconine-\(^{3}H\). The material was well absorbed from the gastrointestinal tract and nearly 22 percent of the label was excreted via urine and feces in 7 days. The excretion was higher in urine (21 percent) than in feces (<1 percent). Tissue concentrations of radioactivity peaked at 12 hours following oral administration, with the highest concentrations found in lungs, liver, spleen, skeletal muscle, kidney and pancreas, with heart and brain containing moderate amounts. Concentrations of radioactivity in tissues following intraperitoneal administration were significantly higher than those observed after oral treatment. Excretion of chloroform-soluble products in the feces was 10 times higher than that of the chloroform-insoluble metabolites after both oral and intraperitoneal administration. In the urine, the activity was predominantly in the chloroform-insoluble form and the chloroform-soluble metabolites were relatively minor in
amounts (0.27, 0.85, and 2.45 percent versus 0.005, 0.14 and 0.19 percent of dose for 12, 24 and 72 hours, respectively). After 7 days, the chloroform-soluble metabolites in urine increased to 20 percent of the excreted radioactivity, while the amount of chloroform-insoluble metabolites was less than 1 percent. Subcellular distribution of the labeled compound indicated the highest concentration of radioactivity in the nuclear and microsomal fractions of brain, liver and heart tissues. A small amount of radioactivity, shown by a minor peak, was also observed in the fractions between the mitochondrial and microsomal fractions on a sucrose gradient. Binding of radioactivity was observed in brain, testes, kidney, lung, liver and heart. All of the label in the brain appeared to be in the bound form. The results indicated that alpha-chaconine is slowly absorbed from the gastrointestinal tract after oral administration, and persists in various tissues, much of it in bound (non-extractable) form (in nuclear and microsomal fractions).

Excretion of alpha-chaconine-(\(^3\)H) and its metabolites was investigated after oral and intraperitoneal administration in hamsters. The separation of the glycoalkaloid and its metabolites in feces and urine was accomplished by thin-layer chromatography. An increase in the concentration of excreted alpha-chaconine metabolites in feces and urine was observed. In urine over 50 percent of the eliminated radioactivity during the initial 24 hours was due to the aglycone, solanidine. The fraction of the total dose administered which was excreted represented only 27 percent (26 percent in feces and less than 1 percent in urine) during the 7 day test period. Contrary to the general belief
that potato glycoalkaloid absorption is poor following oral administration, only 5 percent or less was excreted in feces during the initial 72 hours, a fact explained by the binding of radioactivity to tissues.

Inhibition of acetylcholinesterases by alpha-chaconine was studied. The inhibition of purified erythrocyte acetylcholinesterase and horse serum cholinesterase by alpha-chaconine was found to be a mixed-type with kinetic constants. An inhibition constant (Ki) for both the specific and pseudocholinesterases was $8.3 \times 10^{-6}$ M and $4.0 \times 10^{-4}$ M, respectively. Kinetic constants obtained for both enzymes were as follows: $V_{\text{max}}$ of $7.14 \times 10^{-5}$ and $3.76 \times 10^{-4}$ moles/liter/min, respectively, and $K_m$ of $6.2 \times 10^{-5}$ and $1.33 \times 10^{-4}$, respectively.

The distribution of acetylcholinesterase among the subcellular fractions of rat brain homogenate separated by sucrose density gradient centrifugation was determined, as well as the inhibition pattern of these fractions following *in vitro* incubation with $0.016$ M alpha-chaconine. Enzyme activity was found to be distributed equally between the mitochondrial and microsomal fractions, with the nuclear fraction having the least activity. Percentage inhibition of the various fractions obtained was: whole homogenate 43, nuclear fraction 55, mitochondria 35, and microsomes 33.

Brain acetylcholinesterase activity of animals given intraperitoneal doses (10, 30, 60 mg/Kg) of alpha-chaconine was 79, 55 and 18 percent of the control group. Acetylcholinesterase activity of heart and plasma of animals administered alpha-chaconine did not show the dose-related response observed in the brain. Inhibition of heart acetylcholinesterase was 61 percent, while plasma gave 51 percent for
the rats given a dose of 10 mg/Kg and no inhibition for rats given 30 mg/Kg.

Acrylamide gel electrophoretic separation of cholinesterases in aqueous homogenates from whole brain and heart of adult male rats administered alpha-chaconine was investigated. Brain acetylcholinesterase isoenzymes were found to be inhibited by 30 and 60 mg/Kg dosage levels of alpha-chaconine administered intraperitoneally. Electrophoretic separation of plasma from the treated animals resulted in five anodally migrating zones having properties of cholinesterases. These sites hydrolyze acetylthiocholine and alpha-naphthylacetate, and all were inhibited by alpha-chaconine except the slowest migrating band (band 5). Inhibition of isoenzyme activity of bands 1 and 2 is observed for the groups administered 10 and 30 mg/Kg alpha-chaconine with the percentage inhibition of both bands (1 and 2) being 40 and 77 percent for animals given 10 mg/Kg and 100 and 75 percent for the latter group. Isoenzyme bands 3 and 4 were completely absent in the alkaloid treated animals. Inhibition of non-specific cholinesterase isoenzymes (butyrylthiocholine hydrolyzable bands) by alpha-chaconine was clearly demonstrated.

In vitro inhibition of plasma, erythrocyte and brain esterase isoenzymes was estimated by incubating gels with $10^{-4}$ M alpha-chaconine after the electrophoretic separations. With this concentration of alpha-chaconine, the various isoenzymes in rat plasma, erythrocyte and brain showed some response to the inhibitory potency of alpha-chaconine. The slower-moving isoenzyme bands were inhibited to 100 percent with the different concentrations of inhibitor. The
fast migrating isoenzyme bands in plasma and erythrocytes were least affected by alpha-chaconine ($10^{-4}$ M), with no inhibition. Plasma protein isoenzymes from adult male rats were not affected by alpha-chaconine.

(129 pages)
INTRODUCTION

Considering all the chemicals present in our food supply, including natural components, agricultural chemicals, food additives, and natural and man-made contaminants, it might be said that the greatest unknown aspect involves the natural components of our foods. It is estimated that the normal components of natural food products constitute about 99% of the weight of our daily diet, with food additives, pesticide residues, and other contaminants making up the balance. The normal and natural constituents of foods contribute the greatest amount and variety of the chemical substances consumed by man. In the general toxicological context of the diet of man and other animals, therefore, the natural chemical components of foods can be regarded as a major part of his chemical environment.

The potato (*Solanum tuberosum* L.) tuber is widely used as a major food crop in some parts of the world. The importance of potatoes as a food source is underscored by its nutritive value, being rich in carbohydrates, and containing appreciable amounts of proteins and some minerals. Several compounds are produced in white potatoes, and their presence can be influenced by physiological stress conditions imposed on the tissues. Some of these compounds, which are normal constituents of the potato tubers, increase in concentration due to stress, viz.: (a) solanum glycoalkaloids--solanine, chaconine, leptines and leptinines (*Zitnak, 1964, 1968; Kuhn and Löw, 1954, 1955a, 1955b); (b) phenolic compounds--caffeic and chlorogenic acids; and (c) coumarin-type compounds--scopolin and scopoletin. Some other compounds
produced only as a response to stress include \( \alpha \)- and \( \beta \)-solarmarine, terpenes--rishitin, rishitinol, phytuberin and lubimin. Stress conditions in potatoes may result from exposure to light, injury, chemical toxicants, and other environmental factors. The resultant stress condition can either alter normal metabolic pathways of metabolism, or give rise to new compounds.

The problem of greening of potato tubers with a concomitant bitterness and off-flavor is of great concern to commercial potato growers. From the human and animal health standpoint, it is also of concern to the consumer due to the apparently toxic constituents. The green color is due to chlorophyll development, while the bitter taste is caused by the accumulation of toxic alkaloids. Toxicological and pharmacological investigations have been conducted on solanine, while information on the toxicity and pharmacology of \( \alpha \)-chaconine, representing nearly 60\% of the total potato alkaloids, is scanty. Toxicity of other new potato glycoalkaloids (e.g., \( \alpha \)- and \( \beta \)-solammarine) has not been investigated.

Current interest in the toxicology of these glycoalkaloids has been rekindled by the alleged association of certain congenital malformations in newborn infants with the consumption of blight infected potatoes by expectant mothers. These defects include anencephaly (absence of portions of the brain) and spina bifida (abnormally incomplete closure of the vertebral column) (Renwick, 1972). The exact nature of the substances present in potatoes capable of causing these defects is not known. Most of the terpenoid stress metabolites are known to be toxic to various fungi, but no data on animal toxicity are as yet available.
Inhibition of acetylcholinesterases and other esterases by naturally occurring non-protein inhibitors has been the subject of several investigations (Orgell et al., 1958; Orgell, 1963; Pokrovski, 1956; Harris and Whittaker, 1959, 1962). Inhibition of the esterases by extracts of potato tissue (tubers, sprouts and leaves) was shown to affect the three serum cholinesterase phenotypes in a way similar to the action of dibucaine. Further characterization of the cholinesterase inhibition pattern has shown that α-solanine and solanidine differentially inhibited the serum cholinesterase of persons of usual, intermediate and atypical phenotypes (Harris and Whittaker, 1962). Since acetylcholinesterase (AChE) activity varies in different parts of the rat brain, a possibility, therefore, exists that these differences may be due to specific alterations in the isoenzymes.

Interest in the study of isoenzymes and their responses to physiological and pathological processes in the organism has been tremendous in recent years, especially in the isoenzyme status of brain acetylcholinesterases (AChE, EC 3.1.1.7), and cholinesterase (ChE, EC 3.1.1.8). Both enzymes constitute the choline-ester hydrolases in certain tissues and organs. While the physiological role and natural substrates of AChE are unknown, interest in its ultrastructural localization and molecular behavior has developed. The increased permeability of excitable membranes to ions during electrical activity is known to be triggered by series of reactions initiated by acetylcholine (ACh). Acetylcholinesterase catalyzes the hydrolysis of this ester, thus permitting membrane permeability to return to its resting state (Nachmansohn, 1959, 1966). On account of the vital role of
these enzymes in the processes involved in the conduction of nerve impulses in nerves and muscle fibers, they have received major attention in recent years. Kinetic studies of these enzymes have provided a better understanding of the mechanisms of their reactions with various substrates, inhibitors, and the molecular forces in the active sites. These studies have further contributed to a better understanding of the modes of action of the various drugs which affect the central nervous system.

The use of high resolution polyacrylamide gel electrophoresis in conjunction with quantitative procedures has demonstrated that rat brain AChE can exist in multiple molecular forms: -- isoenzymes (Vijayan and Brownson, 1974; Davis and Agranoff, 1968; LaMotta et al., 1970; Christoff et al., 1966; Chan et al., 1972; McIntosh and Plummer, 1973; Wentold et al., 1974). Physiochemical and enzymatic properties of AChE isoenzymes from rat brain differ (Bajar and Zizkovsky, 1971) in contrast to the identical properties of the two isoenzymes from human brain (Bernsohn et al., 1963). Alterations in esterase isoenzymes including AChE have been reported during development in several animal species, including the rat (Igbal and Talwar, 1971; Lagnado and Hardy, 1967).

Due to the existence of multiple esterase isoenzymes, major differences in the specific isoenzymes may not be reflected in total esterases, including both specific and non-specific cholinesterases. Electrophoretic separation of the isoenzymes, thus, becomes a valuable technique for the qualitative and quantitative study of the esterases, especially the inhibition specificities of various inhibitor compounds.
It, therefore, becomes pertinent to examine electrophoretically as well as otherwise, those tissues in which cholinergic mechanisms are known to function, to establish the characteristics of the various separable cholinesterases with respect to their response to the cholinesterase-inhibiting potato alkaloids.

Most of the studies on mammalian toxicity of potato glycoalkaloids with respect to acetylcholinesterases have been carried out using biochemical assays and very little effort made towards a differential inhibition of multiple molecular forms of the enzyme. Changes have been shown to occur in the isoenzymes in the absence of significant alterations in the total activity of the enzyme (Vijayan and Brownson, 1975). In view of this apparent relationship between AChE activity and solanine (or its structural analogs), a study to identify any inhibitor sensitivities of the isoenzymes becomes necessary.

In consideration of the available information relating to the status of some of the toxic glycoalkaloids in potatoes, the following investigations were undertaken:

1. Absorption, distribution and metabolism of the steroidal glycoalkaloid, \( \alpha \)-chaconine-(\(^3\)H) and tissue binding of radioactivity in hamsters following intraperitoneal and oral administration.

2. Acetylcholinesterase inhibition by \( \alpha \)-chaconine with respect to

   a. Inhibition kinetics of acetylcholinesterase and other cholinesterases by the steroidal glycoalkaloid \( \alpha \)-chaconine (\textit{in vitro} and \textit{in vivo}).

   b. Cholinesterase isoenzyme changes following the administration of several doses of \( \alpha \)-chaconine to adult male rats.
Several excellent reviews on the chemistry of the solanum alkaloids in potatoes have been published (Schrieber, 1968; Heftmann, 1967; Wood, 1976; Jadhav and Salunkhe, 1975). The constituents of potato tubers can be divided into several categories depending on the physiological condition of the tubers.

The first category includes those compounds normally present in tubers and whose concentration may be increased as a result of stress. This group includes the following.

**Solanum alkaloids**

The alkaloids in potatoes are glycosides with the same alkamine, with a steroidal base (aglycone)-solanidine(5-solaniden-3-ol), but differ with respect to their sugar moieties. The aglycone contains an alcohol hydroxyl group, a reducible double bond between C-5 and C-6 and a tertiary nitrogen. The molecular formula is C$_{27}$H$_{23}$O$_{4}$OH (Bently, 1965). $\alpha$-Solanine and $\alpha$-chaconine constitute the major glycoalkaloids in potato tubers, accounting for about 95% of the total glycoalkaloids (Guseva and Paseshnichenko, 1958) in a 40% and 60% ratio, respectively (Allen and Kuč, 1968). Solanine and chaconine differ only with respect to their sugar chain (Figure 1). The sugar portion of $\alpha$-solanine contains galactose, D-glucose and L-rhamnose, while the $\alpha$-chaconine has one mole of D-glucose and two moles of L-rhamnose per mole. Other
Figure 1. Structure of solanidine, α-solanine and α-chaconine.
naturally occurring glycoalkaloids, found in minor or trace amounts, are solacaulin, leptines and leptinines (hydroxy and acetoxy-derivatives of solanines and chaconines) (Zitnak, 1968; Schrieber, 1968). The \( \beta \) - and \( \gamma \)-solanine and the \( \beta \) - and \( \gamma \)-chaconines are regarded as products of the partial hydrolysis of \( \alpha \)-solanine and \( \alpha \)-chaconine, respectively.

The concentration of the above glycoalkaloids present in tubers, peels and leaves has been shown to increase under various conditions of ultraviolet light exposure, fungus infection and, in some instances, mechanical wounding and slicing (Wu and Salunkhe, 1976; McKee, 1955; Salunkhe et al., 1972). An increase in these alkaloids in incubated noninoculated sliced tubers has been noted by Locci and Kuc (1967), thus contributing to the conclusion that their concentration increases in response to physiological stress. Shih et al. (1973) also observed a significant suppression of alkaloid accumulation following the inoculation of the cut surface with Phytophthora infestans. The above observations have led to the suggestion of an alteration of the biosynthetic pathways for glycoalkaloid production by fungus infection (Shih et al., 1973).

**Phenolics**

The prominent phenolic compounds found in potato tubers are chlorogenic acid and caffeic acid (Figure 2). The principal phenol accumulating in a cut tissue is chlorogenic acid, while caffeic acid is formed by hydrolysis of chlorogenic acid, the process facilitated by certain fungi (Kuc et al., 1956; Sakuma and Tomiyama, 1967; Lee and LeTourneau, 1958). Exposure to light of low intensities has been
Figure 2. Phenols and coumarine-like compounds in white potatoes.
shown to double the synthesis of chlorogenic acid in the dark (Zuker, 1963, 1965).

Coumarin-like compounds

These include scopolin and scopoletin (Figure 2). Mechanical wounding in addition to other stress conditions of infection by micro-organisms induces accumulation of these compounds (Clark, 1969).

Stress-induced products of potatoes

Certain compounds, not normally found in healthy potatoes, are produced in response to certain stress conditions including aging of sliced potatoes and inoculation of potatoes with some fungi (Kuć and Currier, 1974; Shih and Kuć, 1974). The stress products identified to date include certain alkaloids, e.g., α- and β-solamarine containing the spirosolane, tomatidine (Tomatid-5-en-3-ol) as the aglycone, and some terpene-like compounds. Tomatidinol is the aglycone of the alkaloids, in the woody nightshade—a toxic member of the potato family, Solanum dulcamara. Structurally, solamarines are related to the solanidine alkaloids with respect to their trisaccharide moiety. α-Solamarine and β-solamarine have identical sugar components with α-solanine and α-chaconine, respectively. Terpenoid compounds produced in response to stress include rishitin, rishitinol, phytuberin, and lubimin vertispirane derivatives (Kuć, 1973) (Figure 3). Levels of accumulating of these compounds are known to be fungitoxic around the site of infection, probably acting as a disease resistance mechanism.

Rishitin (C_{14}H_{22}O_{2}) is a bicyclic norsesquiterpene alcohol (Katsui et al., 1968) originally isolated from the Rashiri cultivar
Figure 3. Structure of some Solanum steroid alkaloids.
of potatoes (Tomiyama et al., 1968). Rishitin accumulates to significant levels in sliced tubers inoculated with some fungi. Traces of rishitin have also been found with another terpene, phytuberin \((C_{17}H_{26}O_4)\) in some potato cultivars inoculated with \(P.\ infestans\) (Varns et al., 1971). The hydroxyl group on carbon 3 in the molecule is important for its antifungal activity (Ishizaka et al., 1969).

Phytuberin, an aliphatic unsaturated sesquiterpene acetate (Hughes and Coxon, 1974) was isolated by Varns et al. (1971). A sesquiterpenoid alcohol, rishitinol \((C_{15}H_{22}O_2)\) has also been identified in potatoes infected with \(P.\ infestans\) with the structure and configuration deduced by a combination of spectral, chemical, and synthetic methods (Katsui et al., 1973).

Other terpenoids include Lubimin \((C_{15}H_{24}O_2)\), a sesquiterpenoid hydroxyaldehyde, with a greater antifungal activity than rishitin (Metlitskii et al., 1971) and two vetispirane derivatives, spirovetival-\((10), 11\)-diene-2-one and spirovetiva-\(1(10)-3,11\)-triene-2-one (Coxon et al., 1974).

**Biosynthesis of Solanum Alkaloids**

On the basis of structural similarity between various steroidal compounds, saponins, terpenes, hormones, and alkaloids, a common biosynthetic pathway has been postulated for these compounds. The sequences involved in the biosynthesis of the steroids and terpenoids proceed via the acetate-mevalonate-isopentenyl pyrophosphate-squalene and cholesterol pathway. The similarity of the carbon skeleton of the aglycone moiety of the alkaloids-solanidine with that of cholesterol,
is evidence for assuming a common precursor for both compounds. Being derivates of nitrogen-free compounds, steroid alkaloids have been considered pseudoalkaloids from the synthetic viewpoint (Wood, 1976).

A thorough review of the biochemistry and biogenic relationship of the steroid alkaloids of the solanum group has been documented by several investigators (Heftmann and Mosettig, 1960; Heftmann, 1963; Schrieber, 1968; Jadhav and Salunkhe, 1975). A brief account of the salient points covered by the above review is discussed below.

Initial tracer experiments on the biogenesis of alkaloids in potatoes, by Guseva and Paseshinichenko (1958), demonstrated the uptake and utilization of acetate-2-$^{14}$C and DL-mevalonic acid-2-$^{14}$C as intermediates. Glycoalkaloids isolated from sprouts grown under normal light illumination showed a concentration of the labeled $^{14}$C in the aglycone, while sprouts grown in the dark revealed the label in the sugar portion. Mevalonate was also found to be a more effective precursor than acetate in potato seedlings (Guseva et al., 1961). Recent studies by Jadhav et al. (1973) have shown the incorporation of labeled carbon from $\beta$-hydroxy-$\beta$-methylglutaric acid (HMG), leucine, alanine, and glucose into the glycosidic steroidal alkaloids from potato sprouts. The glycoside moiety contained a higher amount of radioactivity than aglycone portion, and this was assumed to be indicative of glycosylation following administration of labeled glucose (Jadhav et al., 1973).

Figure 4 shows generalized pathways of biosynthesis of the solanum alkaloids. Glycosylation of solanidine by crude enzyme preparations from potato sprouts or tubers has also been carried out (Jadhav, 1973). On the basis of the above observations, a hypothesis suggesting a
Figure 4. Pathways of biosynthesis of the solanine alkaloids.
\[ \text{ACETIC ACID (C}_2\text{)} \rightarrow \text{MEVALONIC ACID (C}_6\text{)} \]

\[ \text{FARNESYL PYROPHOSPHATE (C}_{15}\text{)} \rightarrow \Delta^3 \text{-ISOPENTENYL PYROPHOSPHATE (C}_5\text{)} \]

\[ \text{SQUALENE (C}_{30}\text{)} \rightarrow \text{CHOLESTEROL (C}_{27}\text{)} \]

\[ \text{GLYCOALKALOIDS (C}_{45}\text{)} \quad (R=\text{SUGARS}) \rightarrow \text{SOLANIDINE (C}_{27}\text{)} \]
stepwise synthesis of α-solanine and α-chaconine from solanidine has been postulated. Synthesis of the stress metabolites in plants, especially potatoes, is postulated to involve several pathways, including: (a) shikimic acid, (b) acetate-malonate, and (c) acetate-mevalonate pathways, or (d) a joint participation of the above pathways (Kuć, 1966). Pathways for the biosynthesis of caffeic and chlorogenic acid from phenylalanine are shown in Figure 5, while Figure 6 shows the synthetic pathways for phenolic and isoprenoid compounds. It has been suggested that the biosynthesis of glycoalkaloids and terpenoid compounds in stressed potatoes involves the same pathways. Using 14C-labeled acetate and mevalonate, it was observed that the accumulation of glycoalkaloids and rishitin in potato tubers inoculated with P. infestans arises from de novo synthesis using the acetate-mevalonate pathway (Shih and Kuć, 1973). The branch point in the synthetic pathway leading to rishitin or the glycoalkaloids appears to be after mevalonate (Wood, 1976).

The unsolved problem in the biosynthetic pathway of the solanum glycoalkaloids is the origin of the nitrogen atom in the aglycone moiety. One hypothesis suggests a cyclization of cholesterol in the side chain prior to the formation of 27-hydroxycholesterol, followed by a direct replacement of the -OH group by an amino function (Heftmann, 1967).

Toxicology and Pharmacology of Solanum Glycoalkaloids

Chronology of poisoning episodes

Potato glycoalkaloids have been implicated, during the last 75 years, as the causative agent in numerous cases of poisoning in man
Figure 5. Biosynthetic conversion of phenylalanine to caffeic and chlorogenic acids.
Figure 6. Generalized pathways for the biosynthesis of phenolic and isoprenoid compounds. [Shih, 1972.]
and farm animals resulting from the consumption of green potatoes. Several reviews contain accounts of poisoning in man and farm animals attributed to potatoes (Harris and Cockburn, 1918). The prominent poisoning episodes due to potatoes will be outlined below.

Leipzig, Germany (1918). An outbreak of poisoning involved 41 persons and all indications implicated potatoes. Investigations revealed an alkaloid content equivalent to 430 mg/kg of tubers, and this represented solanine or other unidentified alkaloids (Rothe, 1918).

Glasgow, Scotland, England (November 1918). One death was reported out of 61 cases investigated. Analysis of potatoes suspected of causing poisoning showed a solanine content of 410 mg/kg potato (Harris and Cockburn, 1918). Other incidences of potato alkaloid poisoning have been reported by Bömer and Matti (1923) and Wiliimott (1933).

United States of America (1924). A well-documented case of poisoning from potato occurred in Illinois, where seven members of a family of nine were poisoned by eating green potatoes. Two deaths were reported in this episode (Hansen, 1925).

Symptoms of poisoning common to all the cases described included nausea, vomiting, abdominal pain, depression, and exhaustion.

Toxicological and pharmacological studies

The earliest experiments establishing the toxicity of solanine, using laboratory animals, was carried out in 1895 by Meyers. Attempts at isolating solanine or its metabolites from the urine of a dog fed 0.1 gm alkaloid mixture per day for 10 days proved unsuccessful. Studies on the distribution, excretion and metabolism of α-solanine-3H have been carried out by Nishie et al. (1971), following oral
administration of labeled α-solanine, residual radioactivity after 4 days was distributed in the following tissues and organs: kidneys, liver, spleen, lungs, abdominal fat, blood and heart in order of radioactivity concentration. Excretion of radioactivity after an oral dose of 5 mg/kg of α-solanine-³H reached a 90% level after 24 hours, with a urine excretion of 6% and 72% in feces. After 4 days, 94% of the radioactive dose was excreted, with urine accounting for 10%, while feces accounted for 85%. On the basis of the disposition of α-solanine obtained from the above studies, the low oral toxicity of α-solanine has been attributed to: (a) poor absorption from the gastrointestinal tract as evidenced by low blood concentration, (b) rapid urinary and fecal excretion of metabolites, and (c) partial and/or complete gastrointestinal hydrolysis of solanine to the less toxic solanidine.

Differences in sensitivity to total potato alkaloid and to α-solanine shown by several animal species have been tabulated in Table 1. A detailed list of symptoms of toxicity has been described by Nishie et al. (1971) and Patil et al. (1972). Studies with rabbits receiving 10-30 mg/kg solanine ip showed an increased rate of respiration of 18-76% above the control within the 1-2 hour period, later (2-6 hours) decreasing to 18-76% below normal. The early tachypnea caused whole body movement, while the drop in respiratory rate was associated with a cyanotic state (Nishie et al., 1971). In order to determine the characteristic pattern of death associated with solanine toxicity, Nishie et al. (1971) also studied electroencephalogram (EEG) and electrocardiogram (ECG) signals along with respiration of rabbits. Death was preceded by a specific initial appearance of an isoelectric
Table 1. Evaluation of α-solanine toxicity.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dose of α-solanine</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Administration</td>
<td>Amount</td>
<td></td>
</tr>
<tr>
<td>Human\textsuperscript{b}</td>
<td>Oral</td>
<td>2.8 mg/kg</td>
<td>Toxic\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>20-25 mg\textsuperscript{c}</td>
<td>Toxic\textsuperscript{d}</td>
</tr>
<tr>
<td>Sheep</td>
<td>Oral</td>
<td>225 mg/kg</td>
<td>Toxic\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>500 mg/kg</td>
<td>Lethal</td>
</tr>
<tr>
<td></td>
<td>Intravenous</td>
<td>17 mg/kg</td>
<td>Toxic\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>Intravenous</td>
<td>50 mg/kg</td>
<td>Lethal</td>
</tr>
<tr>
<td>Pregnant rat</td>
<td>Oral</td>
<td>10% of sprout diet</td>
<td>Death of all pups before weaning age</td>
</tr>
<tr>
<td>Rat</td>
<td>Gastric intubation</td>
<td>590 mg/kg</td>
<td>50% death within 24 hours</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal</td>
<td>75 mg/kg</td>
<td>50% death in a few hours</td>
</tr>
<tr>
<td>Mice</td>
<td>Oral</td>
<td>1000 mg/kg</td>
<td>Nontoxic</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal</td>
<td>42 ± 1.8 mg/kg</td>
<td>50% death in 7 days</td>
</tr>
<tr>
<td></td>
<td>Intraperitoneal</td>
<td>10 mg/kg</td>
<td>Toxic\textsuperscript{d}</td>
</tr>
</tbody>
</table>
Table 1. Continued.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dose of α-solanine</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Administration</td>
<td>Amount</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td></td>
<td>32.3 mg/kg</td>
<td>50% death</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td></td>
<td>50 mg/kg</td>
<td>Lethal</td>
</tr>
<tr>
<td>Chick embryo</td>
<td>Injection into</td>
<td>18.8 ± 1 mg/kg</td>
<td>50% mortality in</td>
</tr>
<tr>
<td>Chick embryo (4-day-old)</td>
<td>yolk sac</td>
<td>18 days</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>Intraperitoneal</td>
<td>20 mg/kg</td>
<td>Overnight death</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Death in 2.5-24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>hours, recovery if</td>
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<td></td>
<td></td>
<td></td>
<td>survived for at</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>least 24 hours</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td></td>
<td>30 mg/kg</td>
<td>Death in 6.25 hours</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
<td>Intravenous</td>
<td></td>
<td>10 mg/kg</td>
<td>Death in 50 minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Death in 2 minutes</td>
</tr>
</tbody>
</table>

Source: Jadhav and Salunkhe (1975).

In a case of potato poisoning (total alkaloid).

Determined from potatoes consumed (total alkaloid).

General symptoms of food poisoning.
EEG tracing with a concomitant decreased respiratory rate. Indication of an initial central nervous system depression was seen in the simultaneous cessation of breathing and EEG signals. Occurrence of a terminal ventricular fibrillation, deep cyanosis, and a near-unconsciousness usually preceded death.

Studies of the synergistic and/or antagonistic effects of some drugs on α-solanine toxicity have been carried out. Administration of atropine sulfate (2 mg/kg), pargyline hydrochloride (5 mg/kg), and amphetamine sulfate (5 mg/kg) ip 30 minutes prior to the administration of 40 mg/kg α-solanine showed death rates of 5/10, 8/9 and 10/10, respectively (Patil et al., 1972). Mice receiving amphetamine were quite active even after solanine injection. Atropine reduced the lethal effects of solanine with a reduction of death rate from 9/10 (without atropine) to 5/10 (with prior atropine). Satoh (1967) showed that hyperglycemic effect with high blood sugar level, induced by solanine, could be prevented by a prior pretreatment with some adrenergic blockers or reserpine.

Postmortem examination results observed in the experiments of Patil et al. (1972) and of Gull et al. (1970) do not reveal any definite lesions directly related to the toxicity of α-solanine. Recent investigations into the toxicity of the steroidal glycoalkaloids, solanine and α-chaconine, have revealed some interestingly new findings with respect to their potency.

Comparative study of the acute and chronic ip administration of alkaloidal (α-chaconine, α-solanine, total glycoalkaloidal extracts) and phenolic compounds (chlorogenic and caffeic acids) from *Solanum*
*tuberosum* L. was carried out in pregnant and non-pregnant (female) rats by Chaube and Swinyard (1976). The acute LC$_{50}$ (95% confidence limits) for $\alpha$-chaconine, $\alpha$-solanine and TGA-extract were 84 (65.6-107.5), 67 (52.3-84.7), and 60 (35.7-100.8) mg/kg, respectively.

Chronic administration of $\alpha$-chaconine, $\alpha$-solanine and TGA-extract to non-pregnant rats for 2 days (40 mg/kg/day) and 8 days (20 mg/kg/day) resulted in 40 and 42% mortality, respectively. Daily injections (ip) of $\alpha$-chaconine (5-20 mg/kg) in days 5-12 to pregnant rats resulted in maternal (40-66%) and fetal death (15-100%). $\alpha$-Solanine and the TGA-extract on the other hand were found to be lethal only to the fetus (17-86%).

Recent studies carried out in our laboratory by Sharma et al. (unpublished) show that $\alpha$-chaconine is more toxic than $\alpha$-solanine. The greater toxicity of $\alpha$-chaconine than that of $\alpha$-solanine is in agreement with the pathological lesions produced in kidney and liver. The histopathological effects observed were: severe congestion and leucocytic infiltration of the kidney and liver; renal tubular degeneration and hepatic centrilobular necrosis were also observed in animals fed chloroform-extracts of *Alternaria solani* (Sharma et al., unpublished).

The toxicity of chlorogenic and caffeic acids, present in plants used as human food (Sondheimer, 1961) is unknown. Coumarin, derivatives of which are used clinically as anticoagulants, is known to be toxic to man (Quennville et al., 1959; Mahairas and Weingold, 1963; Saidi et al., 1965; Fillmore and McDevitt, 1970) and animals (Kraus et al., 1949). Coumarin and scopoletin have been shown to cause a reduction of litter size in rats, without being teratogenic (Ruddick et al.,
1974). A subsequent report by Chaube and Swinyard (1976) has shown negative effects with respect to mortality (maternal and fetal) and incidences of neural tube defects, following daily injections of chlorogenic acid (5-500 mg/kg) and caffeic acid (40-187.5 mg/kg) on days 5-12 of gestation. Some 21-day-old fetuses showed abnormalities, however. The route of administration was thought to contribute to differences in the sensitivities observed.

**Cholinesterase inhibition**

Association of cholinesterase inhibition with the toxicity of α-solanine was established by Orgell et al. (1958), Pokrovskii (1956), Harris and Whittaker (1959, 1962), and Orgell (1963). The presence of cholinesterase inhibitor(s) in aqueous extracts of potato tissue (tuber, sprouts, leaves, flowers, and stem) was established by Orgell et al. (1958). Tuber peel was later shown to contain 10-40 times the concentration of the inhibitor present in the innermost flesh, while extracts of tuber sprouts showed the same activity as the extracts of the tuber peel. Similar inhibitors have also been found in other members of the potato family, foliage and roots of tomato (*Lycopersicon esculentum* Mill cv. Commune Bailey), fruit of eggplant (*Solanum melongena* L.) and leaves of tobacco (*Nicotiana tabacum* L.). Zitnak (1960), on the basis of the possible presence of α-solanine in potato-plant extract, and the similarity of the distribution of solanine with the inhibition pattern of the various parts of the potato plant, associated the potato alkaloid with the cholinesterase inhibitor in the experiments described by Orgell et al. (1958).
Further experiments by Harris and Whittaker (1962) substantiated the anticholinesterase action of α-solanine by showing that these compounds differentially inhibited three genetically-different forms of human serum cholinesterase. The three phenotypes were "usual," "intermediate," and "atypical," with individuals belonging to the atypical groups, presumably, less susceptible to the toxic effects of α-solanine than persons with the "usual" type enzyme. A survey of the relative inhibitory capabilities of 139 alkaloid, glycosides, and similar compounds on human plasma cholinesterase was carried out by Orgell (1963). The carbamates, neostigmine, and physostigmine, were much more toxic to the enzyme system than all other compounds tested with relative inhibitory values of 239.0 and 1850, respectively. The total glycoalkaloids, leptine l, solanine, and demissidine showed values of 9.9 and 8.6 and 7.0, respectively.

Studies by Patil et al. (1972) on the pattern of plasma and erythrocyte cholinesterase inhibition of α-solanine in rabbits, and the effects of cumulative doses of solanine on AChE in a dog, typified solanine as a weak-to-moderate inhibitor of both specific and non-specific cholinesterase in the rabbit. Their study showed less inhibition of cholinesterase in the erythrocytes than in the plasma in vivo. This differential inhibition was attributed to: (a) different distribution of α-solanine at these sites, (b) differences in the mechanism of inhibition of the enzyme erythrocytes and plasma and/or (c) dilution of red blood cells, especially in cases of reversible inhibition. In the dog, small doses of solanine showed a quick inhibition followed by a rapid recovery of serum cholinesterase without inhibition of red-cell cholinesterase.
Esterase inhibition

The toxic action of some AChE inhibitors, organophosphorus (OP), and methylcarbamate chemicals, acting in both insects and mammals is initiated by inactivation of AChE at localized sites in the nervous system. Inhibition of AChE reduces the normal rate of hydrolysis of the neurohormone acetylcholine (ACh), a mediator of synaptic transmission in both mammals, but not in insects (O'Brien, 1967). The localized nature of inhibition makes it difficult to evaluate the degree of inhibition required for either a disruption of normal nerve function or a lethal effect. It does require a combination of data from in vitro enzyme assays, histochemical and neurophysiological studies (Metcalf, 1971; Booth and Lee, 1971; Holmstedt, 1971).

AChE reacts with both inhibitors and normal substrates in accordance with the following equation (Eq. 1):

\[
EH + AB \rightleftharpoons a \rightarrow EHAB \rightleftharpoons b \rightarrow BH + EA
\]

(a) The free enzyme EH combines with the esterifying agent AB to form an intermediate complex EHAB, which may dissociate or undergo reactions to chemically modify the enzyme; (b) the esterified enzyme EA is formed with loss of the leaving product BH; (c) the esterified enzyme is hydrolyzed to generate the free enzyme and liberate the acid AOH; or (d) the acidic group is modified while at the same site on the enzyme, giving EA' (Casida, 1973).

Contrary to the earlier notion on the nature of the active zone in AChE, the currently accepted view shows the existence of several
binding sites (types $\alpha$, $\beta$, $\gamma$) grouped around the esteretic site involved in the complex formation (a). This step involves several binding forces: hydrophobic, van der Waals and coulombic forces and change-transfer complex formation (Casida, 1973; Hetnarski, and O'Brien, 1972). The ($\alpha_1$, and $\alpha_2$) binding site(s) could represent the site formerly called the anionic site and also necessary for binding quartenary ammonium compounds and some alkylating agents: a site at the catalytic center, partly hydrophobic, is known to be involved in the binding of many organophosphorus and methylcarbamate compounds (O'Brien, 1971). This site binds other acylating agents. The esterification of the enzyme in step (b) has also been shown to involve reaction with the esteratic site or subsites involved in each of the esterification and desterifications (O'Brien, 1971).

The existence of multiple molecular forms of both acetylcholinesterases and serum cholinesterases has been confirmed. The different molecular forms, with different molecular weights, has been attributed to sub-unit aggregation (Chan et al., 1972; McIntosh and Plummer, 1973; Wenthold et al., 1974). LaMotta et al. (1968) demonstrated the interconvertibility of the various molecular forms of serum cholinesterases and suggested that the multiple forms were polymers of varying size formed by aggregation of a common polypeptide sub-unit. The molecular forms have been classified as isoenzymes on the basis of their similar substrate specificity, inhibitor susceptibility and their interconvertibility. Molecular weights of the serum isoenzymes as determined by acrylamide-containing density-gradient centrifugation were shown to be 82,000, 110,000, 170,000, 200,000, and 260,000 (LaMotta et al., 1970).
The various isoenzymes have been shown to be inhibited at different rates by organophosphorus compounds and certain carbamates (Main, 1969; Reiner et al., 1965; Chiu et al., 1972).

**Teratogenicity of solanum alkaloids**

The postulated relationship between the incidences of congenital malformations in the offspring of pregnant women consuming blighted potatoes by Renwick (1972) has stimulated the current wave of research activity into the toxicity of the solanum glycoalkaloids in potatoes, as well as the stress products of blighted potatoes. The birth defects of greatest concern are spina bifida and anencephaly. Anencephaly is characterized by the absence of the cranial vault with the cerebral hemispheres completely missing or reduced to small masses attached to the base of the skull. Spina bifida is characterized by a cleft or abnormal opening in the spinal cord. Although the embryogenesis of spina bifida in man is well known (Swinyard et al., 1973), the etiology of this birth defect is unknown.

Results obtained from earlier experiments by Poswillo et al. (1972) initially supporting the above postulated relation have since been refuted by later studies (Poswillo et al., 1973; Chaube et al., 1973; Swinyard and Chaube, 1973; Renwick, 1974). In Poswillo's earlier studies, marmosetts were used as the experimental animals. Four of the 11 fetuses recovered from animals given blighted potato concentrate showed gross abnormalities on macroscopic examination. The severity of the defects observed was related to the duration of the dosing period, and the defects included cranial dysplasia (Poswillo et al., 1972).
Studies on the toxicity of the stress compounds or their metabolites in potatoes subjected to infection with Phytophthora infestans, slicing and aging have been carried out by Wood et al. (1976). Injection of alcohol extracts from the stressed potatoes into incubated and non-incubated fertile chick eggs, via the yolk-sac, produced abnormalities which were not specifically associated with any of the compounds produced in potatoes. Negative results were observed in the embryos injected with alcoholic extracts containing the terpenoid constituents. Embryos injected with glycoalkaloids from non-inoculated, infected and aged tissues showed moderate and scattered abnormalities. The abnormalities included crossed beaks, deformed eye cavities, malformed wings; while spinal and brain defects were absent (Wood, 1976). Doses given were 400 mg/egg for the terpene-containing extracts, and about 80 mg/egg for the glycoalkaloid-containing extracts.

Further studies of the teratogenic effects of solanine and glycoalkaloids from potatoes infected with Phytophthora infestans in early chick embryos have been conducted by Mun et al. (1975). Injections of the above compounds were made into fertile chicken eggs between 0 and 26 hours of incubation. Embryos from eggs injected 130-200 μg/egg (yolk sac injection) showed 22-27% mortality and 16-22% abnormal embryos. Abnormalities observed included absence of the tail or trunk below the wing bud (rumplessness). A significant proportion of the abnormal embryos showed malformations that were related to this condition, including fluid- or blood-filled vesicles in the lower trunk or tail region on either one or both sides of the neural tube (Mun et al., 1975).
Other experiments on the teratogenic potency of these solanum alkaloids and/or the compounds resulting from the infection by fungus have shown negative results. Ruddick et al. (1974) have shown that feeding of freeze-dried blighted potatoes (75 g/kg/day) to rats during days 1-22 of gestation failed to elicit any teratogenic characteristics. Negative results were also obtained with oral administration of α-solanine, α-chaconine, and scopoletin on days 6-15 of gestation.

Experiments to determine the teratogenic potency of blighted potatoes (potatoes infected with *P. infestans*), solanine, and total glycoalkaloids extracted from potato blossoms were conducted by Swinyard and Chaube (1973). Teratogenic effects were not observed in pregnant rats given blighted potatoes on days 7-16 of gestation. At doses of 5 and 10 mg/kg/day (ip), given on days 5-12 of gestation, solanine and total glycoalkaloids (suspended in carboxymethyl cellulose) were found to be non-toxic to maternal and fetal rats; however, minor abnormalities were observed (about 44%). By comparison, on a mg/kg/day basis, the total glycoalkaloid sample was about seven times more toxic to fetuses at 10 mg/kg/day than was solanine. This has led to the suggestion that the total glycoalkaloid contained other toxic substances (Swinyard and Chaube, 1976). Administration of solanine (5 mg/kg, ip injection) to pregnant rabbits on days 0-8 of gestation produced absorption and resorptions with no teratological effects. Also, yolk sac injections of 50-20 mg/kg of solanine in embryonated chick eggs by the above researchers produced 63-90% mortality with no significant teratogenic effects. Adult rhesus monkeys were also killed (48 hour) following a single ip injection of 40 mg/kg of total
glycoalkaloid extract, as was the case with two injections of solanine (20 mg/kg).

Despite the results so far obtained, some of which have refuted Renwick's hypothesis, a great deal of research is being carried out in this area, as well as into other areas of the toxicology of the glycoalkaloids or the toxic metabolites produced in response to stress conditions. Recent studies dealing with the problem of alkaloid toxicity have included the use of such animals as mini-swine and hamsters (Sharma et al., 1977).

Teratogenicity of steroidal alkaloids from the plant Veratrum californicum has been established in fetal rats, rabbits, hamsters and sheep, especially for those alkaloids with a terminal furanopiperidine group (Keeler, 1964, 1970a, 1970b, 1975a). Potential teratogenicity of some solanum alkaloids such as α-solamanarine (spiro-solanes), which is structurally related to cyclopamine, the teratogenic steroid alkaloid in Veratrum californicum has been investigated (Keeler, 1973). The spirosolanes, like veratrum, possess terminal furan and piperidine rings, but with the furan moiety fused to the steroidal portion of the molecule rather than to the piperidine ring. Based on the similarity in structure between both groups of alkaloids, Keeler (1973) postulated that potato alkaloids possessing a terminal furan and piperidine ring could be teratogenic. Early results showed the spiroslane alkaloid, solasodine, to be non-teratogenic in rats (Keeler, 1973). But subsequent tests on solasodine at doses considerably higher than those given to rats produced teratogenic effects in hamsters. Hamsters were gavaged once on days 7 or 8 of gestation with
solasodine suspension (180 mg/3ml water). The compound produced 48 deformed offspring in 23 of 89 litters surviving pregnant dams. Deformities observed were characterized as exencephaly, cranial bleb, and spina bifida, each occurring with the same frequency (Keeler et al., 1975b, 1976). Animals that were 'exencephalic' had disproportionately small and malformed heads with a prominent dorsocranial defect exposing the brain. Exposure extended from the cerebral hemispheres to caudal mesencephalic. Tectum cranial blebs consisted of soft protuberances and discolorations of the skin in the interparietal midline overlying compressed or contused brain and meningeal tissues, and this has been assumed to represent a mild form of exencephaly (Keeler et al., 1976).

Feeding of air-dried ground sprouts of the Kennebec potato to hamsters on days 7, 7½, or 8 at 500 mg has been shown by Keeler et al. (1976) to be teratogenic. The sprout preparations produced 64 abnormal offspring in 26 of 113 (23%) litters from surviving pregnant dams. Deformities observed by the above group included exencephalics and cranial blebs representing 80%, and spina bifida with some traces of microophthalmia, the latter two being less common (Keeler, 1976).
MATERIALS AND METHODS

Chemicals

Erythrocyte acetylcholinesterase, horse serum cholinesterase, butrylthiocholine iodide, acetylthiocholine iodide, 5,5'-dithiobis-2-nitrobenzoid acid (DTNB), $\alpha$-napthyl acetate $\alpha$-napthylbutyrate, and fast blue BB were obtained from Sigma Chemical Company, St. Louis, Missouri.

Acrylamide monomer, $N,N'$-tetramethylethylenediamine (TEMED), bromophenol blue, coomassie brilliant blue R-250, were all obtained from Eastman Organic Chemicals, New York, New York.

Other chemicals and reagents obtained from other sources included: Silica Gel G (Applied Science Laboratories, State College, Pennsylvania), sodium hydride and methyl iodide (J. T. Baker Chemical Company, Phillipsburg, New Jersey), dimethyl sulfoxide (DMSO), ACS certified (Fischer Scientific Company, Fairlawn, New Jersey), Aquasol Reagent (Universal Liquid Scintillation Cocktail), Protosol Reagent (tissue and gel solubilizer), $\alpha$-chaconine-($^3$H) (G) (New England Nuclear Corporation, Boston, Massachusetts).

Physiologic Disposition

Methods

Studies were carried out with male Golden hamsters (Simonson Laboratories, Gilroy, California), 130-150 g body weight. Animals were maintained on commercial laboratory chow throughout the
duration of the experiment. Food was withdrawn 12 hours before treatment.

Randomly labeled α-chaconine-{³⁵H} with an initial specific activity of 2.5 Ci/m mole) was diluted with unlabeled chaconine to obtain the desired specific activity. Unlabeled α-chaconine was obtained by a preparative TLC separation of the mixture of potato alkaloid isolated from freeze-dried potato sprouts by the method of Gull and Isenberg (1960). The mixture of ammonia-precipitable alkaloids, twice recrystallized from ethanol, was dissolved in methanol:water (4:1). Chaconine, solanine and solanidine were separated on preparative thin-layer plates coated with Silica Gel G (1 mm thickness) and developed in a butanol:acetic acid:water (10:3:1) solvent system. Location of the bands was accomplished by spraying 1-inch strips on both edges of the plates with antimony trichloride while protecting the rest of the plate. Identity of the bands was determined on the basis of Rf comparisons (Sinden et al., 1973), and by chromatographic comparisons of the solanine and chaconine from potato sprouts with commercial solanine and labeled α-chaconine-{³⁵H} (Figure 7). Melting point (Thomas-Hover-Arthur H. Thomas Company, Philadelphia, Pennsylvania) was 243°-246°C (literature value 243, Kühn and Low, 1954). The diluted labeled compound was dissolved in a dimethyl sulfoxide-propylene glycol mixture (3:7) (Nishie et al., 1971). The animals received 10 mg/kg tritiated-chaconine (250 mCi/ml) ip or po given at a concentration of 5 mg/ml and at the rate of 2 ml/kg. Each animal was then housed in a metabolism cage which allowed for the separation of feces and urine.
Figure 7. Thin-layer chromatography of some potato glycoalkaloids on Silica Gel G. [Solvent system used: butanol:acetic acid: water (10:3:1). A = α-chaconine from potato sprouts obtained by preparative TLC; A' = standard α-chaconine-(3H); B = commercial α-solanine with traces of α-chaconine and β-chaconine; C = solanine separated in our laboratory.]
At specified times (3, 12, 24, 72, and 168 hours) after administration, the hamsters (three animals per time interval) were anesthetized with ether and exsanguinated by cardiac puncture. The following tissues were removed: heart, lungs, liver, kidney, large intestine (including contents), stomach and contents, spleen, pancreas, skeletal muscle, brain and testes. About 100 mg of each tissue were weighed out in triplicate and carefully dissolved in 1 ml of Protosol at about 55°C for 20 to 24 hours. Following dissolution of the tissue, the solutions were cooled to room temperature (22°C) and decolorized with 30% aqueous hydrogen peroxide (H₂O₂) where applicable (Herberg, 1960). Fifteen ml of scintillation fluid were added and the vials were shaken vigorously. All samples which were decolorized with H₂O₂ were later acidified with 0.5 M HCl to eliminate any chemiluminescence usually associated with decolorization (Herberg, 1960). Blood samples (0.3 ml of whole blood) were dissolved in glass vials with Protosol and treated in a similar manner as the tissues.

Radioactivity of all samples was determined in a Beckman LS-133 liquid scintillation spectrometer (Beckman Instruments, Incorporated, Fullerton, California) using two channel ratios. A linear correlation between channel ratio and counting efficiency was developed and used to obtain the counting efficiency in every sample. Efficiency value obtained was applied to the observed net count rate (count per minute) of the sample to give the sample disintegration rate (dpm). Results were expressed as ng/gm equivalent to α-chaconine based on the specific activity of the dosing solution.
Aliquots of urine (1 ml) were transferred to vials and counted for radioactivity. Thereafter, distilled water was added to the urine specimens to a final volume of 2 ml. The mixture was extracted three times with chloroform and the chloroform layers were combined and evaporated to dryness at 37°C. Radioactivity present in the water layers was measured by liquid scintillation counting. Feces were extracted three times with 20 ml acetone and the acetone layers were combined and counted for radioactivity. The material was dried by evaporation, then the acetone residue was washed twice with petroleum ether, and extracted three times with 10 ml chloroform. The aqueous and chloroform phases were counted for radioactivity.

Subcellular distribution of α-chaconine-(3H) in tissues

Tissues from three animals given chaconine were pooled and homogenized 20% w/v in 0.25 M tris-HCl (pH 7.2) buffered sucrose in a glass homogenizer with a Teflon plunger. The homogenate was centrifuged at 900 g for 10 minutes to remove nuclei and intact cells. The supernatant fluid (4 ml postnuclear fraction) was layered carefully on the top of density gradient at 4°C, prepared as described below (Watanabe and Sharma, 1972).

Discontinuous sucrose density gradients were prepared by successively layered 2.2 ml of decreasing concentrations of sucrose, ranging from 1.5 M at the bottom to 0.5 M at the top at 0.1 M intervals in a cellulose nitrate tube. The gradients were refrigerated (4°C) for a minimum of 4 hours to allow equilibration between the layers. Post-nuclear fractions layered on these gradients were centrifuged at
17,500 x g (10,500 rpm) for 16 hours at 4°C in a Spinco Model L Ultra-centrifuge equipped with a swinging-bucket SW 25 rotor. A gradient with 4 ml 0.25 M sucrose replacing the tissue homogenate was similarly prepared and analyzed for sucrose concentration by the orcinol method (Snell et al., 1961). The absorbance profiles of the centrifuged tissues were determined at 260 nm and at 280 nm for comparison.

**Determination of bound radioactivity**

Bound radioactivity was determined by the methanol wash procedure (Boyd et al., 1975). The method involved precipitating tissue homogenates with an equal volume of cold trichloroacetic acid (20%). Precipitates were washed several times with 5 ml portions of hot methanol (50%) followed by further extractions with water, chloroform, ether, benzene, and hexane, until solvents removed no further radioactivity. After removing all solvents from washed precipitates, the residues were solubilized and counted as previously described for the tissue. Sufficient α-chaconine-(³H) was added to precipitated tissue homogenates from control animals to provide radioactivity levels comparable to those from the test animals. Precipitates from the control animals were treated to the same washing procedure as above.

**Enzyme Inhibitor Studies**

**Animals and treatment**

Male Sprague-Dawley rats (Simonson Laboratories, Gilroy, California) weighing about 200 g were used for the *in vivo* enzyme inhibition studies. Three animals per group were each injected intraperitoneally with 10, 30, 60 and 90 mg/kg of α-chaconine. α-Chaconine
was dissolved in 8% ethanol solution to provide injection volumes of between 0.025 ml and 0.225 ml. The animals were sacrificed 3 hours following the initial injection. In all groups an equal number of control animals were administered appropriate volumes of ethanol solution and were sacrificed along with the experimental animals.

**Tissue preparation**

Venous and cardiac blood was collected from ether-anesthetized male Sprague-Dawley rats and placed in 0.1 M sodium oxalate (9 parts blood:1 part sodium oxalate, v/v). The plasma was removed from the cellular elements by centrifugation and stored by freezing at -85°C with dry ice within minutes after the blood was drawn. Plasma samples were used directly for both enzymatic studies or for electrophoresis, while the erythrocytes were hemolysed with water or saponin, further diluted (1:600), centrifuged, and the supernatant used for enzymatic studies.

Subcellular fractionation of the brain was carried out by density gradient centrifugation (Watanabe and Sharma, 1972; Sosa-Lucero et al., 1969). Individual fractions were assayed for AChE activity according to the colorimetric method of Ellman et al. (1961). The assay system for the pure enzyme included: 3 ml phosphate buffer (0.1 M), pH 8.0, 20.0 ml substrate (acetyl-or butyrylthiocholine), 100.0 ml DTNB and 50.1 ml enzyme. The blank consisted of buffer, substrate and DTNB solution. The change in absorbance with time measured at 412 nm in a Beckman DBG-spectrophotometer, attached to a Beckman 10-inch recorder (Beckman Instruments Inc., Fullerton, California) was calculated from
the slope of the linear curve obtained. Since the extinction coefficient of the yellow anion (II) is known, $1.36 \times 10^4$, the rate was converted to absolute units, viz.,

$$\text{Rate (moles/l per min)} = \frac{\text{absorbance/min}}{1.36 \times 10^4}$$

The assay system for enzyme activity in the tissues consisted of:

- 0.2 μl aliquot of tissue homogenate, 2% homogenate in PO₄ buffer,
- 0.1 M, pH 8.0, 2.8 ml phosphate buffer (pH 8.0, 0.1 M) 100 μl DTNB and 20 μl substrate.

Substrates used were acetylthiocholine and butyrylthiocholine for the specific and non-specific cholinesterases, respectively. The principle of the method is the measurement of the rate of production of thiocholine as substrates are hydrolyzed. This is accomplished by the continuous reaction of the thiol with 5,5'-dithiobis-2-nitrobenzoate ion (I) to produce

\[
\begin{align*}
H_2O + (CH_3)_3NCH_2CH_2SCOCH_3 \longrightarrow (CH_3)_3NCH_2CH_2S^- + CH_3CHO + 2H^+ \\
(CH_3)_3NCH_2CH_2S^- + RSSR \longrightarrow (CH_3)_3NCH_2CH_2SSR + RS^-
\end{align*}
\]

(I) \hspace{2cm} (II)

\[R = O_2N\begin{tabular}{c}COO^- \end{tabular}\]

the yellow anion of 5-thio-2-nitro-benzoic acid (II). The rate of color production is measured at 412 nm in a spectrophotometer. The
The reaction with the thiol is very rapid so as not to be rate limiting in the measurement of the enzyme, and in the concentration that does not inhibit the enzymatic hydrolysis.

Polyacrylamide Gel Electrophoresis

Acrylamide gel electrophoresis was performed in an SE-500 Vertical slab Electrophoresis assembly (Hoeffer Scientific Instruments, San Francisco, California). The gel slabs, 1.5 mm thick, were cast in the sandwich space formed between a pair of matched 140 x 175 mm glass plates separated by 140 mm 1.5 mm thick polyvinyl chloride (PVC) plate spacer strips. The lower gel contained acrylamide at a final concentration of 7.5%. The samples were layered in wells formed in a 4% spacer gel.

Separating and spacer gels were prepared from stock solutions A, B, C, D, E, and F as used in the method of Ornstein and Davis (1962). Separating and spacer gels sufficient for two 1.5 mm x 100 mm x 140 mm cm slabs were prepared as follows separating gel (pH 8.0):

Solution A (Tris-HCl:TEMED) = 6.01 ml
Solution C Acrylamide:Bis (30:0.8) = 12.0 ml
Solution F (0.14% ammonium Persulfate-fresh) = 24.0 ml
Distilled water = 5.52 ml
10% Triton X-100 = 0.48 ml
Total = 48.00 ml
Spacer Gel:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution B (Tris-HCl pH 6.7)</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Solution D Acrylamide:Bis (10:2.5)</td>
<td>9.6 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8.16 ml</td>
</tr>
<tr>
<td>10% Triton X-100</td>
<td>0.24 ml</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Total</td>
<td>24.90 ml</td>
</tr>
</tbody>
</table>

Samples of plasma and tissue supernatant were solubilized in an 80% sucrose solution buffered with Tris-HCl of the same composition as shown below. Samples in sucrose were overlaid with about 0.05 to 0.1 ml of spacer gel to form a cap gel. Routinely, gels were prepared in the evening, stored at 4°C and electrophoretic separation carried out the next morning. The lower gel solutions were mixed and carefully poured into the sandwich spaces in the upright precooled chamber. After filling the space to the desired depth, the upper surface of the lower gel was covered with the layering solution. This step transfers the curved meniscus from the liquid lower gel to the layering solution and is essential for the production of a flat, smooth upper surface (origin) of the separation gel. Polymerization was carried out overnight at room temperature (22°C).

After polymerization of the lower gel was complete, the water layer was poured off and about 12 ml of cold spacer gel solution was poured in with 20-Well Teflon combs for wells (0.75 mm or 1.5 mm thick x 4 mm wide x 15 mm deep) positioned in the sandwich space above the lower gel. Polymerization was initiated by use of a fluorescent (UV) lamp. Electrophoretic separations were carried out at room temperature.
in a 0.005 M tris-glycine buffer (pH 8.9). Power was provided by a constant power supply in two phases. During phase I, a low current 75 volt, 40 mm was provided for about 30 minutes or until the tracking dye, bromophenol blue, had transversed the spacer gel. The low current and the high cooling reserve of the system prevents convection and distortion. The spacer gel swells under the influence of a high current flow, but under this condition it remains at a constant volume, keeping the wells open. In phase II, the voltage was increased to 150-200 volts and electrophoresis continued until the tracking dye was about 1 cm from the bottom of the slab (4 hours). Following electrophoresis, the gel slabs were removed by rimming them with a cannula through which distilled water was run. The slabs were washed with buffer and then strained for total, specific and non-specific esterases.

**Histochemical staining of gels**

Total esterase activity was demonstrated with α-napthyl acetate, dissolved in 50% acetone or in N,N'-dimethylformamide, as substrate according to the method of Gomori (1958). Fast blue RR (dizaonium salt) was used as the coupling agent in the buffer system consisting of Tris-maleate (0.2 M, pH 7.1). Gels were incubated in the dye-substrate mixture for at least 30 minutes at room temperature. Gels were later placed in an acid-alcohol mixture consisting of three parts absolute methanol and two parts 10% glacial acetic acid for a minimum of 30 minutes at 37°C. The acid alcohol mixture served a three-fold purpose: (a) to stop the reaction, (b) to develop the coupled azo
dye from reddish purple to a darker blue-black, and (c) to remove nonspecific staining (Allen et al., 1965).

Selective staining of acetylcholinesterases was performed using acetylthiocholine iodide according to the modification of the procedure of Maynard (1966). The method is based on the enzymatic hydrolysis of the thioanalogues of acetyl- and butylcholine by the isoenzymes, with the precipitation of the liberated thiocholine as a mercaptide, and its subsequent replacement by CuS. The substrate acetylthiocholine (AThCh) is hydrolyzed at a more rapid rate than ACH by both specific ChE and nonspecific cholinesterases, presumably because of the weaker linkage of the -C-S- than of the -C-O bond. Following electrophoresis, the gel slabs were placed in glass troughs containing a reaction mixture modified from the thiocholine method of Koelle (1951) as described by Maynard (1966). Gel slabs were pre-incubated for 30 minutes at 0°C in a freshly prepared solution containing CuSO₄·5H₂O glycine, MgCl₂·6H₂O and maleate-NaOH buffer (pH 6.1) in the proportions used by Koelle (1951). This was followed by incubation for 1 to 20 hours at room temperature in the reaction mixture containing the above reagents plus acetyl- or butyryl thiocholine iodide, as substrate in the proportions used by Koelle (1951). The solutions were saturated with the reaction product by adding a trace of copper thiocholine (CuThCh), stirring to obtain its complete dispersal, and storing in the incubator for 15 minutes prior to addition of the substrates. Proteins were stained with Coomassie Brilliant blue RR250.
Inhibitor studies

The effects of the solanum alkaloids, α-chaconine on the isoenzymes were evaluated. Varying concentrations of α-chaconine were added to the pre-incubation solution and the reaction mixture. In order to avoid hydrolysis of any remaining substrate by the reactivated enzyme (assuming reversibility of inhibition existed with these compounds), it was necessary to place the gels in water containing the appropriate concentration of the inhibitor following incubation. This was necessary with the azo dye method. Concentration of glycoalkaloid used was $10^{-4}$ M α-chaconine.

Protein determination

Protein was determined by the method of Lowry et al. (1951).
RESULTS

Physiologic Disposition

Absorption, distribution, and excretion

The concentration (ng/g) of the label in various tissues, organs, and body fluids is shown in Table 2 for animals administered po, and in Table 3 for the ip administered animals. Peak radioactivity levels were seen in liver, lungs, skeletal muscle, spleen, stomach, pancreas, brain, testes and intestines 12 hours after ingestion of the glyco-alkaloid; while kidney and heart showed their highest levels after 24 hours. With the exception of the heart, kidney, and intestines and contents, a decrease in the concentration of the label within the tissues was observed after 12 hours for the animals administered po. Tissues and organs from animals administered ip showed their highest radioactivity concentration after 24 hours (Table 4). The concentrations of the label in some of the tissues were generally higher (about two times higher) following ip administration than at corresponding times following po ingestion of α-chaconine-(3H). The blood radioactivity profile shows an initial (3 hour) concentration of 0.82 ng/ml with a peak concentration of 1.74 ng/ml at 12 hours followed with a decrease in its concentration reaching 0.30 ng/ml at 168 hours.

The concentration of radioactivity in the tissues with respect to organ/blood ratios at various times after administration of the label is shown in Figure 8. The tissues concerned (kidney, skeletal muscle, heart, and liver) gave tissue/blood ratios of 2-4 (Figure 8) for the
Table 2. Concentration of radioactivity in body organs, tissues and fluids of male hamsters after po administration of 10 mg/kg of α-chalcone(3H).a

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Hours after oral administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Brain</td>
<td>3.35 ± 0.42</td>
</tr>
<tr>
<td>Heart</td>
<td>4.41 ± 0.39</td>
</tr>
<tr>
<td>Lungs</td>
<td>6.68 ± 0.79</td>
</tr>
<tr>
<td>Liver</td>
<td>14.11 ± 3.13</td>
</tr>
<tr>
<td>Spleen</td>
<td>6.25 ± 0.91</td>
</tr>
<tr>
<td>Stomach</td>
<td>75.91 ± 19.17</td>
</tr>
<tr>
<td>Pancreas</td>
<td>7.81 ± 2.33</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.58 ± 0.32</td>
</tr>
<tr>
<td>Intestines and contents</td>
<td>125.41 ± 17.30</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>3.81 ± 0.36</td>
</tr>
<tr>
<td>Testes</td>
<td>3.29 ± 0.40</td>
</tr>
<tr>
<td>Blood</td>
<td>0.82 ± 0.13</td>
</tr>
</tbody>
</table>

aValues in nanograms per gm or per ml blood. Each group consists of three observations. Mean values ± SE.
Table 3. Concentration of radioactivity in body organs, tissues and fluids of male hamsters after ip administration of 10 mg/kg α-chaconine-(3H).a

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Hours after intraperitoneal administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Brain</td>
<td>2.45 ± 1.17</td>
</tr>
<tr>
<td>Heart</td>
<td>4.54 ± 0.58</td>
</tr>
<tr>
<td>Lungs</td>
<td>9.16 ± 0.22</td>
</tr>
<tr>
<td>Liver</td>
<td>11.49 ± 0.30</td>
</tr>
<tr>
<td>Spleen</td>
<td>9.36 ± 3.15</td>
</tr>
<tr>
<td>Stomach</td>
<td>14.71 ± 2.35</td>
</tr>
<tr>
<td>Pancreas</td>
<td>5.77 ± 1.26</td>
</tr>
<tr>
<td>Kidney</td>
<td>8.95 ± 0.50</td>
</tr>
<tr>
<td>Intestines and contents</td>
<td>19.92 ± 9.95</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>41.74 ± 23.69</td>
</tr>
<tr>
<td>Testes</td>
<td>3.84 ± 0.08</td>
</tr>
<tr>
<td>Blood</td>
<td>2.60 ± 0.23</td>
</tr>
</tbody>
</table>

aValues in nanograms per gm tissue or per ml blood. Each group consists of three observations. Mean values ± SE.
Table 4. Cumulative excretion of radioactivity after oral and intraperitoneal administration of α-chaconine shown as percent of administered dose (10 mg/kg).a

<table>
<thead>
<tr>
<th>Tissues</th>
<th>3</th>
<th>12</th>
<th>24</th>
<th>72</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hours after po administration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine b</td>
<td>NDc</td>
<td>0.25 ± 0.24</td>
<td>1.46 ± 0.20</td>
<td>3.64 ± 1.81</td>
<td>20.76 ± 2.81</td>
</tr>
<tr>
<td>Feces d</td>
<td>0.01 ± 0.00</td>
<td>0.15 ± 0.04</td>
<td>0.19 ± 0.06</td>
<td>0.26 ± 0.05</td>
<td>0.24 ± 0.07</td>
</tr>
<tr>
<td>Intestinal contents e</td>
<td>62.71 ± 17.30</td>
<td>37.20 ± 8.56</td>
<td>43.88 ± 1.18</td>
<td>9.71 ± 5.76</td>
<td>0.92 ± 0.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Hours after ip administration</strong></th>
<th></th>
<th>24</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine b</td>
<td>ND</td>
<td>1.42 ± 0.28</td>
<td>2.45 ± 0.28</td>
</tr>
<tr>
<td>Feces d</td>
<td>ND</td>
<td>0.05 ± 0.00</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Intestinal contents e</td>
<td>9.52 ± 4.71</td>
<td>17.08 ± 6.10</td>
<td>3.59 ± 1.06</td>
</tr>
</tbody>
</table>

*Mean values ± SE in percentage of dose administered. Each group consists of three observations.

bα-Chaconine-(3H) excreted with urine.
c**No data**—no urine or feces collected during these periods.
dα-Chaconine-(3H) excreted in feces.
eα-Chaconine-(3H) present in the intestinal contents.
Figure 8. Tissue concentration/blood concentration ratio of $\alpha$-chaconine-$({ }^{3}H)$ in the male hamster after po administration of 10 mg/kg of the glycoalkaloid. [Each value was calculated from the mean of three samples. Bars represent half the standard error (SE).]
various time intervals except the liver with an initial (3 hour) ratio of 18. The tissue/blood ratios for identical tissues in organs in animals administered ip showed the highest value after 7 days, except skeletal muscle with a high initial (3 hour) ratio (16) after injection.

The cumulative \(\alpha\)-chaconine-(\(^3\)H) or its metabolites excreted via urine increased with time after oral ingestion (Table 4), reaching 21% of the administered dose in 168 hours. The amount excreted in feces as shown in Table 4 was much lower (\(<\,1\%\) than through the urine due to the low feces collection during the experimental period. High levels (62% administered dose) of the radioactivity were found in the large intestines and intestinal contents, especially during the initial 24 hours and decreased at subsequent periods. The amount of excreted radioactivity (sum of the radioactivity excreted in urine, feces, and present in the intestines and intestinal contents) was highest (62%) for the initial 3 hours after po administration of the labeled compound and decreased to about 22% after 168 hours (Table 4). The excretion of radioactivity in urine, feces, and intestinal contents is much lower than that observed after oral ingestion (9, 18, and 5% for 3, 24, and 168 hours, respectively).

**Characterization of extracts from urine and feces**

Separation of radioactivity into a chloroform-soluble and insoluble fractions provided the initial differentiation in the metabolism of \(\alpha\)-chaconine-(\(^3\)H). The chloroform/water partition coefficient of \(\alpha\)-chaconine was found to be 0.38. In the urine, the concentrations of both the chloroform-soluble and chloroform-insoluble fractions increased during the experimental period for animals administered po, with the
ratio of CHCl₃-soluble:CHCl₃-insoluble fraction increasing from < 1 for the initial 72 hours to 78 after 7 days (Table 5). Animals administered ip accumulated more of the CHCl₃-soluble metabolites (1.2%) in the urine after 24 hours and more of the chloroform-nonextractable components (1.7%) after 168 hours, giving a ratio of 6.4 and 0.6 for 24 and 168 hours, respectively.

In the feces, the ratio of the chloroform-extractable to chloroform-nonextractable components was as follows: 4, 47, 36, 35 and 115 for 3, 12, 24, 72 and 168 hours after po administration, and 4 and 1.5 for 24 and 168 hours for animals injected ip (Table 6).

Subcellular distribution of labeled α-chaconine

Characteristic profiles of absorbance (260 and 280 nm), sucrose density, and radioactivity from the postnuclear fraction of hearts, brains, and livers of animals administered α-chaconine-(³H) are shown in Figures 9, 10, and 11. Absorbance profiles of the postnuclear supernatants from the tissues showed three main zones. Absorbance profiles at 280 nm follow closely the pattern at 260 nm.

Radioactivity profiles for the various fractions show maximum activity in the subcellular components appearing in fractions 5-13 and 14-20 (liver), fractions 13-21 (brain), and fractions 10-15 and 16-22 (heart). A smaller zone of radioactivity is seen in fractions 4 to 9 in the heart tissue radioactivity profile. Figure 12 shows the total amount of radioactivity with respect to the subcellular components corresponding to the various absorbance peaks, including the nuclear fraction. The zone of absorbance sedimenting between the mitochondrial
Table 5. Percentage of chloroform-soluble and insoluble products excreted in urine at different collection periods following oral and intraperitoneal administration of 10 mg/kg \( \alpha \)-chaconine-(\(^3\)H) in hamsters.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Hours after po administration</th>
<th>Hours after ip administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>CHCl(_3)-soluble</td>
<td>ND(^b)</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>±0.000</td>
<td>±0.090</td>
</tr>
<tr>
<td>CHCl(_3)-insoluble</td>
<td>ND</td>
<td>0.270</td>
</tr>
<tr>
<td></td>
<td>±0.140</td>
<td>±0.440</td>
</tr>
<tr>
<td>CHCl(_3) H(_2)O</td>
<td>ND</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(^a\)Chloroform-soluble and -insoluble fraction as percent of the given dose (three animals in each group).

\(^b\)ND = no data because no urine could be obtained during the first three hours in some groups.
Table 6. Percentage of chloroform-soluble and insoluble products excreted in feces at different collection periods following oral and intraperitoneal administration of 10 mg/kg α-chaconine-(3H) in hamsters.\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Hours after po administration</th>
<th></th>
<th>Hours after ip administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>CHCl\textsubscript{3}-soluble</td>
<td>0.004</td>
<td>0.140</td>
<td>0.180</td>
</tr>
<tr>
<td>±0.000±0.030±0.020±0.060±0.060</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHCl\textsubscript{3}-insoluble</td>
<td>0.001</td>
<td>0.003</td>
<td>0.005</td>
</tr>
<tr>
<td>±0.000±0.001±0.002±0.001±0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHCl\textsubscript{3}/H\textsubscript{2}O</td>
<td>4</td>
<td>47</td>
<td>36</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Chloroform-soluble and -insoluble fraction as percent of the given dose (three animals in each group).
\textsuperscript{b}ND = no data because no feces could be obtained during the first three hours in some groups.
Figure 12. Distribution of radioactivity in the subcellular brain, heart and liver fractions of hamsters, following density gradient centrifugation of tissue homogenates.

[A: Tissues from animals given 10 mg/kg ip α-chaconine-(3H) ip. B: Tissues from animals given 10 mg/kg α-chaconine-(3H) po.]
and microsomal fractions showed a significant concentration of radioactivity.

Tissue binding

Since samples prepared from the control group gave only background levels of radioactivity, it indicated that the methanol wash procedure removed all unbound label in the homogenates from the test animals. The residual activity following the wash represented "non-extractable" or tightly bound $\alpha$-chaconine-$\left(^3\text{H}\right)$ and/or its metabolite(s). Residual activity, tightly bound to tissue macromolecules, found in the tissues of test animals (ip or po) is shown in Figure 13 with the respective percentages of binding for ip and po administration. The percentage of bound radioactivity in the brain was markedly greater (Figure 13) than in any other tissue. Also the percentage of bound radioactivity in the tissues of animals administered po was greater than that in tissues from ip administered animals, with the exception of the brain.

Excretion

Representative thin-layer chromatographic separations of the chloroform-soluble fractions from urine and feces at various time intervals are shown in Figure 14. In addition to the aglycone solanidine (identified by comparison with migration rate of the hydrolysis product) in the excretory products, other metabolites corresponding to the smaller areas of radioactivity were also observed. Composition of the metabolites found in feces showed a slightly different pattern from urine with respect to the minor metabolites, the main component representing the aglycone--solanidine.
Figure 14. Radiochromatographic scan of the thin-layer chromatographic separation of the chloroform-soluble metabolites excreted in the urine of male hamsters given α-chaconine-(3H) (orally). [Numbers identify the various fractions. Solvent system, ethanol:acetic acid (2:1).]
The concentrations of \( \alpha \)-chaconine metabolites in urine and feces and their percentages as fractions of the total radioactivity obtained at different time intervals are presented in Tables 7 (urine) and 8 (feces), respectively. The values were determined from the proportion of the total radioactivity present in the chloroform-soluble fractions, in relation to the activity present in individual peaks from the TLC separations. Maximum excretion of the unmetabolized \( \alpha \)-chaconine was observed at 12 hours, beyond which the concentration decreased from 62\% to 17\% after 7 days. This decrease in the excretion of the main compound was accompanied by a corresponding increase in the excretion of other metabolites. Metabolites 1, 2, and 3 were excreted in greater concentrations during the sampling period. Data for feces showed an increasing excretion of the major metabolite (fraction 1) during the test period. Other metabolites were excreted at much reduced rates when compared to urine fractions.

The results presented showed an increase in the concentration of excreted \( \alpha \)-chaconine and/or its metabolites in both urine and feces during the experimental period. The decline in the percentage of the main excretory product with time, and the corresponding increase in the percentage of other chloroform-soluble products is explained by the metabolism of the administered alkaloid. These represented 38, 81, and 83\% of the total concentration at 24, 72, and 7 days, respectively. The fraction of the total dose administered which was excreted was 26\% in feces and less than 12\% in urine, a total of about 27\% in 7 days.
Table 7. Concentration of excreted radioactivity in urine following administration of α-chaconine-(³H) (10 mg/kg, orally) to male hamsters

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Total excreted radioactivityᵃ</th>
<th>Chloroform-extractable fractionsᵇ</th>
<th>Water-soluble fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>0.25</td>
<td>100.0</td>
<td>--</td>
</tr>
<tr>
<td>24</td>
<td>1.50</td>
<td>62.34</td>
<td>18.89</td>
</tr>
<tr>
<td>72</td>
<td>3.60</td>
<td>18.8</td>
<td>28.87</td>
</tr>
<tr>
<td>168</td>
<td>20.80</td>
<td>16.63</td>
<td>4.88</td>
</tr>
</tbody>
</table>

ᵃValues determined from proportion of total activity present in the chloroform-soluble fractions in relation to the activity present in the various peaks obtained from TLC separations.
ᵇActivity expressed as fraction of dose administered (in percentages). Values represent cumulative excretion of the dose administered.
ᶜND = no data, since no urine was excreted at the time period.
Table 8. Concentration of excreted radioactivity in feces following administration of α-chaconine-(\(^3\)H) (10 mg/kg, orally) to hamsters.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Total excreted radioactivity(^a)</th>
<th>Chloroform-extractable fractions(^b)</th>
<th>Water-soluble fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0.01</td>
<td>50.3</td>
<td>21.1</td>
</tr>
<tr>
<td>12</td>
<td>0.14</td>
<td>86.5</td>
<td>4.3</td>
</tr>
<tr>
<td>24</td>
<td>0.19</td>
<td>94.5</td>
<td>4.5</td>
</tr>
<tr>
<td>72</td>
<td>0.24</td>
<td>96.1</td>
<td>1.8</td>
</tr>
<tr>
<td>168</td>
<td>0.26</td>
<td>73.6</td>
<td>8.7</td>
</tr>
</tbody>
</table>

\(^a\) Values determined from proportion of total activity present in the chloroform-soluble fractions in relation to the activity present in the various peaks obtained from TLC separations.

\(^b\) Activity expressed as fraction of dose administered (in percentages). Values represent cumulative excretion of the dose administered.
Acetylcholinesterase inhibition

The kinetic constants, $K_m$ and $V_{max}$ for the hydrolysis of acetylthiocholine iodide and butyrylthiocholine iodide by purified cholinesterases—bovine erythrocyte cholinesterase and horse serum cholinesterase—are shown in Table 9. Double-reciprocal plots ($\frac{1}{v}$ vs $\frac{1}{s}$) for the inhibition of bovine erythrocyte cholinesterase and horse serum cholinesterase by $\alpha$-chaconine are shown in Figures 15 and 16, respectively. The bimolecular inhibition constants ($K_I$) ($8.3 \times 10^{-6}$ M for inhibition of acetylcholinesterase and $4.0 \times 10^{-6}$ M for inhibition of butyrylcholinesterase) were obtained by replots of the slopes of the double-reciprocal plots versus the inhibitor concentrations used (Dixon and Webb, 1964).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{max}$ (moles/liter-min⁻¹)</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine erythrocyte</td>
<td>$7.14 \times 10^{-5}$</td>
<td>$6.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>Horse serum</td>
<td>$3.76 \times 10^{-4}$</td>
<td>$1.33 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

$^a$Substrate concentrations were 0.010, 0.025, 0.050, and 0.075 M. Enzyme activity was assayed by the method of Ellman et al. (1961).

Distribution of acetylcholinesterase activity in fractions obtained by a continuous density gradient centrifugation of the nuclear-free brain homogenates is shown in Figure 17, with highest activity distributed evenly between the fractions. Also shown in Figure 17 is the
Figure 15. A double-reciprocal plot for the hydrolysis of acetylthiocholine by cholinesterases in the presence of different fixed concentrations of α-chaconine. [Experimental conditions were the same as described in the text. $I_1 = 0.016$ mM; $I_2 = 0.032$ mM.]
Figure 16. A double-reciprocal plot for the hydrolysis of butyrythiocholine by cholinesterases in the presence of different fixed concentrations of α-chaconine. [Experimental conditions were the same as described in the text. $I_1 = 0.016$ mM; $I_2 = 0.032$ mM.]
Figure 17. Subcellular brain acetylcholinesterase activity of the nuclear-free fraction of rat brain homogenate separated by zonal centrifugation on sucrose density gradient. [Activity expressed as micromoles (µmoles) acetylthiocholine hydrolyzed per minute per mg protein. Assay conditions are described in the section under methods. (—— sucrose concentration (M); ·—— control acetylcholinesterase activity; ·—— residual acetylcholinesterase activity following incubation of assay system with 0.016 M α-chaconine).]
Specific Activity of Brain Acetylcholinesterase
(moles substrate hydrolysed/min/mg protein)
pattern of residual activity following in vitro incubation of aliquots of the various fractions with α-chaconine (0.016 M).

Subcellular fractionation of the brain homogenate yielded two main peaks (fractions) with the enzyme activity fairly well distributed between these two subfractions. A comparison of the acetylcholinesterase activity, in fractions incubated with α-chaconine and in control samples, within the subfractions (1 and 2), nuclear fraction and whole homogenates is shown in Figure 18. Percentage inhibitions of the various subfractions were as follows: whole homogenate (43), nuclear (55), fraction 1 (35), and fraction 2 (33).

A dose-related response of brain, plasma and heart cholinesterases following ip administration of α-chaconine (10, 30, 60 and 90 mg/kg) to adult male rats is shown in Figure 19. Acetylcholinesterase activity of the brain homogenate from the treated animals was shown to be 79, 55 and 18% of the control group for the dosage levels 10, 30 and 60 mg/kg, respectively. Acetylcholinesterase activity of heart tissue homogenates, though inhibited by α-chaconine (39%) did not exhibit the dose-related response evident in the brain. Plasma cholinesterase activity of the treated animals was 49% for the groups given 10 and 30 mg/kg ip.

The mortality rate among the treated rats served as a basis for determining the lowest dose necessary to kill the rat within 12 hours. All the rats injected with 90 mg/kg died within 2 hours of administration, while two of three rats died in the 60 mg/kg group and one of the three rats in the 30 mg/kg group died within 2 hours.

All rats administered α-chaconine showed initial signs of depression, convulsions, coma and respiratory arrest, which are definite
Figure 18. Cholinesterase activity of subcellular brain fractions of rats treated (in vitro) with 0.016 mM α-chaconine. [Numbers on the abscissa indicate the fractions obtained from density gradient. Numbers in parentheses represent the percentages of inhibition of the samples assayed. C = control, t = treated (samples were incubated with α-chaconine). Enzyme activity represented as μmoles acetylthiocholine hydrolyzed per minute per mg protein.]
Figure 19. Comparison of acetylcholinesterase activity in brain, plasma and heart homogenates of adult male rats injected intraperitoneally with several doses of α-chaconine (0, 10, 30 and 60 mg/Kg). [Enzyme activity assayed by the colorimetric method of Ellman et al. (1961).]
The diagram shows the activity of acetylcholinesterase in different tissues (plasma, heart, brain) after treatments with different doses of a substance: Control, 10 mg/kg, 30 mg/kg, 60 mg/kg. The activity is measured in micromoles per minute per milligram of protein.
signs of cholinesterase inhibition. Also, blood samples from rats administered the higher doses of α-chaconine (30 and 60 mg/kg) tended to clot easily despite mixing thoroughly with oxalate. This feature was absent in the blood samples from rats in the control group or those administered 10 mg/kg α-chaconine. Since all the rats administered 90 mg/kg α-chaconine died, data for this group are not available.

Table 10. Lethal doses in rats (ip).

<table>
<thead>
<tr>
<th>α-Chaconine dose mg/Kg</th>
<th>Death rate (2 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.3</td>
</tr>
<tr>
<td>30</td>
<td>1/3</td>
</tr>
<tr>
<td>60</td>
<td>2/3</td>
</tr>
<tr>
<td>90</td>
<td>3/3</td>
</tr>
<tr>
<td>Control</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Inhibition of Cholinesterase Isoenzymes

Visual inspection, as well as densitometric determinations, were used in estimating the extent of inhibition of the various isoenzymes by α-chaconine. Bands in the zymograms were assigned numbers, with the fastest moving band being designated as band 1. Complex zones of enzyme activity, without any clear separation of individual bands, have been described as isoenzyme zones, rather than as bands.

Electrophoretic separation of plasma and brain acetylcholinesterase isoenzymes on polyacrylamide gels was not as satisfactory as was
expected, with the bands of enzyme activity not clearly resolved in some cases. This was especially true of the frozen tissue samples. Nevertheless, the results obtained were discernible enough for meaningful comparisons to be made of the in vivo and in vitro effects of α-chaconine on the separable isoenzymes.

In vivo effects

Following electrophoresis in acrylamide gel slabs, homogenates of brain showed three zones of activity against acetylthiocholine (AcThCh) (sites 1, 2 and 3) isoenzyme separation in brain homogenates in response to the intraperitoneal administration of α-chaconine shown in the densitometric tracings (Figure 20). Clearly defined peaks of appreciable activity are indicated, with the effect of α-chaconine clearly demonstrable by the gradual reduction in peak height (bands 1 and 2). A complete elimination of band 1 in the rats administered 30 and 60 mg/Kg of the glycoalkaloid is clearly shown in the densitometric tracings (Figure 20). Incubation of the gels, after electrophoresis, with $10^{-4}$ M diisopropylfluorophosphate (DFP) resulted in the elimination of all the isoenzyme bands 1 and 2 in the brain homogenate (not shown).

Incubation of gels with α-naphthyl acetate as substrate and a diazo salt (Fast blue RR) as the coupling agent, resulted in a number of esterase bands and zones, some of which correspond in mobility to the zones that hydrolyze AcThCh (Figure 21, bands 1-5). Inhibition of these sites by α-chaconine is also shown with only three bands of low staining intensity seen in the esterase zymograms of brain homogenates from rats administered 60 mg/Kg. Isoenzyme bands from rats administered
Figure 20. Densitometric tracings of AChE isoenzymes of the brain homogenates from adult male rats given varying doses of α-chaconine intraperitoneally. [Electrophoretic separation was done on 7.5% acrylamide gel slabs using discontinuous tris-hydrochloride and tris-glycine buffers at pH 8.4. AChE was solubilized with 0.1% Triton-X-100 in tris-hydrochloride buffer pH 8.5. Final dilution of samples represents 1:12 (weight volume). Intensity of staining of the esterase bands was used as a measure of enzyme activity following incubation with substrate-acetylthiocholine. Final staining was accomplished by the histochemical method of Gomori (1958).]
10 and 30 mg/Kg α-chaconine showed a diminished staining intensity as compared to the controls.

A gel photograph of the electrophoretic separation of plasma proteins of rats administered several doses of α-chaconine is shown in Figure 22a. The protein bands in all experimental groups are identical in the number of bands, with a slight change in the intensity of staining. The observed differences, if any, are clearly demonstrated in the densitometric tracing of the protein separations (Figure 22b).

Specific cholinesterase (AChE) zymograms of plasma from the three experimental groups (0, 10, 30 mg/Kg) are shown in the densitometric tracings in Figure 23. Several distinct bands and zones of enzyme activity are clearly distinguishable in the densitometric patterns shown, with the control group showing five to six bands or zones hydrolyzing AcThCh. A reduction in the activity of the isoenzyme bands (1 and 2) is observed for the groups administered 10 and 30 mg/Kg α-chaconine. Percentage inhibitions of the two prominent bands (1 and 2) were 40 and 77% for the group administered 10 mg/Kg glycoalkaloid, and 100 and 75% for rats administered 30 mg/Kg glycoalkaloid. Isoenzyme bands 3 and 4 were absent in the treated animals, with the slowest moving band being resistant to the inhibitory effect of α-chaconine. Cholinesterase activity (ChE, non-specific) of plasma was resolved into one distinct band, in addition to several other zones that hydrolyzed butyrylthiocholine (BuThCh), as shown schematically in Figure 24. A decrease in activity of the isoenzyme bands, shown by decreased staining intensity of the bands, was observed with the experimental groups.
Figure 22b. Densitometric patterns of the plasma protein isoenzymes shown in Figure 22a.
Figure 23. Specific cholinesterase (AChE) zymograms of the plasma isoenzymes of rats given α-chaconine (ip). [Plasma (10 μl) was diluted 1:1 with buffered sucrose. Electrophoretic conditions were the same as those described in Figure 20. Only isoenzyme bands of appreciable activity (peak area) were included and numbering of AChE bands is from the anodic end.]
PLASMA AChE ISOENZYME INHIBITION

CONTROL

10 mg/kg

30 mg/kg
Figure 24. Schematic representation of the BuChE isoenzymes of rat whole blood plasma, showing the relative effect of the dose of α-chaconine on the separable isoenzymes. [The method of separation of isoenzymes was the same as indicated under Figure 20. Activity of ChE isoenzymes was detected by the reaction with the substrate--butyrylthiocholine. The numbers correspond to the isoenzyme bands separated. Zones of weak staining intensity were not included.]
The effect of $10^{-4}$ M $\alpha$-chaconine on the various AChE isoenzymes of brain plasma erythrocyte and heart of adult rats is shown schematically in Figure 25.

A gel photograph of the electrophoretic separation of total esterases of plasma, erythrocytes, and organs of an adult male rat, following incubation with and without $10^{-4}$ M $\alpha$-chaconine is shown in Figure 26. Densitometric tracings of the electrophoretic patterns of the tissues shown in Figure 26 are shown in Figures 27, 38, and 29. Percentages of inhibition obtained are shown for the various isoenzymes in Table 11. The isoenzyme pattern of heart cholinesterases, while following closely that of the control group, showed an increase in the activity of some bands over the controls.

Table 11. Inhibition of total esterase isoenzyme activity of various tissues in the rat following incubation of electrophoretic gels with $\alpha$-chaconine ($10^{-4}$ M).\(^a\)

<table>
<thead>
<tr>
<th>Isoenzymes</th>
<th>Serum</th>
<th>Erythrocytes</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>22.96</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>41.97</td>
<td>27.10</td>
</tr>
<tr>
<td>3</td>
<td>78.49</td>
<td>41.77</td>
<td>15.6</td>
</tr>
<tr>
<td>4</td>
<td>81.73</td>
<td>100</td>
<td>19.5</td>
</tr>
<tr>
<td>5</td>
<td>90.00</td>
<td>100</td>
<td>67.31</td>
</tr>
<tr>
<td>6</td>
<td>100.00</td>
<td>100</td>
<td>100.00</td>
</tr>
</tbody>
</table>

\(^a\)Enzyme activity of isoenzymes was calculated by integration of the peaks obtained by scanning of gels in a densitometer. Electrophoresis was done with samples obtained from pooled homogenates from three rats. Only isoenzyme bands showing definite peaks were considered in the determination.
Figure 25. AChE isoenzyme patterns of plasma erythrocytes and organs of adult male rats following incubation with α-chaconine (10-^4^ M), shown schematically. [P = plasma (10 μl); B = brain (20 μl); E = erythrocyte (10 μl); H - heart (20 μl). Electrophoretic conditions were the same as indicated in Figure 20. Numbering indicates the isoenzyme bands.]
Figure 26. Slab gel electrophoretic patterns of plasma, brain, erythrocytes, and heart AChE following incubation of gels with α-chaconine (10^{-4} M). [Experimental conditions were the same as those indicated earlier in Figure 20. P = plasma; B = brain; E = erythrocytes; H = heart. 1 = Control. 2 = Incubation with 10^{-4} M α-chaconine.]
Figure 27. Electropherogram of esterase isoenzymes in plasma from adult male rats, from incubation of electrophoresis gels with $\alpha$-chaconine ($10^{-4}$ M). [Migration of isoenzyme bands is towards the anode, and the numbering of the isoenzymes is from the anodal end. Electrophoretic conditions are the same as those described in Figure 20.]
Figure 28. Densitometric patterns of rat brain esterase isoenzymes, showing the inhibitory effects of α-chaconine (10⁻⁴ M) on the isoenzymes separated by gel electrophoresis. [Electrophoretic conditions are the same as those described in Figure 21.]
Figure 29. Esterase isoenzyme patterns of adult rat erythrocytes following incubation of electrophoretic gels with $\alpha$-chaconine (10$^{-4}$ M).
ERYTHROCYTES

CONTROL

10^{-4} M \alpha-CHACONINE
DISCUSSION

Physiologic Disposition

Distribution

Following the administration of the labeled glycoalkaloid, a considerable amount remained unabsorbed, and probably was eliminated in feces as the metabolized components which were found in the CHCl₃-soluble fraction. The high radioactivity concentration observed in the stomach and intestines shortly after administration might suggest a low absorption within the gastrointestinal tract. This initial phase of low absorption and possible elimination could be followed by the binding to the tissues as evidenced by large 'residual' activity in the tissues.

High concentrations of the administered α-chaconine-(³H) were eliminated either as the unmetabolized form, or as the metabolized form in urine and feces, the most prominent being solanidine. The radioactivity appearing in the feces represented mostly the unidentified metabolites. Residual activity found in tissues, following the elimination phase, may represent unextractable glycoalkaloids or aglycones or metabolites that are probably bound to macromolecules.

Solanine, a structural analogue of α-chaconine has been shown to be poorly absorbed from the gastrointestinal tract, eliminated in the urine and feces, and reaching peak tissue concentration in 12 hours (Nishie et al., 1971). Based on data presented in this study, α-chaconine has different absorption characteristics within the
gastrointestinal tract with solanine, but a rather low elimination in urine and feces, as evidenced by the low elimination of radioactivity.

Levels of the labeled glycoalkaloid or its metabolites were found in the intestinal contents after ip and po administration, probably resulting from biliary excretion. The latter possibility has been suggested based on data concerning concentrations of the cardiac drug, K-strophanthoside. The concentrations were higher in the intestinal walls at the early stages than were observed in the intestinal contents (Marzo et al., 1974). Orally administered radioactivity was apparently cleared rapidly from the blood as shown by the data on the tissue distribution of the administered label which showed no noticeable peak. The rapid rate of blood clearance is probably conditioned by the ability of the liver and other tissues to bind the glycoside. The difference in the tissue concentration of radioactivity, found to be 3-10x higher with the ip route than the po, seems to be that expected of most drugs which could be indicative of incomplete absorption via the po route. High organ/blood ratios indicate possible accumulation in intracellular spaces, a fact which is expected for most alkaloids, since strong bases (pKa > 5) tend to accumulate in stomach and tissues; i.e., having a high tissue/plasma ratio by virtue of the "ion trapping" mechanism (Goldstein et al., 1974). Data shown in Figure 8 with a concentration ratio of approximately 4 tend to support accumulation in these tissues. The ratio of the concentration of the agent in the tissue over the concentration in the blood at any given time, represents an index of the effectiveness or lack of effectiveness of the membranes to influence translocation of the compound.
In subcellular distribution studies, identification of the various zones associated with the absorbance profiles was made on the basis of previous studies on subcellular fractionation of tissues by density gradient centrifugation (Sosa-Lucero et al., 1969). Zone I is shown to contain exclusively mitochondria with the intermediate zone in the upper layers containing fibrillar material and a few granules as cross-contamination between layers. Fractions of maximal absorbance in zone II contain mostly specific granules and some large vesicles (Sosa-Lucero et al., 1969). Based on electron micrograph correlation studies of the fractions obtained from density gradient centrifugation of similar tissues, zone II would contain some mitochondria and lysosomal membrane while zone III contained mostly the microsomes and soluble proteins (Sosa-Lucero et al., 1969; De Robertis et al., 1962; Michaelson, 1967).

A correlation of the radioactivity counts in the various fractions following density gradient centrifugation of the nuclear-free fractions from the tissues with the absorbance profiles gave an indication of the subcellular location of the labeled glycoalkaloid or its metabolites. Isotopic α-chaconine and/or its metabolites in vivo appeared, therefore, to be distributed within the various subcellular fractions, with the greatest activity found in the nuclear, microsomal and soluble fractions. Also, the fraction of the radioactivity which could be isolated with the microsomes showed little change within the various tissues investigated. Accumulation of radioactivity by these tissues was clearly shown by the data in Figures 9, 10 and 11.
Metabolism

The major metabolite of the steroidal glycoalkaloid, solanine, after po administration, is known to be solanidine, which exhibits poor absorption from the gastrointestinal tract (Nishie et al., 1971). In addition to the hydrolytic cleavage of the sugar side chain, and possible hydrolysis of some hexose units in the glycosidal moiety (Nishie et al., 1971) and the metabolism of the steroidal glycoalkaloid could possibly include a turnover of the chloroform-soluble to the insoluble metabolite, and a hydroxylation of the glycoside molecule.

From the results it would appear that chloroform-insoluble metabolites were excreted in both urine and feces following ingestion of α-chaconine. The decreasing ratio of the CHCl₃-soluble to -insoluble components observed may be suggestive of an increasing excretion of the polar metabolites. Biliary excretion and enterohepatic circulation of cardiac glycosides have been shown to be closely related to the polarity of the glycoside molecule (Okita, 1967; Doherty et al., 1970; Kolenda, 1971). The aglycone fraction of α-chaconine, with a single OH group can be classified as a polar compound.

Rate of metabolism data for α-chaconine would help in explaining the pharmacokinetic behavior of this glycoside. The low concentration of radioactivity in feces may be due to the percentage of chloroform-insoluble and chloroform-soluble metabolites absorbed in the gut, and partly to tissue binding. The length of time spent within the gastrointestinal tract as reflected in the rate of absorption would be an important factor in the excretion or elimination of this glycoalkaloid or its metabolites.
Binding

Binding to tissue fractions (microsomes, vesicles, and soluble proteins), in addition to the metabolism of α-chaconine could provide a mechanism of detoxification in the tissues. A correlation of the content of labeled α-chaconine-(\(^3\)H) with the protein sedimentation pattern of tissue components, as a measure of the concentrating capacity of these organelles for the glycoalkaloid, demonstrates the possible affinity of α-chaconine for these tissue components. The inability of the organic solvents to remove all of the radioactivity in the tissue homogenates, as shown by the residual 'bound' activity in these tissues after the solvent wash, is the basis for suggestion of the involvement of some interactions. Binding of α-chaconine (or its metabolites) to tissue macromolecules further corroborates the data on the subcellular distribution. Solanine, a steroidal glycoalkaloid in the same class as α-chaconine, has been shown to cause death, due to central nervous system depression, and its cardiotonic properties are shown to be roughly the same as K-strophanthoside (Nishie et al., 1971). The binding of α-chaconine (and its metabolites), a close structural analogue of α-solanine, to the brain, heart, and other tissues could become a factor of importance while considering the possible toxicity hazards of potato glycoalkaloids.

Excretion of metabolites

The patterns of excretion of the metabolic products in urine and feces differed with respect to their composition. In the urine, over half of the eliminated radioactivity during the initial 24 hours was due to unaltered α-chaconine. In feces, the principal chloroform
soluble metabolite is the unaltered glycoalkaloid, a fact which is indicative of very low absorption within the gastrointestinal tract. Contrary to the general belief that the absorption of the potato glycoalkaloid is poor following oral administration, only 5% or less was excreted in the feces during the first 72 hours, a duration greater than the average transit time of ingested material within the gastrointestinal tract for hamsters. The low recovery in excretory products of the administered glycoalkaloid is a strong indication of accumulation of the compound or its metabolites in body tissues.

In view of the toxic hazards associated with the consumption of potatoes high in glycoalkaloids, the cumulative property may be very important since these glycoalkaloids are normally found in small amounts in normal potato tubers. The eating of blighted or stressed (damaged) potatoes should thus be avoided since glycoalkaloids are known to be increased in tubers under these conditions (Allen and Kūć, 1968; Ishizaka and Tomiyama, 1972) and their effects could be cumulative. The cumulative property of this glycoalkaloid is shown by the binding data.

Acetyl Cholinesterase Inhibition

Kinetics

Enzyme inhibition. Based on results obtained from the inhibition of the cholinesterases by α-caconine, as well as previous information on the inhibitory effects of solanine on this enzyme system, it becomes apparent that such inhibition is crucial in evaluating the toxic effects of potato glycoalkaloids. The bimolecular inhibition
constants ($K_i$) obtained from both acetylcholinesterase ($K_i \ 8.3 \times 10^{-6} \ M$) and butyrylcholinesterase ($K_i \ 4.0 \times 10^{-6} \ M$) show the relative inhibitory capacity of $\alpha$-chaconine as a natural cholinesterase inhibitor. $\alpha$-Solanine has been described as a weak to moderate inhibitor of both specific and non-specific cholinesterase (Patil et al., 1972). Of the natural inhibitors of acetylcholinesterases evaluated, eserine has been described as the "most potent" with a $K_i \ 5 \times 10^{-8} \ M$ (Dixon and Webb, 1964). On the basis of $K_i$ values obtained for $\alpha$-chaconine in this study, $\alpha$-chaconine might thus be regarded as a fairly potent inhibitor of the cholinesterases.

The double-reciprocal plots for the hydrolysis of acetylthiocholine and butyrylthiocholine at various $\alpha$-chaconine concentrations suggests the classification of $\alpha$-chaconine as a partially non-competitive mixed-type inhibitor, with both $V_{\text{max}}$ and $K_m$ of both cholinesterases altered by $\alpha$-chaconine. Studies of other cholinesterase inhibitors have shown that mixed-type effects are obtained (Krupka and Laidler, 1961). Inhibition by these compounds was shown to arise by combining with a later intermediate in the reaction pattern, rather than the initial enzyme substrate complex. Most acetylcholinesterase inhibitors of natural origin contain basic nitrogen atoms in a heterocyclic ring, and in some cases are methylated. A structural comparison of $\alpha$-chaconine with these cholinesterase-inhibiting alkaloids shows only the presence of N-atom in a fused ring position, with very little methylation. This structural dissimilarity between $\alpha$-chaconine and other natural cholinesterase inhibitors does not, therefore, rule out the possibility of $\alpha$-chaconine having a similar mode of interaction with the enzyme.
Further studies on the inhibition kinetics of \( \alpha \)-chaconine are, therefore, necessary in order to elucidate the inhibition pattern of this apparently toxic potato glycoalkaloid.

Subcellular fractionation of the brain homogenate showed that acetylcholinesterase activity was not localized in any particular fraction or subcellular unit, with the microsomes (fraction II) and mitochondria (fraction I) containing the highest amount of enzyme activity. Earlier reports of Michaelson (1967), De Robertis et al. (1962) indicated that major acetylcholinesterase activity is located in the subcellular fraction containing membranous and vesicular elements in the brains of guinea pigs and rats, respectively. The mitochondrial region had a diminished ability to hydrolyze acetylcholine. Reports by Watanabe and Sharma (1972) also indicated that the greatest cholinesterase activity in the subcellular fractions from brain and heart tissues of rats were in the vesicular and soluble regions of the cells. The results obtained in this study seem to agree with studies by McIntosh and Plummer (1976) on the localization of brain acetylcholinesterase in the subcellular fractions. Their results showed that over 75% of the acetylcholinesterase activity was concentrated in the mitochondrial and microsomal fractions. Low enzyme activity in the nuclear fraction has been attributed to the activity in the nuclear envelope (Lewis et al., 1965) and to partly disrupted cells (Whittaker, 1969). This could also explain the low activity observed in the nuclear fraction.

The physiological role of the isolated subcellular cholinesterase fractions has not been fully elucidated, but the susceptibility of these fractions to \( \alpha \)-chaconine inhibition is clearly illustrated in
this study. Localization of radioactivity within the microsomal and mitochondrial fractions following the administration of α-chaconine-(3H) and the observed inhibition of subcellular acetylcholinesterases (mitochondria and microsomes) might suggest a possible site of action for α-chaconine in the brain.

Variation of inhibition sensitivity in different areas by brain subcellular fractions makes it difficult for the use of total cholinesterase activity as a measure of toxicity. It has been estimated, theoretically, that only 6 to 10% of the amount of hydrolyzable acetylcholine is required to maintain 1 to 1.5 million impulses per hour (Nachmanson, 1959). The implication of this, therefore, in relation to the subcellular inhibition studies, is the apparent importance of the inhibition of acetylcholinesterase at a physiologically active site over the inhibition of the enzyme at other less important sites. This is further underlined by the fact that toxicity of most compounds which act on the central nervous system (e.g., organophosphates) is influenced greatly by the inhibition of the respiratory centers.

The possible role of α-chaconine in the blood clotting mechanism was observed in this study. The ability of α-chaconine (60 mg/Kg) administered ip, to induce the clotting of oxalated blood, suggests the diminution of the anticoagulant activity in the blood by this glycoalkaloid. Further investigations into the clotting effects of α-chaconine and possibly other glycoalkaloids are necessary in order to make any definite conclusions in this regard.
Isoenzyme Inhibition

The results obtained in this study showed definite changes in the response of cholinesterase isoenzymes of rat brain, plasma, erythrocytes and to a lesser extent, heart, to systemically administered α-chaconine. This aspect of cholinesterase inhibition by a solanum glycoalkaloid per se has received very little attention.

The complexity of the behavior of esterase isoenzymes from various animal tissues has been observed in earlier studies identifying varying isoenzyme bands. Some reports had established the presence of only arylesterases (EC3.1.1.3) and acetylesterases (EC 3.1.1.6) in brain aqueous extracts (Holmes and Masters, 1967), while Davis and Agranoff (1968) have reported the presence of up to 10 bands in a detergent extract of rat brain. Vijayan and Brownson (1974), using experimental conditions similar to those used in the present investigation, detected three zones of AChE activity from rat brain homogenates, with the possibility of the existence of subzones within the identified bands. The variation in the number of isoenzyme bands obtained for brain, plasma erythrocytes in this study could be justified in view of such complexity in the behavior of AChE isoenzymes. Several factors liable to contribute to such complexity or differences would include preferential binding of certain ChE forms by tissue components and the degree of completeness of migration into the gel. Also, most neuronal AChE is believed to be membrane-bound, thus making its identification in aqueous extracts dependent on the effective disruption of the membranes during homogenization.
The behavior of cholinesterase isoenzymes (e.g., $K_i$ values) in the gel has been shown to differ from that observed of the enzyme in solution (Chiu et al., 1972). This observation has led to the suggestion that enzymes immobilized in the gel could behave differently from those in solution.

The ability of the slower moving isoenzyme bands, separated from brain, plasma, erythrocytes and heart tissues, to hydrolyze both acetylthiocholine and $\alpha$-naphthylacetate was clearly demonstrated in this study. The AcThCh hydrolyzing zones have strong affinities for other choline esters and are also inhibited by DFP, a factor in their identification as reported by Vijayan and Brownson (1975) and Eranko et al. (1962).

Electrophoretic differences in the enzyme patterns of the tissues studied demonstrated the susceptibility of the isoenzymes to the inhibiting action of $\alpha$-chaconine. The fastest migrating AChE isoenzyme bands in brain and plasma appeared to be more susceptible to $\alpha$-chaconine inhibition than the slower migrating bands in the in vivo determination. Isoenzyme band 5 in plasma showed very little change in activity (peak area) at the 30 mg/Kg dose level as compared to almost 100% inhibition of band 1. With the esterases, greater susceptibility to $\alpha$-chaconine inhibition was seen in the slower migrating bands, corresponding in mobility to the AChE isoenzyme bands. $\alpha$-Chaconine, therefore, might be classified as a specific cholinesterase inhibitor. Resistance of the slowest migrating AChE isoenzyme band to DFP and eserine inhibition has been reported (Vijayan and Brownson, 1975). The relationship between the AChE isoenzyme inhibition
characteristics of organophosphate and carbamate compounds with those of \( \alpha \)-chaconine might suggest a possible similarity in their mode of action. Complete inhibition of those isoenzyme bands contributing or possessing the greatest activity would be of some significance in the overall evaluation of the inhibitory action of \( \alpha \)-chaconine and other solanum glycoalkaloids in general. The progressive increase in the activity of the isoenzyme band 3 in the brain homogenate with increasing dosage of the glycoalkaloid is also worthy of mention, as this might influence the overall picture of the total enzyme inhibition by this compound.

In addition to the inhibition of the specific and pseudocholinesterases by \( \alpha \)-chaconine, other esterases which hydrolyze \( \alpha \)-napthyl acetate respond to the inhibiting action of the glycoalkaloid, while some are unaffected by it. Esterases, other than the cholinesterases, include the amylesterases and the aliesterases.

Inherited variations in human serum cholinesterase activity have long been recognized. The demonstration that potato cholinesterase inhibitors exerted a differential action on the usual, intermediate and atypical phenotypes similar to that produced by dibucane (Harris and Whittaker, 1959) may well have important significance as a result of the differential isoenzyme inhibition obtained in this study. It is difficult, though, to speculate on the implications of the present result in terms of its genetic implications.

Very little information is available on the physicochemical basis or even the functional significance of brain and tissue AChE isoenzymes. Any assessment of the in vivo effects of the importance of the
inhibition of isoenzymes would, therefore, be purely speculative. Differential sensitivity of the isoenzymes to various inhibitors could represent structural differences between molecules controlling the rate of reaction with the inhibitor. The various isoenzymes of AChE may be representing enzymes existing at several sites within the cell, either bound to molecules in one form or other, or in some state of aggregation, a possibility which makes the enzyme subunit inhibition a factor of importance while considering overall inhibition of the enzyme system. The response of the tissues to α-chaconine could be a function of the spatial location of the subunits.

Another source of variability in tissue AChE response worth considering is the suggested existence of AChE in two fractions within the autonomic ganglion cells, an externally oriented (functional) enzyme and an internally oriented (reserve) enzyme with different degrees of accessibility to inhibitors (Koelle, 1963). The overall effect of the inhibitor (α-chaconine) in the AChE system, and the possible outcome of such an inhibition, could be determined by the inhibition of the isoenzymes in the various tissues. Further studies on the kinetics of inhibition of the various isoenzymes by α-chaconine would be necessary in order to make any meaningful conclusions on the importance of the isoenzyme inhibition by the glycoalkaloid, in relation to other toxicological properties.
SUMMARY AND CONCLUSIONS

The absorption, distribution, metabolism and tissue binding of radioactivity in male hamsters were studied after oral and intraperitoneal administration of α-chaconine-(^3\text{H}). Absorption of the labeled compound from the gastrointestinal tract was minimal, while its elimination via urine and feces was less than 25\% of the excreted radioactivity. Tissue concentrations of radioactivity reached their highest peak after 12 hours following oral administration, with the highest concentrations being in lungs, liver, spleen, skeletal muscle, kidney and pancreas. Concentrations of radioactivity in tissues following ip administration of the labeled compound were higher than those of observed after oral treatment.

Subcellular distribution of the labeled compound showed the highest concentration of radioactivity in the nuclear and microsomal fractions of brain, liver and heart tissues. Binding studies with tissues from the treated animals showed substantial binding of radioactivity in brain, testes, kidney, lung, liver and heart, with the highest percentage binding (80-100) in brain and testes. Binding of radioactivity to the various tissues is assumed to be a probable cause of the low elimination of the administered label in the experimental animals.

Excretion of α-chaconine-(^3\text{H}) and its metabolites was investigated after oral and intraperitoneal administration in hamsters, with the chloroform-soluble metabolites being excreted in greater amounts (100 times). In urine, over half of the eliminated radioactivity during
Inhibition of acetylcholinesterases by α-chaconine was studied. The inhibition of purified erythrocyte acetylcholinesterase and horse serum cholinesterase by α-chaconine was found to be a mixed-type with a bimolecular inhibition constant ($K_i$) for both the specific and pseudo-cholinesterases of $8.3 \times 10^{-6}$ M and $4.0 \times 10^{-4}$ M, respectively.

The distribution of acetylcholinesterase among the subcellular fractions of rat brain homogenate separated by sucrose density gradient centrifugation was determined, as well as the inhibition pattern of these fractions following in vitro incubation with 0.016 M α-chaconine. Enzyme activity was found to be distributed equally between the mitochondrial and microsomal fractions, with the nuclear fraction having the least activity. Percentage inhibition of the various fractions obtained was: whole homogenate 43, nuclear fraction 55, mitochondria 35, and microsomes 33.

Brain AChE activity of animals given intraperitoneal doses (10, 30, 60 mg/Kg) of α-chaconine were 79, 55, and 18% of the control group. Acetylcholinesterase activity of heart and plasma of animals administered α-chaconine did not show the dose-related response observed in the brain. Inhibition of heart AChE was 61%, while plasma gave 51% for the rats given a dose of 10 mg/Kg and 0% for rats given 30 mg/Kg.

Acrylamide gel electrophoretic separation of aqueous homogenates from whole brain and heart of adult male rats administered α-chaconine
was investigated. Brain acetylcholinesterase isoenzymes were found to be inhibited by 30 and 60 mg/Kg dosage levels of α-chaconine administered intraperitoneally. Electrophoretic separation of plasma from the treated animals resulted in five anodally migrating zones having properties of cholinesterases. These sites hydrolyzed acetylthiocholine and α-naphthylacetate, and all were inhibited by α-chaconine except the slowest migrating band (band 5). Inhibition of isoenzyme activity of bands 1 and 2 is observed for the groups administered 10 and 30 mg/Kg α-chaconine with the percentage inhibition of both bands (1 and 2) being 40 and 77% for animals given 10 mg/Kg and 100:75% for the latter group. Isoenzyme bands 3 and 4 were completely absent in the alkaloid treated animals. Inhibition of non-specific ChE isoenzymes (butyrylthiocholine hydrolyzable bands) by α-chaconine was clearly demonstrated.

*In vitro* inhibition of plasma, erythrocyte and brain esterase isoenzymes was estimated by incubating gels with $10^{-4}$ and $10^{-3}$ M α-chaconine after the electrophoretic separations. With both concentrations of α-chaconine, the various isoenzymes showed some response to the inhibitory potency of α-chaconine. The slower-moving isoenzyme bands were inhibited to 100% with the different concentrations of inhibitor. The fast migrating isoenzyme bands in plasma and erythrocytes were least affected by α-chaconine ($10^{-4}$ M), with 0% inhibition. Plasma protein isoenzymes from ip injected rats were not affected by α-chaconine.
LITERATURE CITED


Nachmansohn, D. 1966. Role of acetylcholine in neuromuscular trans-

Nishie, K., M. R. Gumbmann, and A. C. Keyl. 1971. Pharmacology of

Press, New York.


Oettingen, W. F. von. 1952. Poisoning. Harper Hoeber Medical Divi-
sion. Harper-Row (Hoeber), New York.

Okita, G. T. 1967. Species differences in duration of action of

Orgell, W. H. 1963. Inhibition of human plasma cholinesterases in
vitro by alkaloids, glycosides and other natural substances.
Lloydia 26:36-43.

Orgell, W. H., K. A. Vaidya, and P. A. Dahm. 1958. Inhibition of
human plasma cholinesterase in vitro by extracts of solanaceous

I and II. Distillation Products Industries, Rochester, New York.

Evaluation of solanine toxicity. Food Cosmet. Toxicol. 10:
395-398.

Pokrovskii, A. A. 1956. The effect of the alkaloid of the sprouting

induction of fetal malformation with "blighted" potato: A


Quenneville, G., B. Barton, E. McDevitt, and I. S. Wright. 1959. The
use of anticoagulants for thrombophlebitis during pregnancy.

Reiner, E., W. Seuferth, and W. Hardegg. 1965. Occurrence of cholin-


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Doctor of Philosophy

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Scientific Publications:


Alozie, S. O., R. P. Sharma, and D. K. Salunkhe. Absorption, distribution, and metabolism of the steroidal glycoalkaloid, α-chaconine-(3H), and tissue binding of radioactivity in hamsters following intraperitoneal and oral administration. (Submitted for publication to Tox. Appl. Pharmacol.)