ISOLATION, PURIFICATION, AND STRUCTURAL CHARACTERIZATION OF THE SAPONINS FROM *GLOTTIDIUM VESICARIUM*

By

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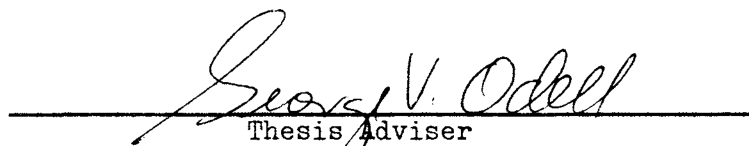
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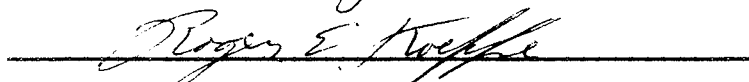

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CHAPTER I

INTRODUCTION

Glottidium vesicarium (Jacq.) Harper, variously known as castle bean weed, coffee bean weed and bladder pod, has been recognized as a toxic range plant in the area of the Coastal Plain from North Carolina and Florida to Oklahoma and Texas (1,2). Loss of cattle has been known from the late 1800's (1). Experimental feedings have been made to cattle by several investigators. Figures for toxicity of seeds range from 0.15 percent, which produced chronic poisoning and death after 14 days (3) to 2 percent, which produced symptoms in 24 hours and death in 2 days (4). Considerable variability was reported by Featherly and coworkers (5), they noticed that the bean had lost its toxicity while being stored in the laboratory.

In 1940, tests performed on the dried seeds by Foote and Stamling (6) indicated the presence of saponins and the absence of alkaloids. Later Nuessle and Lauter (7) reported the isolation of three phytochemical constituents from the mature seeds which showed great similarity in their infrared spectra. The presence of saponins was discussed, but they did not make any further characterization and the purity of their isolates is questionable.

Chemically, saponins are non-cardiac plant glycosides which yield on hydrolysis (a) one or more sugar units and (b) sugar-free aglycones which are derived from polycyclic ring systems and are generically

referred to as sapogenins. The distribution of saponins in the higher plants is very wide; they are known in 400 species from 50 families (8). Although a great many sapogenins have been studied, relatively few glycosides have been characterized. When compared with other plant glycosides such as cardiac glycosides, this group of natural products is rather poorly characterized, due mainly to the following complications:

(1) Saponins are difficult to crystallize, the sapogenins are more stable and more readily isolated in pure condition.

(2) The saponin content of a particular plant species commonly varies with part of plant, season, and stage of growth. Marker and coworkers have repeatedly observed that the composition of the sapogenin fraction in plants undergoes certain changes (9). Krider and Wall report that the leaves of agave and yucca species, which contain saponins, also contain enzymes which are capable of hydrolyzing these glycosides (10).

(3) Saponins which are structurally closely related often occur together in the same plant, each in relatively small amounts. This has rendered the isolation of chemical individuals exceedingly difficult.

The chemical characterization of saponins involves acid or enzymatic hydrolysis followed by the identification of the sugar units and the sapogenin portion. The basic carbon skeletons of sapogenins are established from dehydrogenation and degradation studies. The structure of newly isolated sapogenins is invariably obtained from correlation with known compounds, from physical constants, from elemental analysis, from NMR spectra, and from infrared interpretations and comparisons (11, 12, 13, 14, 15). The shortcomings of some of these approaches have been

mentioned by Wall et al (16). Neither the chemical study nor the infrared data can be used as convincing evidence of chemical structure.

This problem can be exemplified by the work of Wall et al.(17). They isolated a new sapogenin, markogenin, from certain yucca species. Chemical and infrared studies proved conclusively that this sapogenin had the structure assigned by Marker to texogenin (18), but this new sapogenin (which melts some 80 degrees higher than texogenin) is distinctly different from texogenin.

The work done by Djerassi and coworkers has established the usefulness of mass spectrometry in the structure elucidation of sapogenins (19). The mass spectrum is very sensitive to both positional isomerism and stereoisomerism, and is exceedingly useful for the location of functional groups in this class of natural products. Such location can often be very arduous when only conventional chemical techniques are available (20).

The high sensitivity of mass spectrometry makes possible the elucidation of structure from microgram amounts of sample, but the requirement for sample purity is also high. As a result, the technique of preparation of a sapogenin sample with over 99 percent purity comes before the meaningful elucidation of structure can be made.

Several biological actions have been described as caused by saponins: hemolytic effects, ruminant bloat, toxicity to fish etc. The effect of various saponins on plants, animals, and especially on membranes, has served as the subject of many investigations. Our understanding of this aspect had laged far behind the accumulation of chemical information available.

The purpose of the research reported in this dissertation was to

establish the presence of saponins in *Glottidium vesicarium* through their isolation, purification, and structure determination. Certain experiments were also undertaken to demonstrate their biological effects.

CHAPTER II

LITERATURE REVIEW

Early Investigations on *Glottidium vesicarium*

The plant *Glottidium vesicarium* is a large annual legume, two to twelve feet tall, with pinnately compound leaves composed of many small leaflets. The yellow, pea-type flowers are usually borne in pairs in the axils of the leaves. The pods are two to two and one half inches long. They consist of a grayish-brown outer hull and a thin, white, papery inner hull enclosing two brown, reddish-brown or tan colored beans. The woody stem remains erect after the leaves have fallen and bears the persistent pods and toxic beans. Losses of cattle presumably from eating this bean have been reported in Oklahoma (2,5) and Florida (3).

Experimental feedings have been made to chicks (21), fowl (22), cattle (4,5), sheep, goats, etc. In fowl, Emmel (21) found 100 seeds toxic; 150 seeds lethal. Death occurred in about a week after ingestion of 200 seeds (also from 2 to 7 weeks when the seeds were fed at rates between 5 and 20 seeds per day) with considerable variation shown.

Symptoms in fowl included diarrhea, prostration, and dark, congested combs. Postmortem examinations showed necrotic enteritis, inflammation and necrosis in the gizzard.

Cattle poisoned experimentally showed rapid pulse and respiration, muscle trembling, diarrhea or constipation, loss of weight, and finally

paralysis and death (5). Postmortem examinations revealed severe or hemorrhagic inflammation of the abomasum and small intestine. Histologically, generalized albuminous degeneration was found in the kidney and liver, with some necrosis in the latter (4).

Tests performed with dried seeds by Foote and Stamling (6) indicated the presence of saponins and the absence of alkaloids. They succeeded in isolating two sapogenins in very small quantities, whereas other workers were unable to find any alkaloids or glucosides in the bean (5). Nuessle and Lauter (7) reported the isolation of three phytochemical constituents from the mature seeds by fractional precipitation of the ethanol extracts. The extract showed saponin activity. The infrared spectra of these three compounds showed a great similarity. They did not make any further chemical characterization. Their attempts to isolate crude sapogenin as well as the saponins were unsuccessful.

Saponins - Some Historical Aspects

The term saponin (Latin sapo, soap), though originally restricted to a specific substance obtained from the root of *Saponaria rubra* and *Saponaria alba*, is now applied to a large group of compounds all of which have properties similar to those possessed by the original saponin (23). They are hemolytic, toxic to fish, and surface active.

Chemically, saponins may be classified into two broad groups; glycosides of triterpenoid alcohols and glycosides of non-cardioactive steroids. Biologically both groups appear to have the same effects.

The wide occurrence of saponins in plants has long been recognized. The discovery of holothurin, a toxic steroid tetrasaccharide isolated from sea cucumber, has extended the presence of saponins to the animal

kingdom. This "animal saponin" differs structurally from the plant saponins in that it also contains a sulfate ester group (24).

Saponins are often grouped together with other naturally occuring compounds which include the steroid alkaloids, and the cardioactive and cardiotoxic glycosides (25) since they all contain a phenanthrene ring system as their fundamental structure. As a group, they are generally gastrointestinal irritants, causing emesis, and catharisis.

The present knowledge of saponin occurrence, structure and function is both fragmentary and speculative. The significance of the presence of saponins in plants is completely unknown. Their distribution in plants is undefined, since usually only those saponins which are present in sufficient quantity for isolation have been studied. They are usually recognized through their "toxicity" (hemolytic effect, growth depressing effects, ruminant bloat, respiratory inhibition, effect on fish, retardation of seed germination etc.).

Physiological Action and Biochemical Effects

Much information on saponins has been obtained on the basis of their physiological and biochemical effects. The effects of holothurin on "pearl" fish has been studied (26). Lethal concentrations were found to vary from 100 ppm in 3 to 15 minutes to 1 ppm, or less, in 4 to 8 hours. Some neurological properties have also been associated with holothurin A.

The growth depressing effect of saponin in chicks has been noted since the late 1940's (27). Heywang and Bird (28) fed graded levels of alfalfa saponin to chicks. The 0.2 percent level was the lowest that would produce an unmistakable inhibition of growth. A study with grow-

ing chicks and adult roosters indicated that dietary saponin can depress blood plasma cholesterol previously elevated by feeding low protein levels in the presence and absence of dietary cholesterol (29). A saponin isolated from broomweed was shown to have abortifacient properties when administered orally to pregnant rabbits (30). Evidence has been presented which indicates that saponins are responsible for the toxicity of several bloat-producing plants (31,32). Direct evidence is lacking as a result of the difficulty involved in the isolation and resolution of related saponin fractions (32).

The inhibitory effect of soybean saponins on the proteolytic activity of chymotrypsin, pepsin, trypsin, and cholinesterase has been noticed for some years. This effect turned out to be biologically unimportant since this non-specific inhibition is just a result of protein - saponin interaction and high concentrations of soybean saponins are needed for an inhibition of this type (33).

The effect of various saponins on plants has been the subject of a considerable amount of research. From 1943 to 1945, Balansard and Pelissier published a series of short papers in which they reported that leaf application of saponin solutions stimulated the development of shoots and roots in begonia, induced tumors in *Hedera helix* (34), and influenced chlorophyll synthesis in *Euonymus japonicus* (35). They also found that saponin inhibits root growth in tomatoes (36). Also, saponin treatment of excised wheat embryos increases their growth rate (37,38). As pointed out by Heine in 1953 (39), the surface activity of saponins may be responsible for their effect in accelerating water absorption and germination which have been observed in pea, maize, tomato, and other seeds. Usually, low concentrations promote germina-

tion, while high concentrations are inhibitory (40). In some cases, seed treatment with saponins had a lasting effect on subsequent plant growth (36). Their effect in increasing the polyploidising efficiency of colchicine has also been reported (41).

A study on the growth-regulating activity of digitonin showed that it can significantly enhance the growth of sectioned *Avena* coleoptile in the concentration range 0.0001 - 10 mg/l with a maximum response at 0.1 - 1 mg/l. Digitonin is therefore more active than indoleacetic acid, a naturally occuring auxin in *Avena* coleoptiles (42).

Membrane Effects

When polymorphonuclear leucocytes are treated with saponins, they exhibit changes in oxygen uptake and in carbohydrate oxidative metabolism (43). The most relevant change induced by such saponin treatment appears to be an enormous increase in the granule bound NADPH oxidase activity. Cellular integrity is required and saponin seems to exert its effect by producing alterations in the cell membrane. Many investigations have been performed in recent years on membrane alterations produced by saponin either in cellular or in artificial systems.

In 1912 (44), saponin, in high dilution, was found to inactivate *Rous sarcoma* virus by damage of the outer membrane of this virus particle in a characteristic manner. Fifty years later (45), electron microscopic observations revealed that the action of saponin on the outer membrane of *Rous sarcoma* virus, on chicken liver cell membranes and on human or guinea pig erythrocyte ghosts is to produce a hexagonal array of pits each of which is surrounded by a more or less distinct circular, or hexagonal rim. With increasing amounts of saponin, these

membranes are destroyed and masses of strands, or rings, are produced. It has been suggested (46,47) that the hexagonal structure that is produced by adding saponin to preparations of cell membranes represents primarily the molecular arrangement of a complex of saponin with cholesterol, and does not represent any pre-existing arrangement of lipid and protein in the cell membrane. The incorporation of saponin into the cell membrane probably forms a structure that is more permeable than the original membrane. The production of holes through the lipid component of the cell membrane probably constitutes the hemolytic mechanism of saponin but the exact relation of these structural changes to hemolysis remains unclear. This is partially due to the fact that our present knowledge of the structure of the cell surface is still fragmentary.

Pharmacological Effects

The general pharmacological behavior of saponins has been studied in some systems (48).

In East Africa, extracts of members of the plant genus *Albizia* have been used to accelerate labour and procure abortion (49). Partial characterization indicates that the active principle is a saponin. Lipton (50) has presented evidence showing that this oxytocic saponin elicits powerful responses in isolated strips of uterine muscle from different mammalian types. It acts to induce contractions when the tissue is quiescent and to increase the frequency and force of contractions when spontaneous activity is present.

The pharmacological effects of glycyrrhizin, the saponin of licorice root, have been evaluated quite extensively. The strong pharmaco-

logical resemblance between glycyrrhizin and desoxycorticosterone (DOC) has been recognized in the early 1950's. The DOC - mimetic action of ammoniated glycyrrhizin on electrolyte and water metabolism and on pituitary - adrenal function was demonstrated by Kraus (51) in rats. In intact rats, the antidiuretic effect of glycyrrhizin was immediate and cumulative. Rat receiving glycyrrhizin prior to histamine stress showed a marked decrease in adrenal ascorbic acid depletion, suggesting that glycyrrhizin had suppressed the output of adrenocorticotropin (ACTH). Also, tests with mice, rats, cats and rabbits showed (52) that NH_4 glycyrrhizate and Na glycyrrhetinate nearly equal cortisone in treating inflammations, either in the exudative or in the proliferative stage. The detoxifying effect of glycyrrhizin against the toxic action of strychnine nitrate (53) was reported.

Although most saponins are poorly absorbed from the alimentary tract it has been established that the administration of some saponins simultaneously with drugs increases the absorption of the latter from the intestines. There is also a reported adjuvant effect of saponins on the vaccine for "foot and mouth" disease (strains of virus A, O and C) (54).

Another observation which may be of clinical interest is the finding that mice treated with sublethal doses of saponin can produce a new acute phase plasma component and become resistant to the lethal activities of saponin and of streptolysin O (55).

The complete value and significance of saponins in therapeutics is still not established at the present time.

Biogenesis

In saponin biosynthesis, the attachment of sugar units to their aglycone is believed to follow the general mechanism for all other known glycosides.

The biosynthesis of the aglycone portion of saponins is intimately associated with that of the steroids, squalene, the common precursor, can give rise to various steroids and triterpenes depending on the type of folding and cyclization (56,57). The stepwise reactions which lead to the biogenesis of squalene have been well established. Although the biological conversion of squalene to steroids has also been confirmed over and over, the mode of the cyclization processes involved in this conversion has been the subject of considerable research and controversy.

It is generally agreed that both steroids and triterpenes arise biosynthetically by cyclization of a folded squalene chain and that the stereochemical configurations of the products are all predetermined by the way in which the squalene chain is folded.

In 1953 Ruzicka and his collaborators (58,59), on the basis of a well defined system of arbitrary assumptions, developed a system leading from squalene to the formulae of the basic representatives of all known cyclic triterpene groups (e.g. euphol, tirucallol, lupeol, taraxasterol, germanicol, beta-amyrin, taraxerol, friedelin, alpha-amyrin, lanosteron) in full structural and configurational detail.

In this system, the cyclization is a concerted or non-stop process, i.e. it occurs without formation of stabilized intermediates, and it is initiated by the attack of an hypothetical electrophilic OH^+ (or its biochemical equivalent). The simplest example for this postulation is the cyclization of the "all chair" coiling squalene to give a

product corresponding to hydroxyhopenone in all stereochemical details (57) (Fig. 1).

Later, Bloch and his associates (60) provided experimental evidence in support of a concerted reaction mechanism.

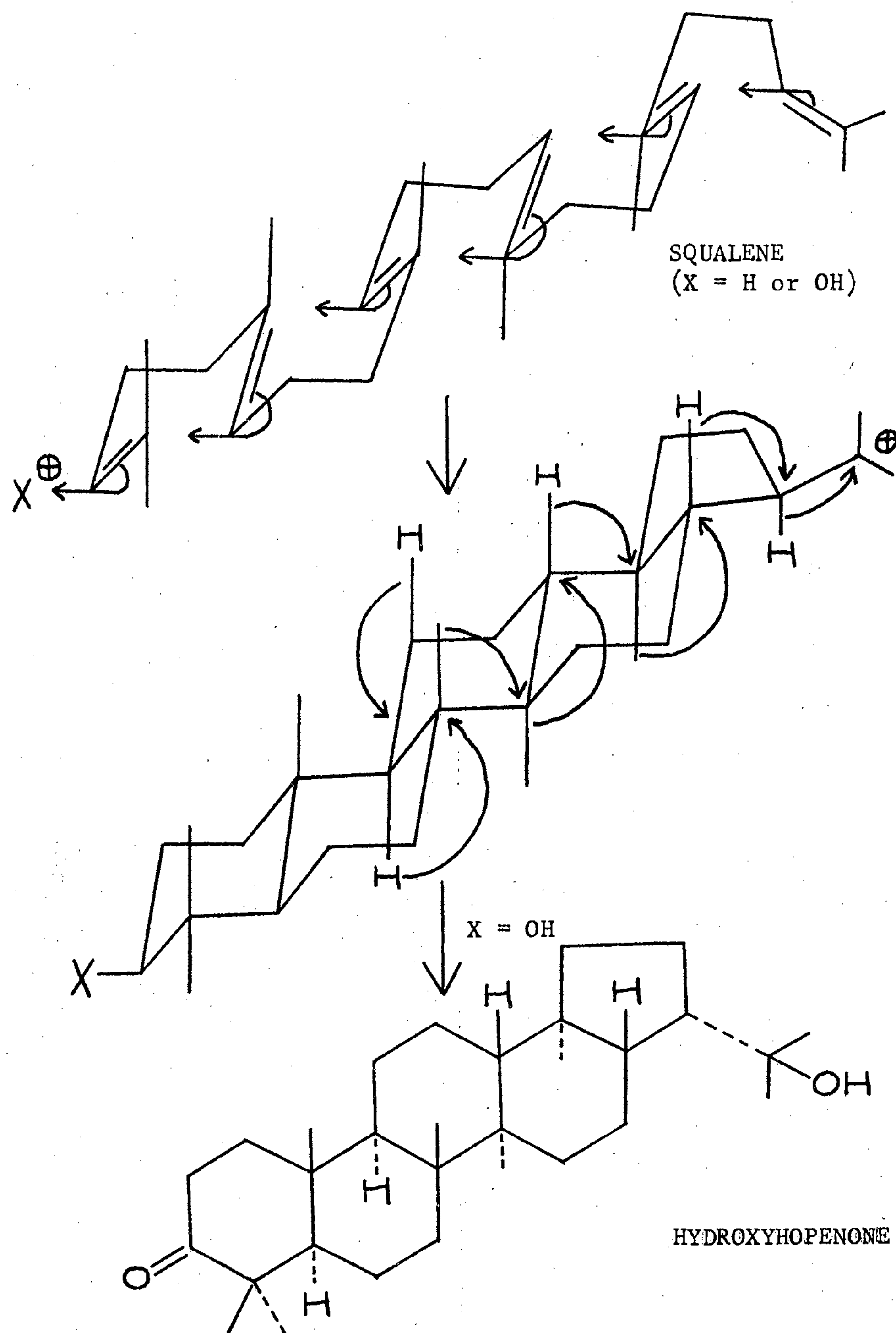
Recently, consideration has been given to squalene oxide as the cyclization intermediate, which suggests that sequential epoxidation and cyclization processes might be involved in the biochemical conversion of squalene to polycyclic terpenoids (61). Evidence for such a stepwise mechanism has been provided by the work of van Tamelen's group (62,63,64). They succeeded in demonstrating the enzymatic conversion of squalene and squalene 2,3-oxide varients to a lanosterol-like system (65).

A few tracer studies on the biosynthesis of sapogenins have been reported. The biosynthesis of pentacyclic triterpenes has been followed in germinating soybeans (66). Radioactive squalene and soyasapogenol A, among other products, were isolated after allowing soybean seedlings to grow in the presence of radioactive acetate or mevalonate.

Heftmann *et al.* (67) demonstrated the incorporation of radioacetate into diosgenin by homogenates of *Dioscorea floribunda* through rigorous purification of the isolated sapogenin. Dissection of a *Dioscorea* tuber revealed significant differences in the diosgenin content and biosynthetic activity of morphologically different parts of the tuber. The upper portion near the shoot is the richest in the crude diosgenin fraction, while the incorporation of acetate by the lower portion of the tuber is less than in the case of the other portions.

While radioactive mevalonic acid was not incorporated into diosgenin in tuber homogenates (67), the *in vivo* incorporation of mevalonic

Figure 1. Hypothetical Scheme for the Cyclization of Squalene


SQUALENE
(X = H or OH)
X⊕
H
H
H
H
⊕
H
X
X = OH
H
H
OH
H
H
O
HYDROXYHOPENONE

acid into four sapogenins of *Dioscorea spiculiflora* plants has been demonstrated (68). Inferences of doubtful value must be made from structural analogies, concentration changes, and simultaneous occurrence of steroids in plants (56).

The Chemistry of Sapogenins

The aglycone portion released by the hydrolysis of saponins is generically referred to as sapogenin. The sapogenins of steroid saponins are C_{27} steroids carrying a spiroketal side-chain, and are derived from the same tetracyclic ring system found in sterols, bile acids, and cardiac aglycons. The structure of some typical steroid sapogenins are shown in structures (I) to (IV) (Fig. 2).

The sapogenins of triterpenoid saponins consist of a 30 carbon pentacyclic skeleton which in principle is divisible into isopentane units (isoprene rule). The triterpenoid sapogenins are conveniently sub-divided into three groups based on their relationships with beta-amyrin (V), alpha-amyrin (VI) and lupeol (VII) (Fig. 2). The basic skeletons of both types of sapogenins were obtained from the same approach, viz. dehydrogenation, but the history of these chemical investigations follows two different lines, and will be treated separately in this review.

Steroid Sapogenins

The investigation on steroid saponins can be traced back to 1875, when the presence of digitonin along with cardiac glycosides in commercial preparations of digitalis from *Digitalis purpurea* was recognized by Schmiedeberg. In the period 1890 to 1918, digitonin prepara-

Figure 2. Some Sapogenin Compounds

(I) Digitogenin

(II) Gitogenin

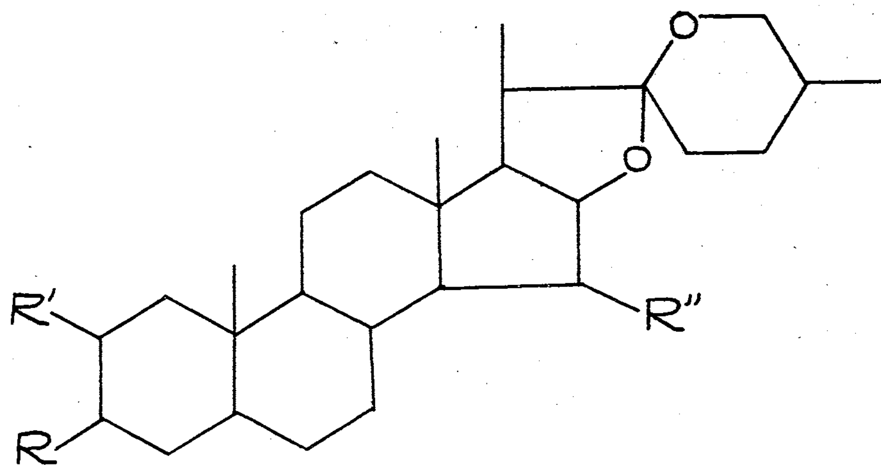
(III) Tigogenin

(IV) Sarsasapogenin

(V) beta-Amyrin

(VI) alpha-Amyrin

(VII) Lupeol

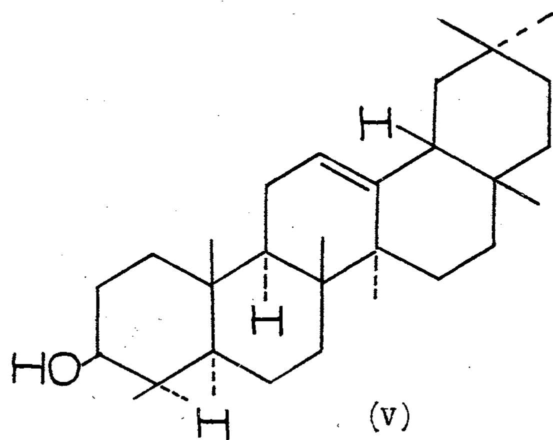


(I) R,R',R" - OH

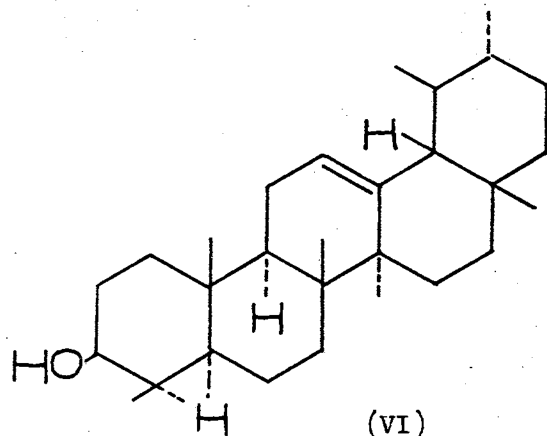
(II) R,R' - OH; R" - H

(III) R - OH; R',R" - H

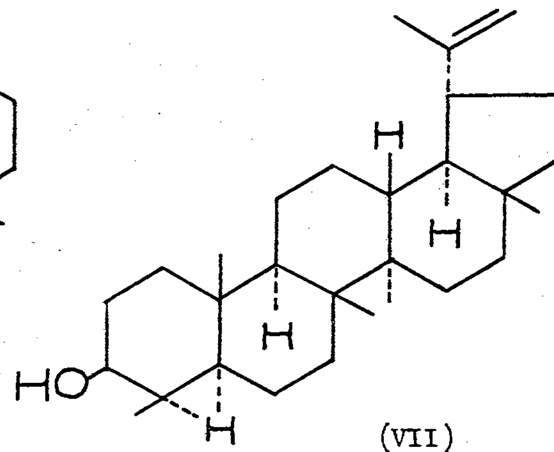
(IV) R - OH; R',R" - OH



(V)



(VI)

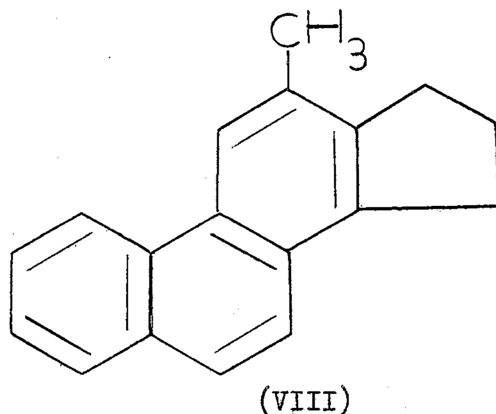


(VII)

rations were examined by Kiliani. But a reputedly pure sample was shown by Windaus and Schneckenburger in 1913 (69) to contain 10 to 20 percent of a saponin, which they named gitonin. Further saponins, tigonin and sarsasaponin, were then isolated from plants other than those of Digitalis species (70).

Although the sapogenins are more stable and more readily isolated in pure conditions, digitogenin, gitogenin, sarsasapogenin, and tigogenin were regarded as C_{26}-compounds until 1935. It was only in 1935 that Simpson and Jacobs (71) concluded that the preponderance of analytical evidence favored revision to a C_{27} formula, and the C_{27} -character of the sapogenins was then established by precision analysis of 1 gram samples of sarsasapogenin and chlorogenin, and more generally by dehydrogenation and degradation (72,73).

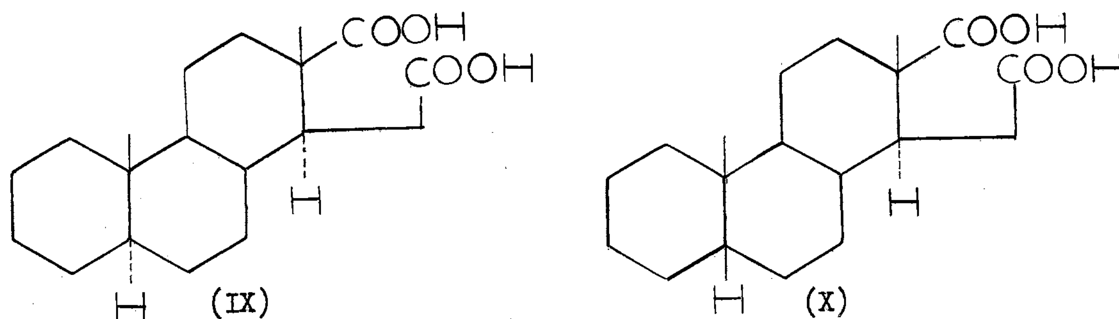
The first indication of the steroid structure of the sapogenin was the isolation of Diel's hydrocarbon (3'-methyl-1,2-cyclopentenophenanthrene) from sarsasapogenin and gitogenin as a product of selenium dehydrogenation (71). Diel's hydrocarbon contains a tetracyclic cholane skeleton related to steroid type compounds (VIII).



(VIII)

The first proof of the presence of the steroid nucleus was given

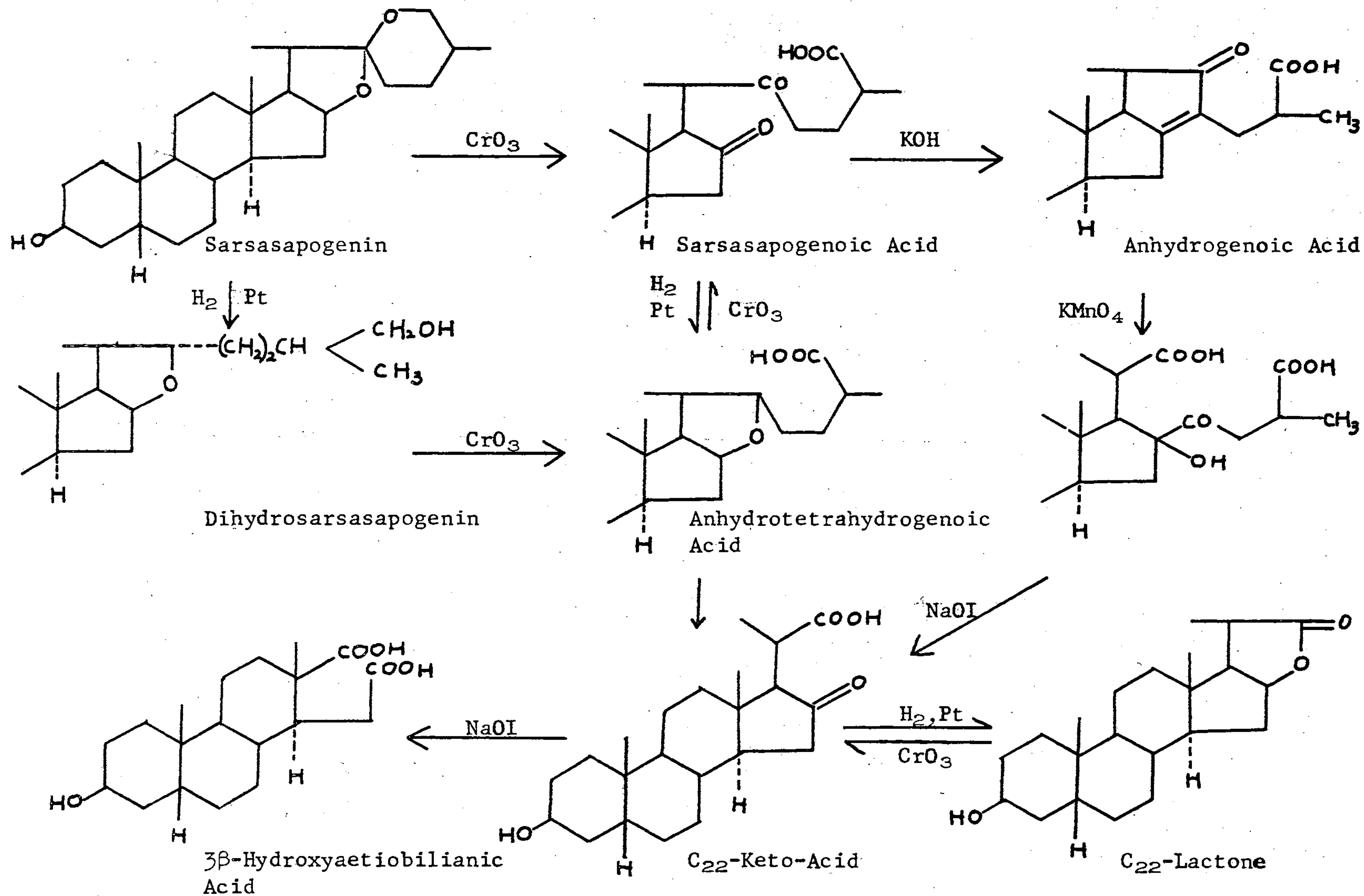
by the demonstration of Tscheche (74,75) that tigogenin, gitogenin, and digitogenin are, respectively, mono-, di-, and tri-derivatives of the same ground substance, and that tigogenin acetate yields aetioallobilianic acid (IX) by non-pyrolytic degradation.



Similar degradation of sarsasapogenin acetate gives aetiobilianic acid (X) (76). Tigogenin, gitogenin, and digitogenin thus belong to the 5 alpha-cholestane series, while sarsasapogenin belongs to the 5 beta-cholestane series. Numerous other degradations of sapogenins and their derivatives to aetiobilianic acids have been reported. In 1941 Marker <u>et al.</u> (77) reported the conversion of diosgenin to cholesterol, thereby providing conclusive proof that the steroidal sapogenins have the same carbon skeleton as cholesterol. This concluded the elucidation of the nuclear structure of steroid sapogenins.

The structure of the spiroketal side chain for the terminal part of the sapogenin was established by Marker in a series of investigations initially concerned with transformations of sarsasapogenoic acid which can be obtained by controlled chromic acid oxidation of sarsasapogenin acetate. Reactions involved in the establishment of the spiroketal side chain structure are described schematically in Fig. 3 (78). Largely through the work of Marker and his associates, the

Figure 3. Elucidation of the Spiroketal Side Chain Structure of Steroid Sapogenin


Sarsasapogenin
CrO3
Sarsasapogenoic Acid
KOH
Anhydrogenoic Acid
H2 Pt
Dihydrosarsasapogenin
H2 Pt
CrO3
Anhydrotetrahydrogenoic Acid
KMnO4
NaOI
H2,Pt
CrO3
C22-Keto-Acid
C22-Lactone
NaOI
3β-Hydroxyaetiobilianic Acid

structure of these type of compounds is for the most part well established (79,80,81,82,18).

Later, the study of Djerassi and coworkers led to the elucidation of the stereochemistry of digitogenin (83). From degradative evidence they were able to show that digitogenin is a 22a, 25a, 5 alpha-spirostane-2 alpha, 3 beta, 15 beta-triol.

The various sapogenins are closely related. Variations in structure arise from (a) the number, position, and configuration of nuclear hydroxyl groups, (b) configuration at C_5 and C_{25}, (c) the occasional presence of a carbonyl group at C_{12}. Numerous interconversions and correlations were achieved by Marker and his coworkers (18,78).

Infrared spectroscopy is useful for characterization of steroid sapogenins because several strong bands between 1350 to 875 cm^{-1} are characteristic of the spiroketal side chain (70). A systematic identification procedure has been set up by Wall and associates at the Eastern Regional Laboratory (16,84). They found that the infrared spectra are highly characteristic of the individual sapogenins and constitute a more positive identification of unknown sapogenins than any other combination of physical constants (84).

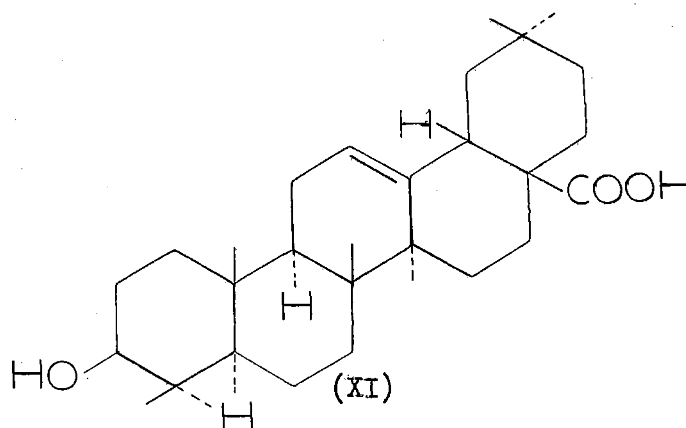
Since the 1940's, the steroid sapogenins have received considerable attention as precursors for the synthesis of sex hormones and cortisone to be used in medicine (16). The synthesis of progesterone from diosgenin (85), testosterone from sarsasapogenin (86), and cortisone from hecogenin (87) are examples for such commercial developments.

Terpenoid Sapogenins

Triterpenes are the most widespread terpenes in plants, where they are almost invariably pentacyclic compounds, e.g. terpenoid sapogenin, with few functional groups and thus difficult to attack chemically for structural deduction (57). Accordingly, their structures were not known until after those of the biogenetically related steroids.

The pentacyclic triterpenes afford the most characteristic picene dehydrogenation products only in meager yield if at all, and the major structure deductions, largely from the work of Ruzicka's group, were based on the more common naphthalenic dehydrogenation products arising from cleavage in the center of the molecule.

The early structural work was done principally with the beta-amyrin group: hederagenin and oleanolic acid. Due to the mass of experimental data, it is easier to explain this type of work in terms of one structure for oleanolic acid (XI) (88).



As was pointed out above, the assignment of a hydrogenated picene nucleus is based primarily on the structure of the aromatic hydrocarbons formed on dehydrogenation with selenium at relatively high temperature (320 to 350 degree). The structure of ring A and the portion of ring B through C_7 have been proven by stepwise oxidative degradation. The

location of the double bond in ring C is based on the pyrolysis of the mono-methyl ester of isooleanonic lactone dicarboxylic acid which is formed from oleanolic acid by a series of reactions including oxidative fission of the ring containing the double bond (89). The position of the carboxylgroup relative to the double bond is indicated from studies which involve the conversion of oleanolic acid to a lactone and bromo-lactone (in these reactions the hydroxyl group is not involved) (90,91).

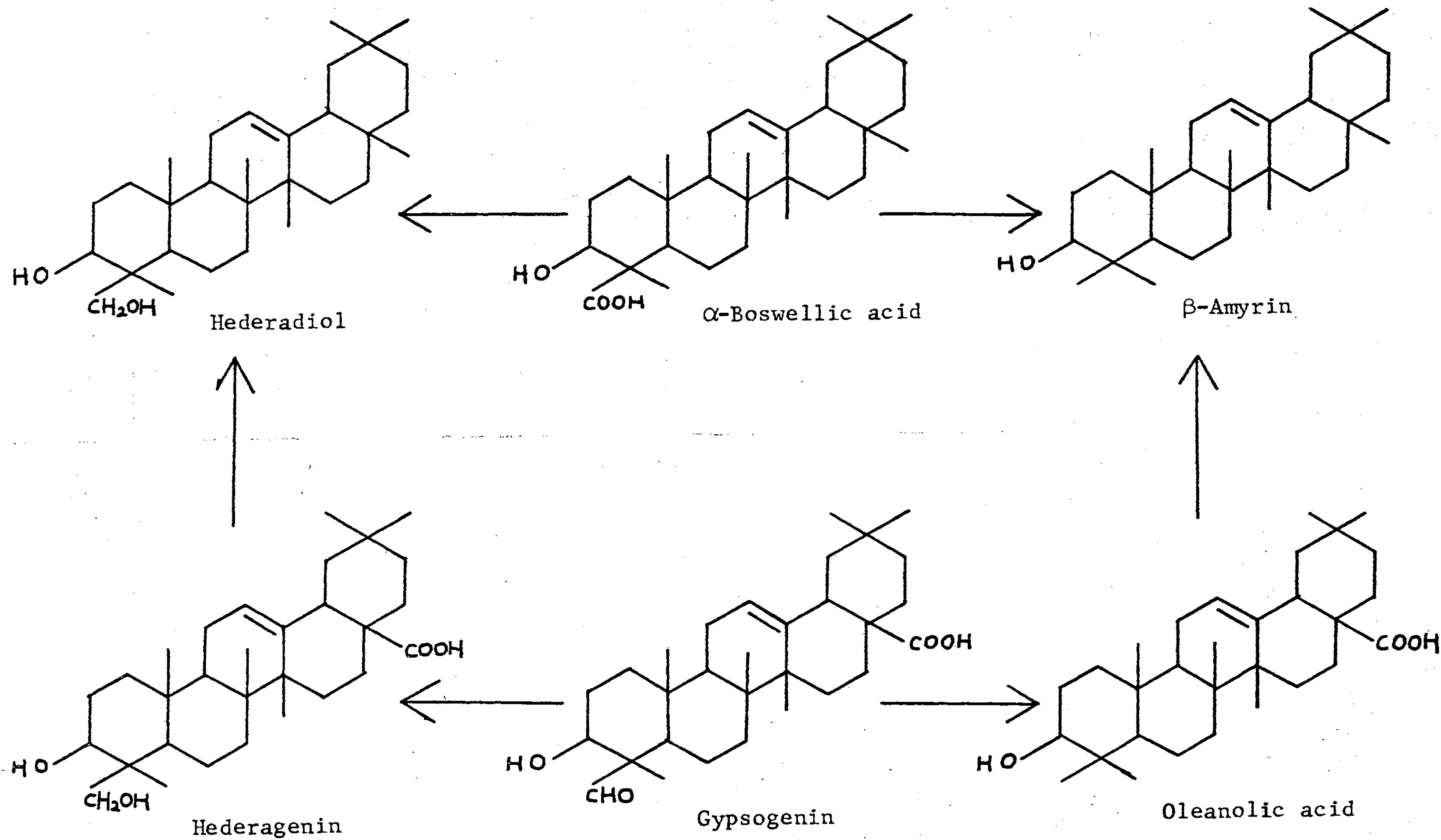
As described in a classical review by Noller (88), probably the most important advance in the field of the triterpenes has been the establishment of the suspected relationships between various individual compounds and their assignment to different groups.

The interconversions have depended for the most part on the conversion of a carboxyl group to a methyl group, or the reduction of an aldehyde or ketone group to a methyl group. The interconversions that were accomplished in the early 1940's are represented schematically in Fig. 4. From this type of work the reactions of each compound in a given group have a bearing on the structure of all members of the group, and the complete determination of structure for one member will establish the constitution of most members of the group.

Later, the structure of the alpha-amyrin group was established from detailed degradation experiments similar to those performed on oleanolic acid (92). The structure of the lupeol group was established by Ames <u>et al.</u> (93) on the basis of interconversions which provide a direct association with the beta-amyrin group.

In triterpenoid sapogenins, the parent hydrocarbon skeleton contains eight centers of asymmetry giving rise to 256 possible configurations which would be further augmented when substituents were intro-

Figure 4. Interconversions of Triterpenes in beta-
Amyrin Series


HO
CH₂OH
Hederadiol
HO
COOH
α-Boswellic acid
HO
β-Amyrin
COOH
HO
CH₂OH
Hederagenin
COOH
HO
CHO
Gypsogenin
COOH
HO
Oleanolic acid

duced in the alicyclic system (48). The resolution of the stereochemistry in this group of compounds involves the application of conformational analysis (94), molecular optical rotation (95), X-ray evidence (96), and stereospecific reactions (97).

The stereochemistry of beta-amyrin was derived from chemical evidence in 1952 (98); that of the lupeol group followed from its relationship with the beta-amyrin group by interconversion (93). The complete stereostructure of alpha-amyrin was established on the basis of both chemical and physical data (99), and was further proved when alpha-amyrin was synthesized from a beta-amyrin derivative by a series of stereospecific reactions (97).

As shown in (V) and (VI), both alpha- and beta-amyrins possess trans-anti-trans-trans-syn-cis arrangement of the rings A, B, C, D, and E.

Physical Methods - Application to Structural Problems

Glycosidic compounds such as the saponins are readily extracted from plant material with hot water or alcohol. The aqueous solution can then be extracted with benzene to remove fatty material. The saponins are then extracted with butanol, leaving proteins and carbohydrates in the aqueous phase. More specifically, saponins may also be precipitated from alcoholic solution as the molecular compounds with cholesterol or other 3 beta-hydroxysteroids.

In contrast to this easy isolation procedure, saponins are difficult to purify. In purification, they are usually first hydrolyzed to their aglycones which are more stable and more readily isolated in pure condition. In the early studies, fractionation of sapogenins was

based principally on differences in solubility of the various sapogenins or their acetates in solvents such as ether, acetone, methanol, or acetic anhydride (18). These procedures were carried out with large quantities (100 to 500 grams) of sapogenins and were difficult to duplicate with quantities of total sapogenins ranging from 1 to 25 grams (16).

From a study of more than 1000 sapogenaceous plant samples, Wall et al. (16) reported column chromatography on alumina as a fractionation method. In a typical isolation experiment, 10 kilograms of fresh agave leaves gave a total of 70 grams sapogenin, which was then separated by column chromatography into 28 grams of hecogenin, 22 grams of tigogenin, 11 grams of gitogenin, and 9 grams of manogenin.

When the isolation of small amounts of pure compounds from complex mixture is attempted, more selective techniques such as paper and thin-layer chromatography are required. Pure compounds are often available only in very small quantities, and provide structural problems of a new order of complexity. The classical chemical approach, which involves functional group tests, derivative preparation, combustion analysis etc., is impractical for handling samples obtained in only milligram amounts. Modern natural products chemistry, in terms of isolation, structural elucidation, and determination of configuration, depends mainly on the application of physical methods.

A brief introduction on the application of physical data, e.g. ultra-violet, infrared, ORD, circular dichroism, and especially NMR and mass spectrometry, to stereochemical, conformational, and structural problems in sapogenins is given below.

(1) Ultraviolet spectrometry, which depends on the electronic

structure of the molecule, can provide information on the presence and nature of unsaturation. Micheli and others (100) have examined the ultraviolet absorption due to non-conjugated double bonds in derivatives of triterpenoids. The lack of agreement between different research groups were reported (101,102).

(2) Infrared spectrometry indicates the presence and environment of functional groups and is very useful for characterization of steroid sapogenins because several strong bands between 1350 to 875 cm^{-1} are characteristic of the spiroketal side chain (103,104). The correlation of infrared absorption frequencies with equitorial or axial orientation of hydroxyl groups was studied by Willex _et al_.(105). They described a significant difference in the stretching frequency of equitorial (3629 - 3630 cm^{-1}) and axial (3637 - 3639 cm^{-1}) 3-hydroxy groups in triterpenoids.

(3) Optical rotatory dispersion and circular dichroism are useful for the determination of relative and absolute configurations of asymmetric centers. They are especially sensitive for detection of ketonic carbonyl group. According to the study of Djerassi (106), a proper evaluation of the Cotton effect curves of carbonyl-containing triterpenes can resolve the problem of the recognition and especially the location of various oxygen functions. These are most frequently carbonyl or hydroxyl substituents. The latter can be oxidized to the former and rotatory dispersion is an excellent method in the placement of carbonyl groups. When applied with caution, rotatory dispersion can also be employed for supporting stereochemical assignments of methyl groups adjacent to carbonyl functions.

(4) Nuclear magnetic resonance depends on the environment of

nuclei (especially protons) and hence gives unambiguous information of certain functional groups as well as their molecular environments. The NMR spectra of a series of pentacyclic triterpenoidal derivative have been studied by Shamma *et al.* (107). The correlation between spectra and the presence of C-28 carbomethoxyl function, a C-28 methyl ester function, or a vinyl methyl function was made.

(5) Mass spectrometry is sensitive and accurate for the determination of molecular weight and for the elucidation of structure (functional groups, length of side chains, nature of skeleton). In favorable cases complete structure can be deduced. The application of mass spectrometry to the field of steroid and pentacyclic triterpenes is mostly based on the work of Djerassi and coworkers (108,19). From the mass spectra of saturated and unsaturated members of the alpha- and beta-amyrin group as well as of representatives of the lupane series, assignments have been made to the principal fragments. In general, the presence of a nuclear double bond controls the fragmentation behavior, and characteristic mass spectral features have been noted which frequently allow assignment of membership of a given triterpene in one of the major classes by this criterion. Also, the location of functional groups can often be narrowed down by consideration of the fragmentation pattern (109).

The above mentioned methods are based on relationships between structural features and physical properties, but the application of such physical methods in the field of natural products is due only in a minor degree to further knowledge of their fundamental theory. In almost all cases, an empirical approach is taken, as it depends on the empirical correlation of certain physical properties with the known

structural features.

CHAPTER III

EXPERIMENTAL

Chemicals

Cholesterol was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Activated carbon (Darco grade G-60) was from Atlas Chemical Industries Inc., Wilmington, Delaware. Oleanolic acid was from Penick and Company, New York-Chicago. Sodium borohydride was from Matheson Coleman and Bell, Ohio-New Jersey while glucuronic acid was from Sigma Chemical Company, St. Louis, Mo. Sorbose was obtained from Mann Research Laboratories, New York, N. Y. Reagents which are not listed above were mostly obtained from Fisher Scientific Company, Fair Lawn, N. J.

Eastman chromagram sheets type K301R2 (coated with silica gel with a polyvinyl alcohol added as binder, size: 20 x 20 cm) were purchased from the Eastman Kodak Company, Rochester, N. Y. Silica gel G (particle size: 10 to 40 μ, contains calcium sulfate as binder) and potassium bromide (ultra pure) were products of E. Merck, A.G., Darmstadt, Germany, distributed by Brinkmann Instruments Inc., Westbury, N. Y. Cellulose powder MN 300 (particle size: 10 μ, no binder) was obtained from Macherey, Nagel and Co., (Germany), distributed by Brinkmann Instruments Inc. Sephadex G-15 (particle size: 40 to 120 μ) and Sephadex G-25 (superfine, particle size: 10 to 40 μ) were products of Pharmacia, Uppsala, Sweden. Bio-Gel P-2 (200 to 400 mesh) was obtained

from Bio-Rad Laboratories, Richmond, Calif. and Gas Chrom Q and QF-1 were products from Applied Science Laboratories, Inc., State College, Penn.

Source of Plant Material

The mature seed samples were collected near Maud, Oklahoma, in November, 1964 and 1965, from plants growing in sandy loam 1/2 mile from the river. The root tissues were collected from the same location, in June and July, 1967.

The Isolation of Crude Saponins

Oven dried plant samples were ground to pass a 40 mesh screen in a Wiley mill and 0.5 to 2 kilograms of this material was extracted three times with 85% aqueous ethanol (volume to cover dried sample). The combined extracts were concentrated at 40 to 50 ^{o}C to about 1/10 of the original volume in a rotory evaporator and extracted three times with equal volumes of water-saturated n-butanol. The combined butanol extracts were evaporated to dryness. The dry residue was dissolved in a minimum volume of 50% aqueous ethanol and a 1/3 volume of saturated cholesterol solution in acetone-ethanol (1 : 1) was then added. This mixture was heated in a boiling water bath for one minute. Upon cooling the reaction mixture in a refrigerator for two hours, a fine gelatinous precipitate formed. This precipitate was collected by gentle suction on a Whatman No. 42 filter paper. The filter cake was dissolved with anhydrous pyridine. To this solution four to five volumes of anhydrous ether were added and the crude saponins were reprecipitated. The precipitate was filter-collected and the filter

cake was washed alternately with anhydrous ether, chloroform, and anhydrous ether. The product was removed from the filter paper and dried in a desiccator. The final product was a white powder.

Saponin Detection by Hemolytic Activity

Experiments in which crude and purified preparations were tested for hemolytic activity were conducted in the following manner: one ml of the material to be tested in 0.9% NaCl solution was mixed with three to five drops of a 50% (v/v) suspension of washed erythrocytes. The test suspensions were allowed to stand at room temperature and the extent of hemolysis was made at intervals by centrifuging and by evaluating both the red color in the supernatant liquid and the volume of unhemolyzed erythrocytes on the bottom of the test tube. A negative result was indicated if no hemolysis was observed in 12 hours. The erythrocytes used in these tests were prepared either from human or bovine blood.

Paper Chromatography of Saponins

Aqueous ethanol solutions of saponin isolates were applied to Whatman No. 3 MM paper and developed at room temperature in the specified solvent system. The following detection systems were employed: 1) the chromatogram was sprayed with 0.005 M periodic acid, prepared by dilution of 1 ml of 0.1 M periodic acid with 19 ml acetone (this reagent should be used within 3 hours after preparation), dried in air at room temperature for 4 - 5 minutes, and sprayed with 0.01 M benzidine (in a mexture containing 0.6% glacial acetic acid, 4.4% water, and 95% acetone), 2) or the chromatogram was sprayed with a saturated

antimony trichloride solution in carbon tetrachloride (freshly prepared) and heated to 100°C for 10 minutes. The benzidine-sprayed chromatogram was inspected under visible light (orange spots) while the $SbCl_3$ chromatogram was inspected under both visible (blue-violet spots) and ultraviolet light (fluorescent spots).

Thin-Layer Chromatography of Saponins

Eastman chromagram sheets type K301R2 were employed for analysis. These chromatograms were developed at room temperature. For preparative scale, thin-layer plates were prepared according to Stahl (110): glass plates of 20 x 20 x 0.4 cm were coated with a 0.75 mm layer of silica gel G using a commercial spreader apparatus. The layer was applied as a slurry consisting of 50 gram of silica gel G and 100 ml of distilled water, which had been shaken together for one minute. The chromatoplates were allowed to air dry, and were then activated in an oven at 70 - 80°C over night. The following detection systems were employed: 1) the chromatogram was placed in an iodine chamber and checked for yellow spots, 2) or the chromatogram was sprayed with an improved benzidine reagent (111) which was prepared by diluting a mixture containing 10 ml of 5% benzidine in glacial acetic acid and 10 ml of 40% aqueous trichloroacetic acid to 100 ml with 95% ethanol. After spraying, the chromatogram was heated at 70 - 80°C for 10 - 15 minutes, and examined under visible (brownish orange spots) and ultraviolet light (white or orange fluorescent spots).

Infrared Spectrophotometry

Absorption spectra were obtained using a Perkin-Elmer Model 457

Grating Infrared Spectrophotometer. Samples were prepared in KBr pellets using the Perkin-Elmer IR micro sampling accessories.

Complete Acid Hydrolysis of Saponins

Saponin isolates were placed in a round bottom flask fitted with a reflux condenser. A 50% ethanol-water solution containing 3N hydrochloric acid was added to this residue to yield a concentration of 500 mg sample per 100 ml of reaction mixture. Glass beads were added and the solution refluxed for approximately 90 hours. Insoluble residues which appeared during hydrolysis were removed. Two volumes of ethanol were added to the resulting solution which was then treated with activated carbon. The volume was reduced in an rotary evaporator. Water was added and the resulting precipitate was collected on a Buchner funnel, washed once with hot water, oven dried, dissolved in absolute ethanol, and decolorized with activated carbon. The sapogenin was then recovered by evaporation of the ethanol solution and then oven dried. The carbohydrates, which were released during acid hydrolysis, remained in the aqueous phase. This aqueous fraction was evaporated to dryness in a rotary evaporator. The residue was dissolved in water and evaporated to dryness a second time. This last process was repeated until the last trace of hydrochloric acid was removed. The carbohydrates were redissolved in water and lyophilized to give the final product.

Thin-Layer Chromatography of Carbohydrates from Saponin Hydrolysates

The same Eastman chromagram sheets were employed as described earlier with the follow pretreatment: the chromagram sheets were developed once in a solution consisting of 0.3 M sodium monohydrogen

phosphate in 0.1 M phosphoric acid (pH = 6.8), and were air dried over night before use. The improved benzidine reagent, which has been described earlier, was employed for detection.

Preparation of the Alditol Acetate Derivatives

Carbohydrate samples (approx. 50 mg) were dissolved in 2 ml water and 15 mg sodium borohydride in 2 ml water were then added. The reaction mixture was maintained at room temperature for at least three hours and then evaporated to dryness. The dry residue was refluxed for 4 hours with a mixture containing 1 ml of pyridine and 1 ml of acetic anhydride. The solution was cooled and an aliquot was injected directly into the gas chromatograph for analysis. Often this solution had to be concentrated to give a detectable concentration of sugar alditol acetates.

Gas-Liquid Chromatography of the Alditol Acetate Derivatives of Carbohydrates

The column was packed according to the technique developed by Horning et al. (112). Gas Chrom Q (silanized), 100 to 120 mesh, was used as the supporting material for the liquid phase. QF-1 was used as the stationary liquid phase. One hundred ml of a 3% solution of QF-1 in toluene were added to 20 gram of Gas Chrom Q. Vacuum was applied for 30 minutes, and the excess solution was removed by filtration. The packing material was spread on a filter paper to air dry, then dried in the oven at 80°C. A coiled glass column (10 ft x 1/4 in.) was used, which had been washed and silanized before packing. The coated supports were packed into the column with light tapping and the aid of

a water aspirator connected to the opposite end of the column. The packed column was first conditioned at 300°C for 72 hours without gas flowing through the column, and then for another 24 hours with a slow flow of helium. A modified Barber-Coleman Model 5000 gas chromatograph equipped with a hydrogen flame ionization detector was used. The samples were chromatographed at a column temperature of 215°C, detector temperature of 260°C, and injector temperature of 235°C. The helium flow rate was 55 ml/min. at a pressure of 48 psi. For the flame detector, hydrogen was generated at a pressure of 12 psi and mixed with air at 38 psi. In all cases where direct comparison of retention times was made, the samples compared were consecutively analyzed to minimize parameter variations. Injection volumes ranged from 3 to 5 µl.

Thin-Layer Chromatography of the Sapogenins

Eastman chromagram sheets were used as described earlier. The chromatogram, after being developed in the specified solvent system, was first investigated by setting in an iodine chamber. After the spots were marked, the chromatogram was set at room temperature until all of the adsorbed iodine had evaporated. The same plate was then sprayed with 25% ethanolic phosphotungstic acid, heated at 115 to 118°C for 2 minutes, and checked under visible and ultraviolet light.

Micro-scale Purification of Glottidigenin A and Glottidigenin B

The sapogenin fraction obtained from acid hydrolysis of the crude saponin isolates was dissolved in ethanol. This ethanol solution was applied as a thin strip to a 0.75 mm thick plate (10 mg sample / plate) of silica gel G. The preparation of plates has been described earlier.

The plate was developed with toluene - ethyl acetate - formic acid (57 : 40 : 3). The developing chamber was previously saturated with the developing solvent. When the solvent front was about 17 cm from the origin, the plate was removed from the chamber, air dried, and then dried in an oven at 40°C. Usually, different fractions can be located under ultraviolet light (this was not possible when a sample was applied as a single spot), or can be visualized by exposing to iodine vapor. The portions of the plate containing glottidigenin A and glottidigenin B were scraped clean of silica gel G and leached with ethanol. The ethanol extract containing the individual sapogenin was concentrated and then chromatographed once again following the above process. The individual sapogenin was then recovered from its ethanol solution by evaporating to dryness.

Mass Spectrometry of Sapogenins

The spectra were measured using a prototype of the LKB 9000 combination gas chromatograph-mass spectrometer instrument (Karolinska Institute, Stockholm, Sweden). The sample in its solid state was introduced directly into the ion source through a direct inlet system. The mass spectrometry was carried out at an electron energy of 70 eV, ion source temperature 310°C, multiplier high voltage 2.1 kV, trap current 65 milliamps, and accelerator high voltage 3.5 kV. The mass spectra were computer-plotted from tabular intensity data with a Cal Comp 565 plotter driven by an IBM 1620 computer using a Fortran II-D program. The mass spectra were reported in terms of relative intensity, the most abundant ion being taken as 100%.

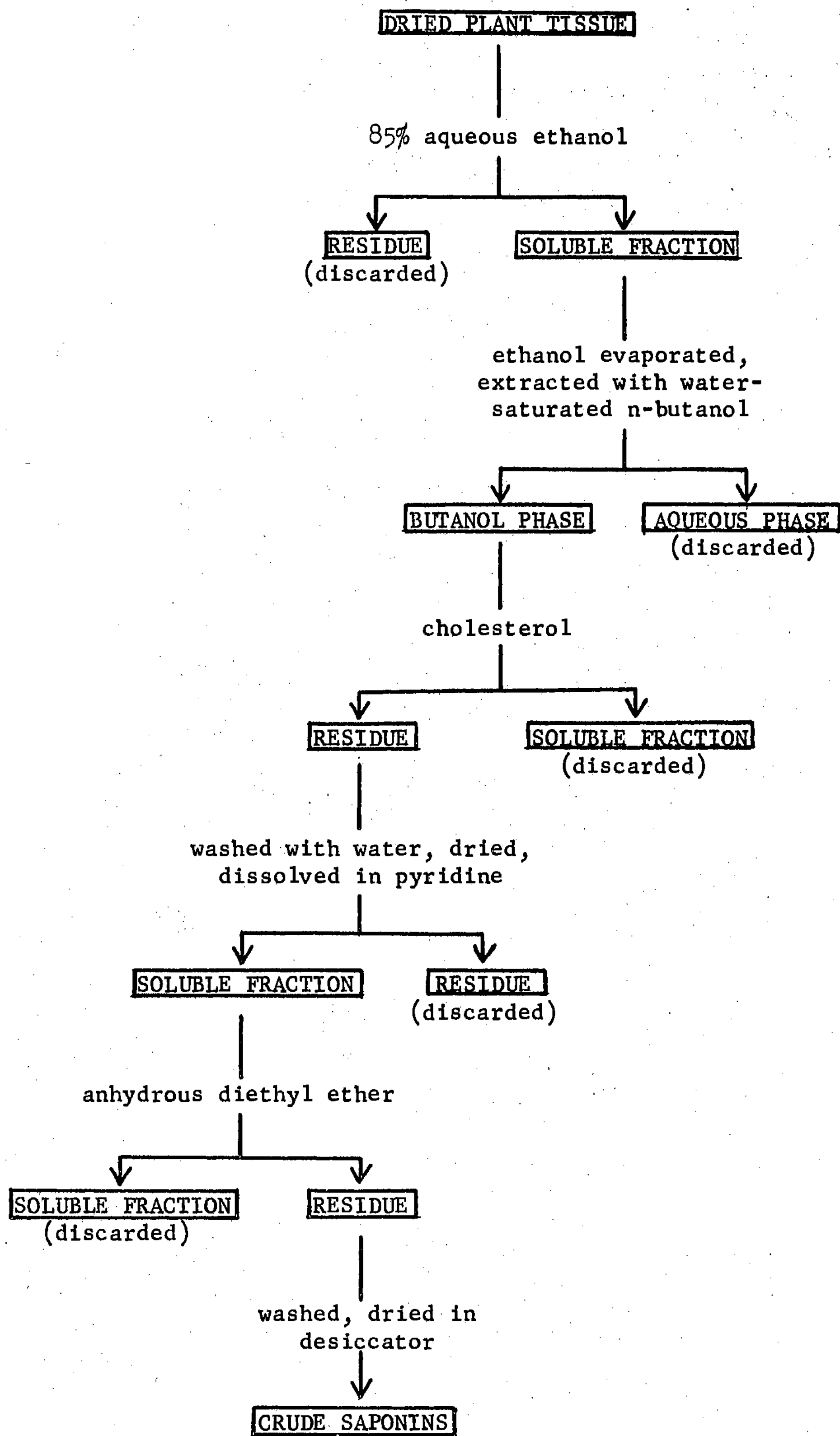
CHAPTER IV

RESULTS AND DISCUSSION

Isolation of Saponin Fraction from Glottidium vesicarium

An outline of the isolation procedure is given in Fig. 5. There are two key steps. A butanol extraction step separates saponins from carbohydrates and amino acids. Solubility experiments with pure digitonin (16) showed that 97% of this compound was found in the butanol phase when equal volumes of mutually saturated butanol and water were used. The cholesterol step is both selective and sensitive for separating saponins from other phytochemical constituents, since saponins are known to form stable molecular complexes with cholesterol. This property has been used for determining whole blood cholesterol (ranging between 0.02 and 0.15 mg) (113). A detailed description of the isolation procedure can be found in the experimental section. Several different approaches were taken in the preliminary studies (114). A variation in solubility properties of saponin isolates prepared by different, as well as identical, procedures has been observed. Some are more soluble in absolute ethanol while others are more soluble in distilled water. Nevertheless most are saponin active samples as indicated by their erythrocyte hemolytic activity. This is not totally unexpected as similar property variations have been reported by other workers (115). There are several possible explanations for such variations; 1) The saponin content of a particular species varies with

Figure 5. Outline of the Isolation of *Glottidium vesicarium* Saponins


DRIED PLANT TISSUE
85% aqueous ethanol
RESIDUE
(discarded)
SOLUBLE FRACTION
ethanol evaporated,
extracted with water-
saturated n-butanol
BUTANOL PHASE
AQUEOUS PHASE
(discarded)
cholesterol
RESIDUE
SOLUBLE FRACTION
(discarded)
washed with water, dried,
dissolved in pyridine
SOLUBLE FRACTION
RESIDUE
(discarded)
anhydrous diethyl ether
SOLUBLE FRACTION
(discarded)
RESIDUE
washed, dried in
desiccator
CRUDE SAPONINS

part of plant, stage of growth, and season. Marker and coworkers (9) have repeatedly observed that the composition of the sapogenin fraction in plants undergoes certain changes. They observed that young agave and yucca plants contain fewer and more oxygenated saponins than older plants. Also, during the winter the complexity of the sapogenin mixture increases. Apparently the number of oxygen functions and double bonds in these molecules decreased. 2) Krider _et al._ (10) reported that leaves of agave and yucca species, which contain saponins, also contain enzymes which are capable of hydrolyzing these glycosides. The possible presence of similar enzyme systems in _Glottidium vesicarium_ may account for some of the above mentioned variations. 3) Complicated ring systems like saponins, which contain several asymmetric centers, are capable of existence in many optically active forms. In mature plant tissue usually only one of the possible optically active forms is found. The enzyme systems responsible for the biosynthesis of these compounds are stereospecific in their action, so that the creation of new centers of asymmetry results in one stereochemical arrangement only and not its enantiomorph (57). Such naturally occurring forms may not be energetically favored. During the isolation procedures modification of molecular configurations may have occurred.

The isolation procedures applied by other workers usually include an activated carbon treatment step for the removal of pigments (14,116). Experiments performed on _Glottidium vesicarium_ showed that the saponins were also absorbed by charcoal. In the procedure adopted, the pigments were removed satisfactorily in the cholesterol step by washing the saponin-cholesterol complex with hot water. The yield of this isolation procedure was 0.03% for unshelled dry bean samples and 0.09 to

0.19% for dried root tissue.

Fractionation of Saponin Isolates

The major difficulty in this process is the resolution of individual saponins present in the crude saponin isolate. Such difficulty is understandable from the example of lucerne saponins (32). Initial fractionation of lucerne saponins by paper electrophoresis revealed at least four constituents, later fractionations which employed paper chromatography (ascending chromatography, multiple development); Carr-Price reagent (antimony trichloride in chloroform) indicated seven spots, while the Liebermann-Burchard reagent revealed ten, possibly twelve constituents. The results in fractionating the crude *Glottidium vesicarium* saponin isolate are summarized as follows.

Column Chromatography

Silicic acid, DEAE cellulose column chromatography, Sephadex G-15, G-25, G-75 and Bio-Gel P-2 column chromatography were ineffective in fractionation of the saponin isolate.

Paper Chromatography

The paper chromatographic separation of saponins has been widely applied (117,32,118,119,120) but no generalization of the data on this question has ever been made. Difficulty in obtaining reproducible results has been reported even for the simplest saponins which contain only one or two carbohydrate units. In this study, the following solvent systems have been employed: isopropyl alcohol - pyridine - glacial acetic acid - water (8 : 8 : 1 : 4); chloroform - n-butanol -

acetone (3 : 6 : 1); n-butanol - acetic acid - water (5 : 4 : 1); iso-propanol - n-butanol - water (3 : 2 : 1); n-butanol - acetic acid - water (6 : 1 : 3); pyridine - n-butanol - water (3 : 2 : 1). The latter solvent system gave a greater number of detectable spots than the others listed above. The chromatographic behavior of some Glottidium vesicarium dry bean saponin isolates is summarized on Table I.

Aside from the inconsistancy of detectable spots which may arise from the inconsistancy of the constituents in the sample itself, paper chromatographic separation of saponins has two major disadvantages; its resolving power and its sensitivity. The paper chromatography of Glottidium vesicarium saponin isolates showed that well-formed spots can be obtained on the chromatograms only with great difficulty. Futhermore, the presence of saponin activity between two well-shaped spots has been detected by hemolytic tests. Another difficulty arises from the low sensitivity of the reactions for the detection of this type of compound. When reagents based on their reactions with the genin portions are used, the relative content of the genin decreases with the increase in seze of the carbohydrate chain of the saponin and the sensitivity of the qualitative reaction decreases. A similar situation occurs when reagents based on their reactions with the carbohydrate portion were applied.

In Table I , samples I and II were easily detected with periodate-benzidine, while sample III showed no response to periodate-benzidine and could only be detected, with difficulty, by antimony thicholoride.

An additional complication was discussed by Khorlin et al. (121). They have reported a great dependence of chromatographic separation of saponins on temperature and the quality of the chromatographic paper.

TABLE I

CHROMATOGRAPHIC BEHAVIOR OF SAPONIN ISOLATES FROM *GLOTTIDIUM VESICARIUM* ON PAPER CHROMATOGRAPHY[1]

Isolate[2]	Detection Reagent	Number of Spots	R_f (range)
I	Periodate-benzidine Reagent	4	0.00 0.18-0.19 0.32-0.35 0.55-0.56
II	Periodate-benzidine Reagent	4	0.00 0.51-0.57 0.68-0.70 0.81-0.83
III	Hemolysis test and Antimony trichloride Reagent	2	0.48-0.58 0.75-0.81

1 Ascending paper chromatography on 3 MM Whatman paper at room temperature.

2 I: Water soluble fraction of saponin isolate from unshelled dry beans collected on November, 1964.
II: Ethanol soluble fraction of saponin isolate from unshelled dry beans collected on November, 1964.
III: Saponin isolates from unshelled dry beans collected on November, 1966.

In nearly all cases, these workers obtained the best separations below 20°C.

Thin-Layer Chromatography

Thin-layer chromatography on silica gel in the following systems has been employed (commercial Eastman chromagram sheets were used for all analytical studies): isopropyl alcohol - pyridine - glacial acetic acid - water (8 : 8 : 1 : 4); pyridine - n-butanol - water (3 : 2 : 1); chloroform - methanol (7 : 3); chloroform - ethanol (2 : 1). In most cases diffuse spots with "tails" were obtained. The maximum number of detectable spots for the saponin isolates was two.

Thin-layer chromatography on a mixture of MN 300G cellulose - silica gel G (5 : 2) in the following two systems has also been tried: chloroform - tetrahydrofuran - pyridine (5 : 5 : 1); pyridine - n-butanol - water (3 : 2 : 1). In the first solvent system saponin isolates from dry seeds gave one spot when detected with the periodate - benzidine reagent and two spots when sprayed with antimony trichloride. In the second solvent system two well-shaped spots were revealed with the antimony trichloride spray and three well-shaped spots by the periodate-benzidine spray. Later when the same system was employed on newly prepared saponin isolates from root tissues, reproducible results were not obtained.

Many solvent systems were tested; the system applied by Khorlin *et al*. (121) produced the best results with respect to resolving power and sensitivity (Fig. 6). This system was then adopted for fractionating individual saponins to be used in the chracterization studies. In Fig. 6, both samples were prepared from the root tissue of *Glottidium*

Figure 6. Thin-Layer Chromatographic Analysis of Saponin Isolates from _Glottidium vesicarium_ on Silica Gel

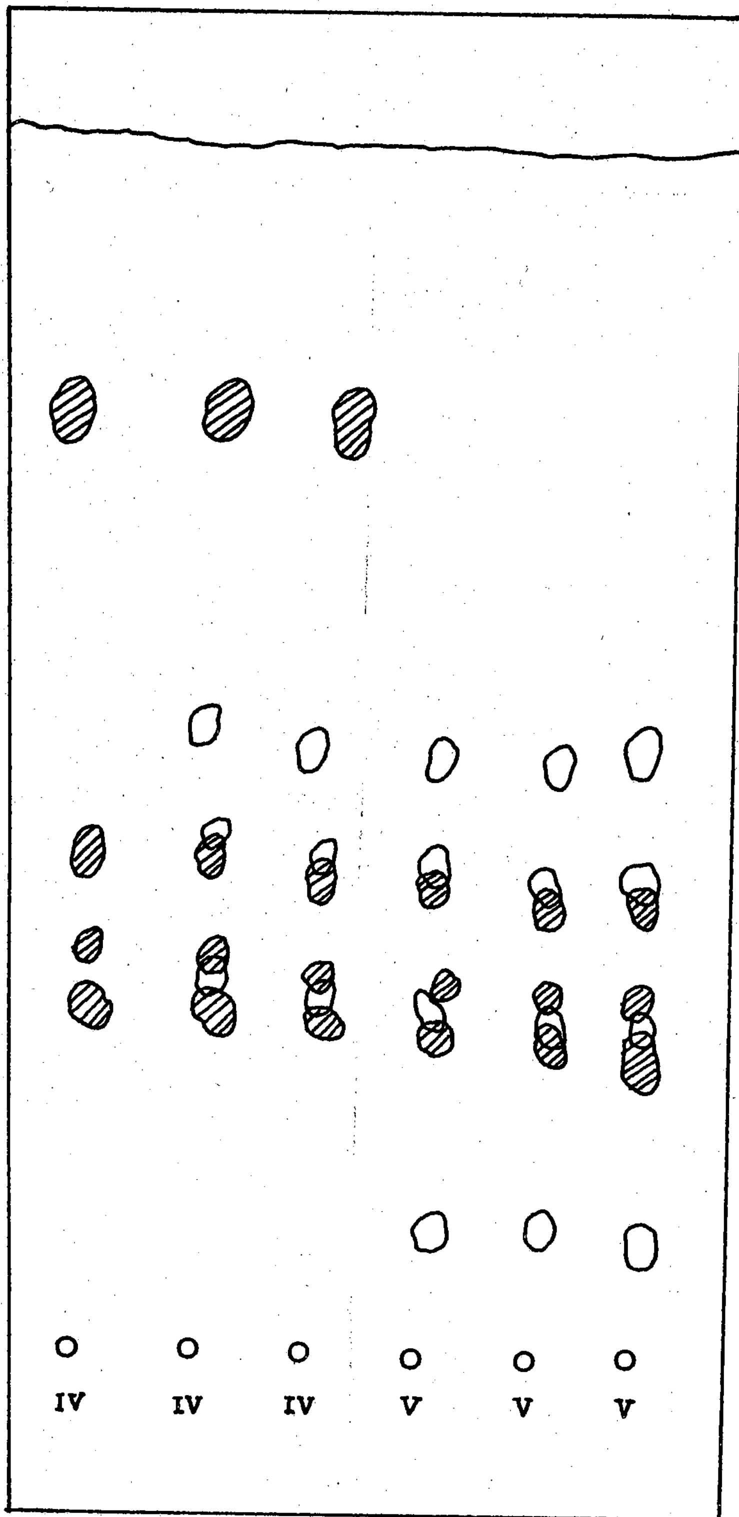
Solvent System: n-butanol - ethanol - 25% aqueous ammonium hydroxide (10 : 2 : 5, v/v)

: Detected by iodine vapor

: Detected by benzidine reagent

IV : Saponin isolates from root tissue collected on June, 1967

V : Saponin isolates from root tissue collected on July, 1967


IV
IV
IV
V
V
V

vesicarium. Identical isolation procedures were applied, the only difference is that sample IV was harvested one month earlier than sample V. Yet, their chromatographic patterns are different. Similar variance in constitution, both qualitatively and/or quantitatively, has been observed repeatedly during this study.

Characterization of the Unfractionated Saponins

Based on the following considerations, it was decided that partial characterization of unfractionated saponin isolates was necessary: 1) The occurence of structurally related saponins was evident from preliminary fractionation studies which demonstrated that chromatography was the only practical fractionation approach which could provide the required sensitivity and resolution. 2) The separation of saponin mixtures by chromatography has two disadvantages: The solvent systems required for the resolution of saponins of acidic character is different from that required for neutral saponins; the former requires a basic system while the latter requires a neutral or an acidic system (121). The detection reagent required for simple saponins is different from that required for complex saponins (reagents based on their reactions with the genin portions are required for saponins with fewer carbohydrate units, while reagents based on their reactions with the carbohydrate portions are required by saponins with more carbohydrate units). 3) There is one known case where a chromatographically homogeneous saponin yields as many as six sapogenins after hydrolysis (112). Thus it was clear that a general understanding on the saponin isolate composition is needed for both the complete fractionation and the characterization of individual saponin constituents.

The following studies were conducted to obtain an acceptable resolution and characterization of individual saponins. In view of the inconsistent properties observed for separate saponin isolates, characterization studies reported here were performed on the saponin isolate from root tissue collected on June, 1967.

Spectrometric Determination of the Type of Saponins Present

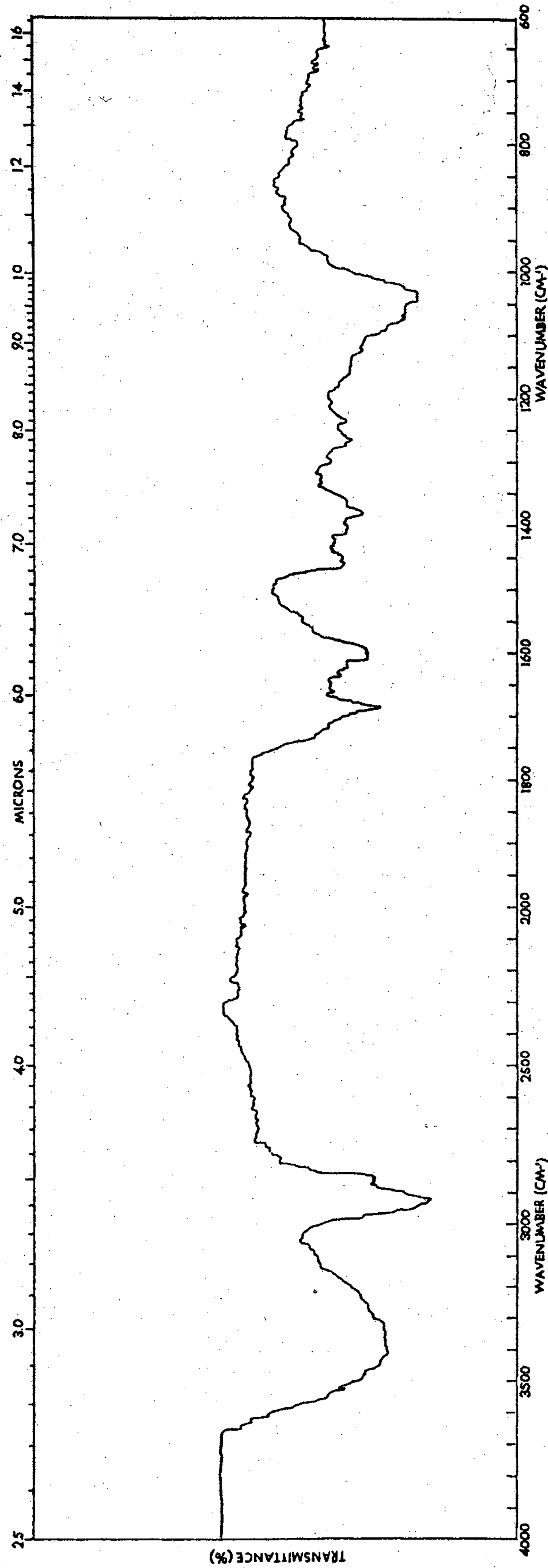
The infrared spectrum of the unfractionated root saponin isolate indicated that only triterpenoidal type saponins were present in significant amounts.

The work of Wall et al. (103) has established that the infrared spectra of steroidal type sapogenins as well as their saponins are characterized by four strong bands in the regions: 852 – 867 cm^{-1}, 897 – 900 cm^{-1}, 920 – 925 cm^{-1}, and 982 – 983 cm^{-1}. The presence of the entire system of these four bands is highly characteristic for the spiroketal side chain. The spectrum given on Fig. 7 shows no such absorption system. This does not exclude the possible presence of steroid saponins in Glottidium vesicarium even though the triterpenoid saponins appear to predominate. A cholesterol precipitation step was involved in the isolation procedure and it is known (103) that not all saponins form insoluble complexes with cholesterol.

Characterization of the Carbohydrate Fraction

Paper chromatography was tried unsuccessfully. The application of thin-layer chromatography established the presence of glucuronic acid, whereas gas-liquid chromatography revealed the presence of seven other sugars – deoxyribose, rhamnose, arabinose, xylose, galactose, glucose

Figure 7. Infrared Absorption Spectrum of *Glottidium vesicarium* Saponin Isolates


MICRONS
2.5
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
12
14
16
WAVENUMBER (CM-1)
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
TRANSMITTANCE (%)

and sorbose.

The first attempts to separate sugar mixtures by thin-layer chromatography was made by Stahl et al. in 1961 (123). In most laboratories this technique did not gain general acceptance for two reasons: poor resolution with certain common sugars and a low capacity of the chromatoplates. Recently thin-layer chromatography on silica gel impregnated with sodium dihydrogen phosphate or sodium monohydrogen phosphate in phosphoric acid was successfully applied (124,125). The system employed in this research was based on the technique developed by Ovodov et al. (125) with one minor variation. Ovodov and coworkers applied an inorganic-salt-solution impregnated silica gel for the preparation of chromatoplates. Studies reported here used commercial Eastman chromagram silica gel sheets with the following pretreatments: the sheets were developed once in the inorganic salt solution and then allowed to stand for at least 24 hours at room temperature beform samples were applied.

In preliminary studies, it was observed that the solvent system n-butanol - pyridine - water (8 : 4 : 3) have good resolution with mixtures of known monosaccharides but saponin hydrolysate gave only one elongated spot. Later, the system of water-saturated s-collidine was used. The resolution of a known sugar mixture was poor, but the presence of glucuronic acid was indicated (Fig. 8A). It has been reported that sugar, sugar acid mixtures separate on silica gel only in the presence of an acid solvent system (125). So an acidic solvent system: n-butanol - ethal - 0.1M phosphoric acid (1 : 10 : 5) was employed. In this system glucuronic acid is known to produce two spots: the lower one came from the acid form, the higher one from the lactone form. The

Figure 8. Thin-Layer Chromatographic Analysis of the Carbohydrates from Saponin Hydrolysate on Silica Gel Layers Impregnated with 0.3 M Sodium Monohydrogen Phosphate in 0.1 M Phosphoric Acid.

Detected by benzidine reagent.

A. Solvent System: Water-saturated s-collidine

B. Solvent System: n-butanol - ethanol - 0.1M phosphoric acid (1 : 10 : 5, v/v)

1. Glucose
2. Arabinose
3. Glucuronic Acid
4. Carbohydrate Fraction from Saponin Hydrolysate
5. Mixture of Rhamnose, Xylose, Arabinose, Glucose, Galactose
6. Galactose
7. Rhamnose

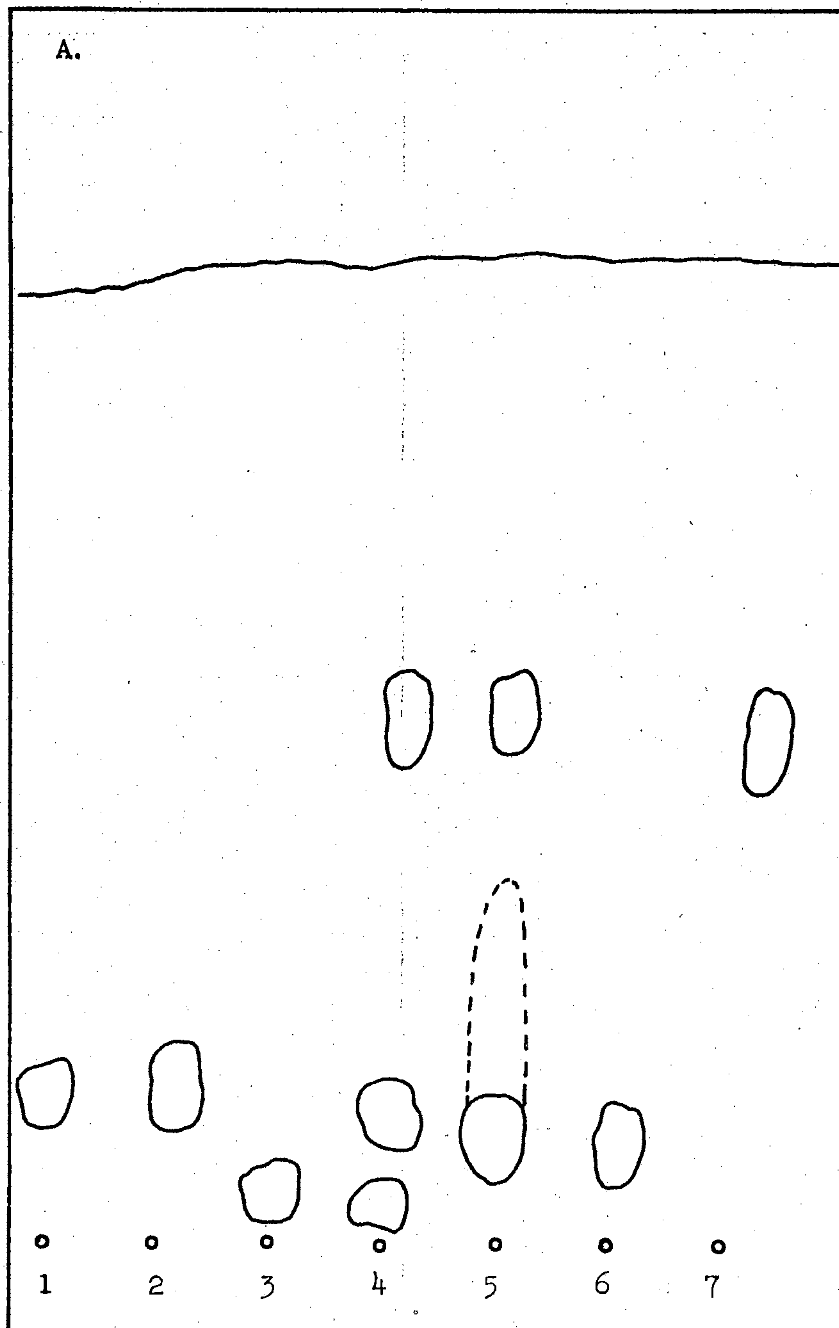
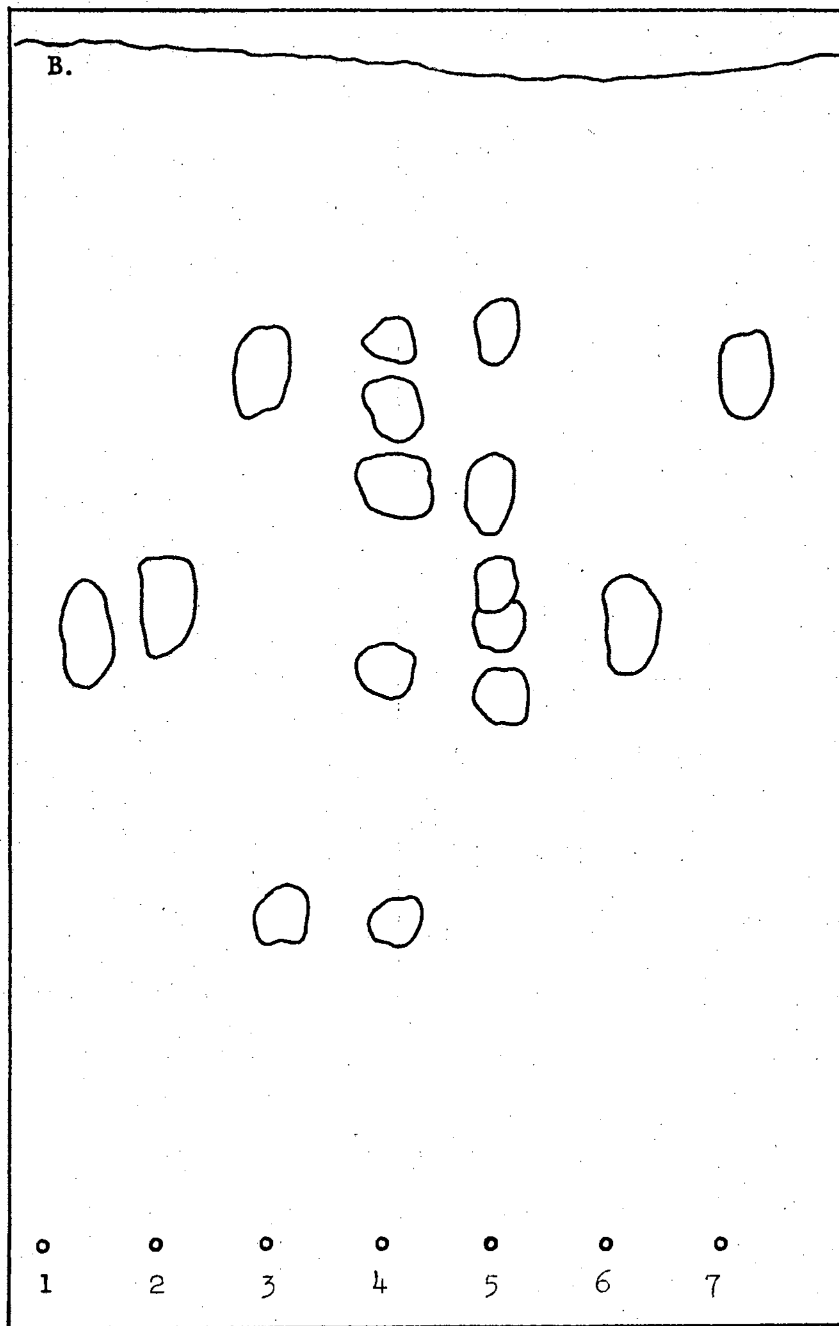

A.
1
2
3
4
5
6
7

Figure 8. (Continued)


B.
1
2
3
4
5
6
7

presence of glucuronic acid in saponin hydrolysate was established by direct comparison with standard glucuronic acids. (Fig. 8B).

From the thin-layer plate, a reproduction of which is shown in Fig. 8B, the presence of at least three other sugars was indicated. Attempts to identify these sugars by thin-layer chromatography were unsuccessful. This was due to the fact that the concentration of glucuronic acid in the saponin hydrolysate is much higher than that of other sugars as it appeared as strong spots while the other components were comparatively weak spot. Application of the sugar sample in smaller amounts showed only glucuronic acid as a clearly detectable spot, whereas application of the sample in greater amounts gave poor resolution.

The gas-liquid chromatographic analysis of sugars required the preparation of carbohydrate derivatives which were sufficiently volatile. The preparation of trimethylsilyl (TMS) derivatives for such work has been described by Sweeley's group (126). This method involves solution or suspension of the sugars in pyridine, followed by reaction with hexamethyldisilazane and trimethylchlorosilane.

Preliminary experiments with known sugar mixtures as well as saponin hydrolysates demonstrated that, in accordance with the reports of Sweeley _et al_. (126), gas-liquid chromatography of TMS sugar derivatives on a SE-30 (methyl silicone gum) column has the following advantages. Trimethylsilylation occures rapidly at room temperature on a micro scale; good resolutions were observed with anomeric pairs as well as configurational isomers. The main difficulty in using TMS sugar derivatives is the formation of as many as four glycosides per monosaccharide from anomeric and ring isomerization. Each of these may produce a peak on the chromatogram.

In view of this difficulty, reduction of monosaccharides to their alditols and then separation of the alditol derivatives is more desirable. This procedure eliminates the problem of multiple peaks as the alditols can not anomerize. The separation of alditols as their TMS derivatives was excluded since it was known to be unsuccessful (127). The alditol acetate derivatives of the carbohydrate fraction from saponin hydrolysate were prepared and gave a clear separation of seven peaks after passing through a QF-1 (fluorosilicone) column. This data is shown in Fig. 9. The first six peaks were identified as deoxyribose, rhamnose, arabinose, xylose, galactose, and glucose, respectively, by a direct comparison of the elution pattern with the pattern given by mixtures of known sugars. The retention time of the seventh peak indicated that it was sorbose (there is still some doubt as to the identification of this peak as the sorbose standard appeared as two peaks, the major peak had the same retention time as the seventh peak).

For general application this method has several disadvantages. The reaction conditions are more prolonged; larger amount of sample are required; glucuronic acid can not be detected. Nevertheless, its application in this given case was satisfactory.

Characterization of the Sapogenin Fraction

The purification of glottidigenin A and glottidigenin B was approached in the following manner. Since enzyme hydrolysis with beta-glucosidase was tried without success, acid hydrolysis in hydrocholoric acid was employed as described in the experimental section. The resolution of individual sapogenins from this acid hydrolysate was obtained by thin-layer chromatography on silica gel plates.

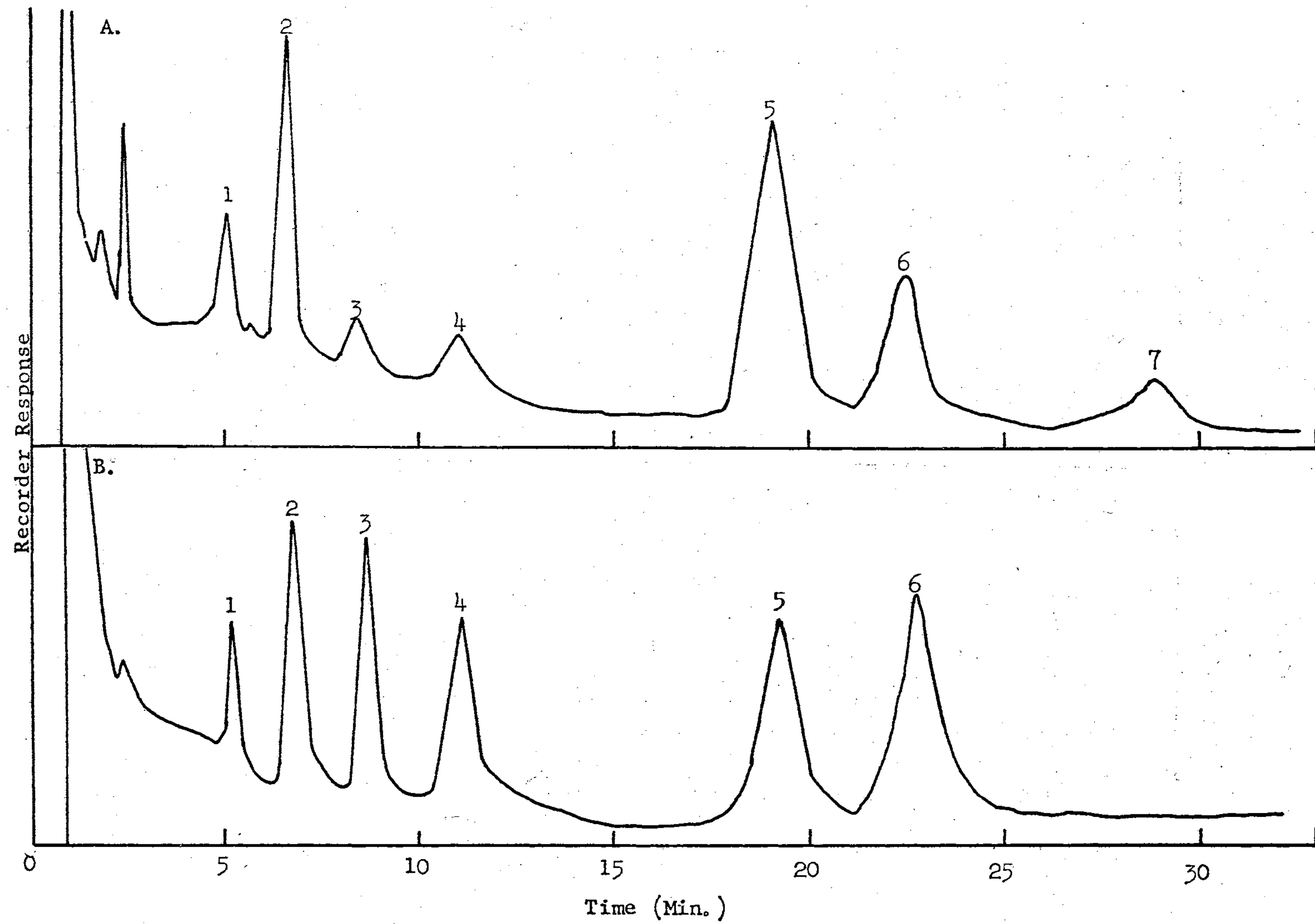
Figure 9. Gas-Liquid Chromatographic Analysis of Alditol Acetates of Carbohydrates on 3% QF-1 Column at 215°C

Column Size: 10 ft x 1/4 in.

A. *Glottidium vesicarium* saponin hydrolysates

B. Mixture of known sugars

1. Deoxyribose
2. Rhamnose
3. Arabinose
4. Xylose
5. Galactose
6. Glucose
7. Sorbose


A.
B.
1
2
3
4
5
6
7
Recorder Response
0
5
10
15
20
25
30
Time (Min.)

The following approaches were taken. A series of solvent systems was tested in a preliminary study. The resolutions are summarized in Table II. From Table II, it may be seen that two solvent systems were appropriate: toluene - ethyl acetate - formic acid (57 : 40 : 3) and benzene - ethanol (49 : 1). A two-dimensional thin-layer chromatography in these two systems on an Eastman chromagram sheet revealed seven spots (Fig. 10). Preparative thin-layer chromatography in the first solvent system (toluene - ethyl acetate - formic acid, 57:40:3) followed by extraction of constituents I to V into ethanol indicated that the components giving spots denoted by I and IV in Fig. 10 were the two constituents present in significant amounts. These two fractions were then isolated by repeated preparative thin-layer chromatography, and characterized by both infrared and mass spectrometry. Samples so prepared have a purity of over 99% as indicated from their mass spectra study. For purposes of recognition Spot IV is referred to as glottidigenin A and spot I as glottidigenin B.

The infrared spectrometric characterization of glottidigenin A and glottidigenin B was based on spectral data from 600 - 4000 cm^{-1} (Fig. 11 and Fig. 12). The spectrum of oleanolic acid is also shown (Fig. 13), since an analysis of mass spectral data indicated that glottidigenin A is either identical with or closely related to oleanolic acid. The similarity between the infrared spectra of these three compounds is striking, a peak-by-peak correlation can be made. The infrared data were analyzed on the basis of one general (128) and several specific (129,130,131) references. The conclusions are as follows:

a. Absorption near 3400 cm^{-1}: This broad absorption is characteristic of O-H bonds in an intermolecularly bonded alcohol group.

TABLE II

CHROMATOGRAPHIC BEHAVIOR OF GLOTTIDIUM VESICARIUM SAPOGENINS ON THIN-LAYER CHROMATOGRAPHY[1]

Solvent System	Developing Time (Minutes)	Number of Spots[2]	
		I	II
Chloroform	130	2	2
Chloroform-Methanol-Water (188 : 12 : 1)	300	2	1
Benzene-Ethanol (9 : 1)	60	4	3
Benzene-Ethanol (49 : 1)	70	5	5
Toluene-Ethyl Acetate-Formic Acid (57 : 40 :3)	50	5	4
Benzene-Ethyl Acetate (9 : 1)	50	3	5

1 Eastman chromagram sheet (silica gel) were employed. The sample applied was isolated from the root tissue collected on June, 1967

2 I: Spots detected with iodine vapor.
II: Spots detected with 25% ethanolic phosphotungstic acid.

Figure 10. Thin-Layer Chromatographic Analysis of the *Glottididium vesicarium* Sapogenins on Silica Gel

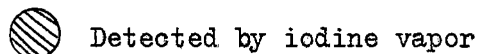
A 1st Dimension: Benzene - Ethanol (49 : 1)

B 2nd Dimension: Toluene - Ethyl Acetate - Formic Acid (57 : 40 : 3)

O Origin

S Solvent Front

Detected by 25% ethanolic phosphotungstic acid


Detected by iodine vapor

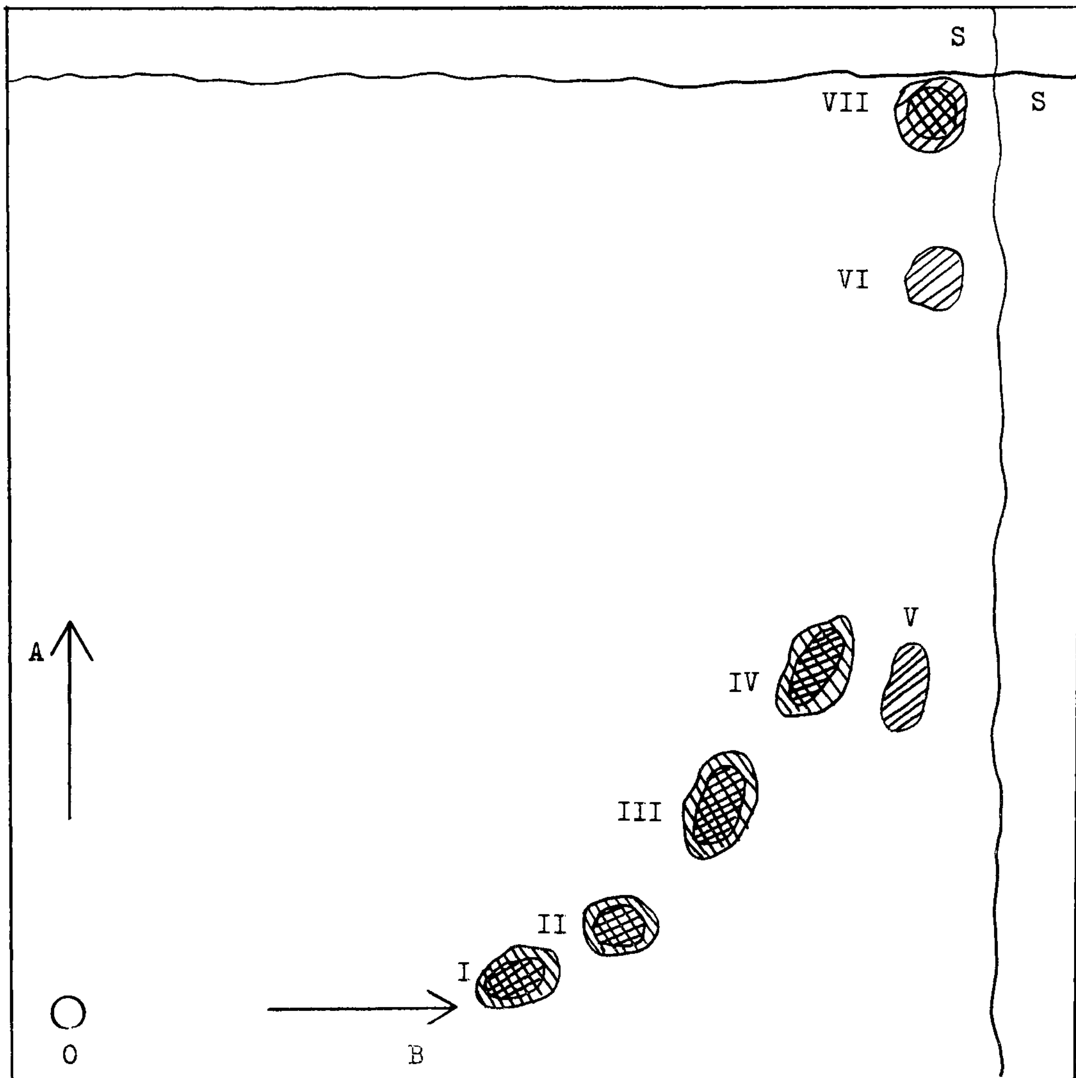
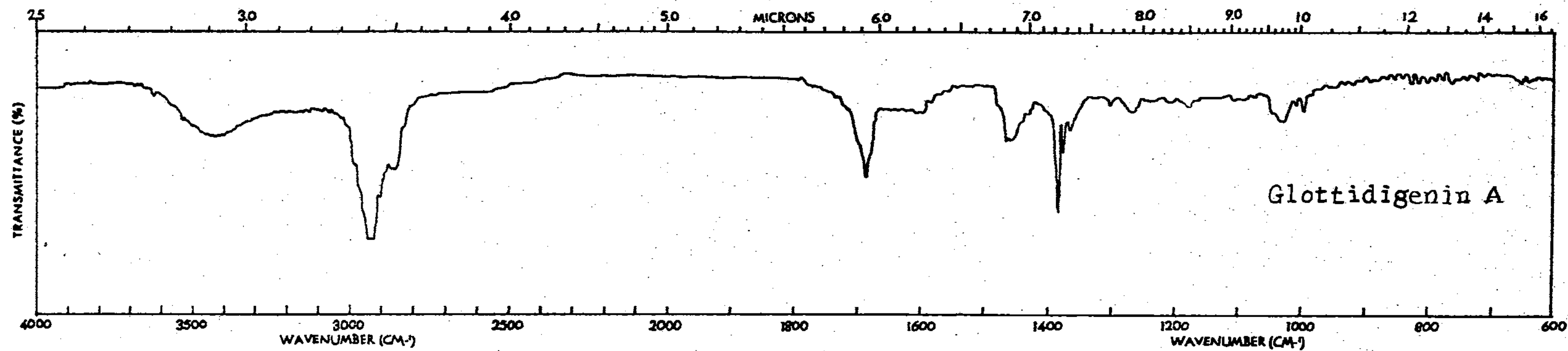
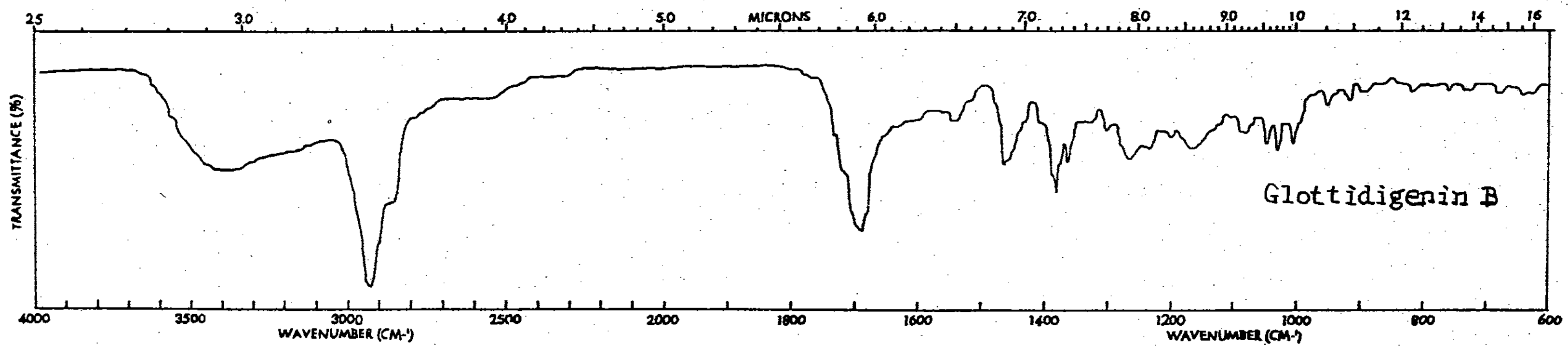

S
S
VII
VI
V
IV
III
II
I
A
B
O

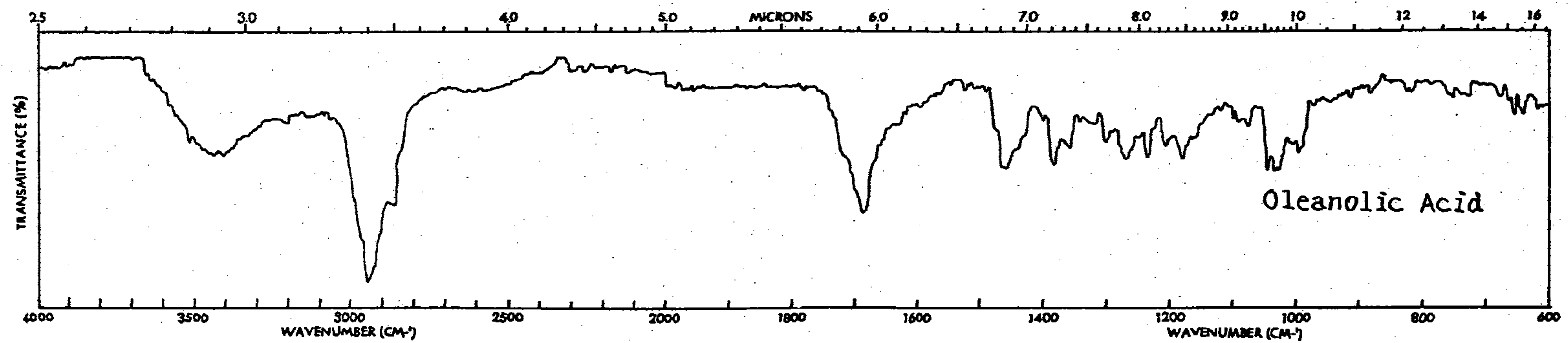
Figure 11. Infrared Absorption Spectrum of Glottidigenin A

Figure 12. Infrared Absorption Spectrum of Glottidigenin B

Figure 13. Infrared Absorption Spectrum of Oleanolic Acid


MICRONS
TRANSMITTANCE (%)
WAVENUMBER (CM-1)
Glottidigenin A


MICRONS
TRANSMITTANCE (%)
WAVENUMBER (CM-1)
Glottidigenin B


MICRONS
TRANSMITTANCE (%)
WAVENUMBER (CM-1)
Oleanolic Acid

b. Absorption in the 2700 - 3100 cm^{-1} region: The broad band centered near 3000 cm^{-1} is due to the O-H bond in interhydrogen bonded acid dimers. Also incorporated in this broad band are the various aliphatic C-H stretching bands which occur at the 2800 - 3100 cm^{-1} region.

c. Absorption near 1690 cm^{-1} region: This band is from the C=O bond of carboxyl groups in hydrogen-bonded acid dimer.

d. Absorption near 1465 cm^{-1} region: The C-H bonds in the methylene groups always show absorption in this region.

e. Absorption in the 1360 - 1390 cm^{-1} region: The C-H bond of dimethyl groups which are attached to the same carbon atom exhibit a distinctive absorption characterized by a doublet at 1365 - 1370 cm^{-1} and at 1380 - 1385 cm^{-1}.

f. Absorption in the 1150 - 1350 cm^{-1} region: The series of bands in this region is due to methylene twisting and wagging vibration.

g. Absorption in the 1050 - 1085 cm^{-1} region: The secondary alcohol of an alicyclic 6-membered ring system gives C-O bond absorption in this region.

h. Absorption in the 650 - 1000 cm^{-1} region: Olefins always show out-of-plane C-H bending absorption in this region.

Based on the above informations, the infrared spectra data indicates the presence of

O-H, C-O, C-H, C=O, and C=C bonds

as well as the

$(CH_3)_2{>}C-$, $-CH_2$, $-CH_3$, ${>}C-OH$, and $-COOH$ groups.

One point is evident from the above studies. In contrast to the steroid sapogenins, which produce infrared spectra highly characteris-

tic of the individual sapogenins (84), the application of infrared spectra to the structural work of triterpenoid sapogenins is very limited. In this case the direct comparison of the spectrum given by oleanolic acid with those given by glottidigenin A and B provided no clue for identification. The qualitative similarity can not be taken as positive evidence due to the lack of specific absorption bands. The quantitative differences can not be used as negative evidence since these acidic compounds may exist either as monomers or dimers. The ratio of these two forms will influence the relative intensity of absorption bands.

The application of mass spectrometry to pentacyclic triterpenes was initiated as early as 1958 (132). Its potential in structural work on this type of compound was not recognized for many years. There are a number of reasons for this situation. The low volatility of these compounds limits the use of the metal inlet system, since the metal may catalyze decomposition at the relatively high temperatures required to furnish adequate sample vapor pressure; the complexity of the molecule and the polycyclic nature of triterpenoidal sapogenins often requires a complex process for fragmentation. The majority of fragments consist only of alicyclic portions which frequently give no unambiguous clue as to the part of the molecule from which they originate. In 1963, the general application of mass spectrometry in the structural elucidation of pentacyclic triterpenes was established from the comprehensive study of a consistent series of compounds (108,133,109,19). Several criteria have been derived which were shown to be generally applicable to the fragmentation pattern of this type of compound: 1) When a direct inlet system is used, which permits the vaporization in an undecomposed

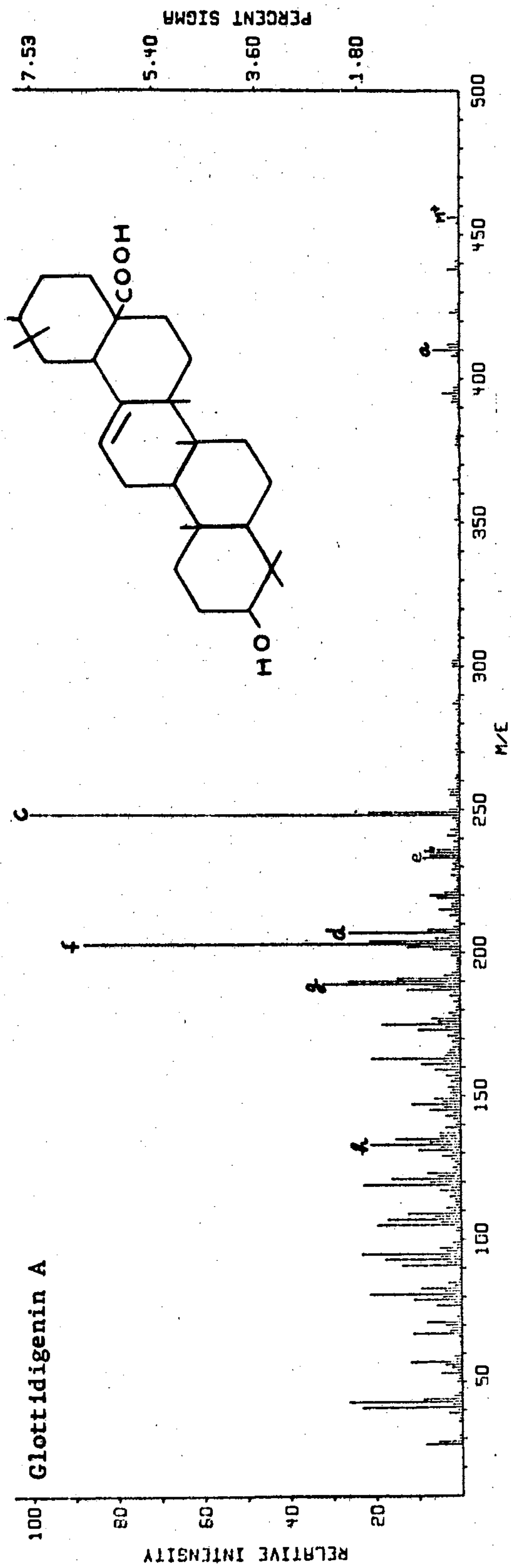
state of relatively non-volatile substances, the probability of recording a molecular ion is greatly increased. 2) In general, the presence of a nuclear double bond controls the fragmentation behavior. In the case of the amyrin series which are characterized by the presence of a 12 - 13 double bond this feature has proven to be readily recognizable by mass spectrometry, since the molecular ion undergoes the equivalent of a retro-Diels - Alder fragmentation and produces a very characteristic peak. 3) The spectrum is shown to be very sensitive to both positional isomerism and stereoisomerism in this group of compounds. In general, the loss of angular substituents ($-CH_3$, $-COOCH_3$, $-CH_2OAc$) from C - 17 is much more pronounced than the removal of other substituents.

Efforts in interpretating the mass spectra data of glottidigenin A (Fig. 14) and glottidigenin B (Fig. 15) are in principle based on the work of Djerassi and coworkers (109,19). Their assignment of principle fragments has been accepted by other workers (134,135,136) though their approach to the mechanism involved in the formation of these fragments is debatable. The mechanisms proposed by Djerassi _et al_. (19) called for the localization in the molecular ion of the missing electron (lost in ionization by electron impact) at favoured sites. This localized positive charge was viewed as being capable of triggering subsequent decomposition reactions leading to the fragment ions obserbed in the mass spectrum. Recently McLafferty (137) proposed another approach which involves the separate consideration of the effect of the positive ion and radical sites.

The structural information from Fig. 14 and Fig. 15 can be itemized as following. The fragmentation pattern which was in most parts based on reference 19 is described in Fig. 16.

Figure 14. Mass Spectrum of Glottidigenin A

Figure 15. Mass Spectrum of Glottidigenin B


Glottidigenin A
RELATIVE INTENSITY
PERCENT SIGMA
M/E
COOH
HO

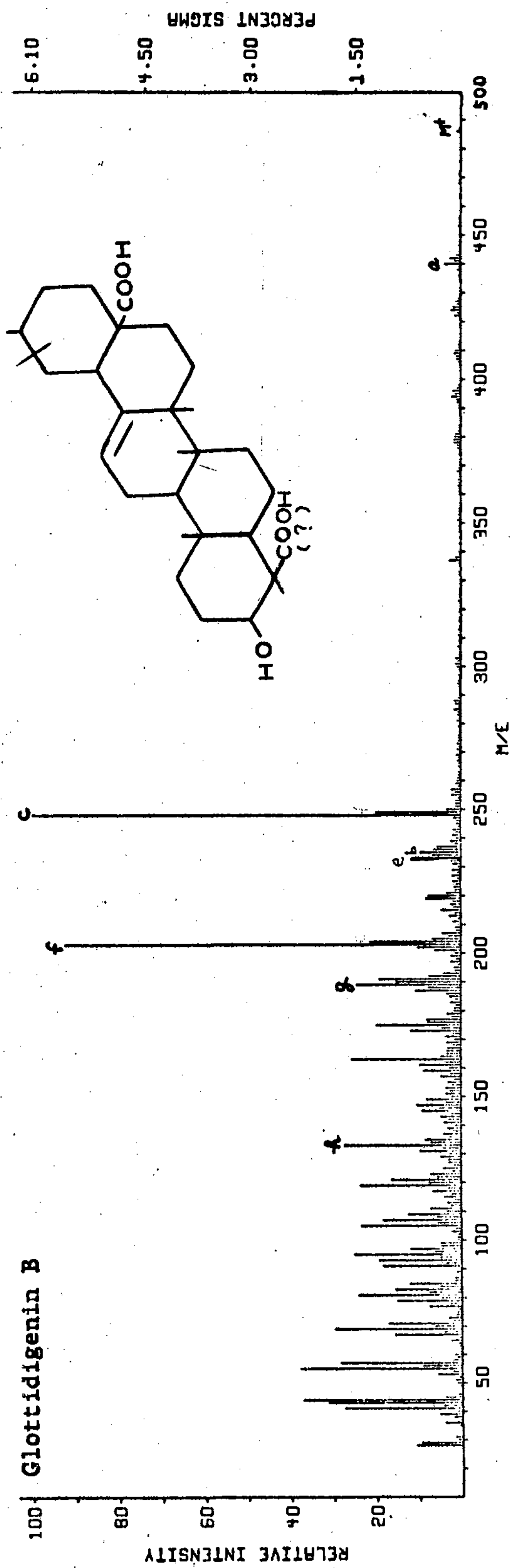

Glottidigenin B
RELATIVE INTENSITY
PERCENT SIGMA
M/E
COOH
COOH
(?)
HO

Figure 16. Fragmentation Patterns of Glottidigenin A and Glottidigenin B

(The fragmentation pattern given here is based on those proposed by Djerassi *et al*. The structure of glottidigenin A is given to illustrate the possible fission mechanism. Glottidigenin B has the same structure as glottidigenin A except that one of the four methyl groups attached to rings A and B is replaced by a carboxyl group)

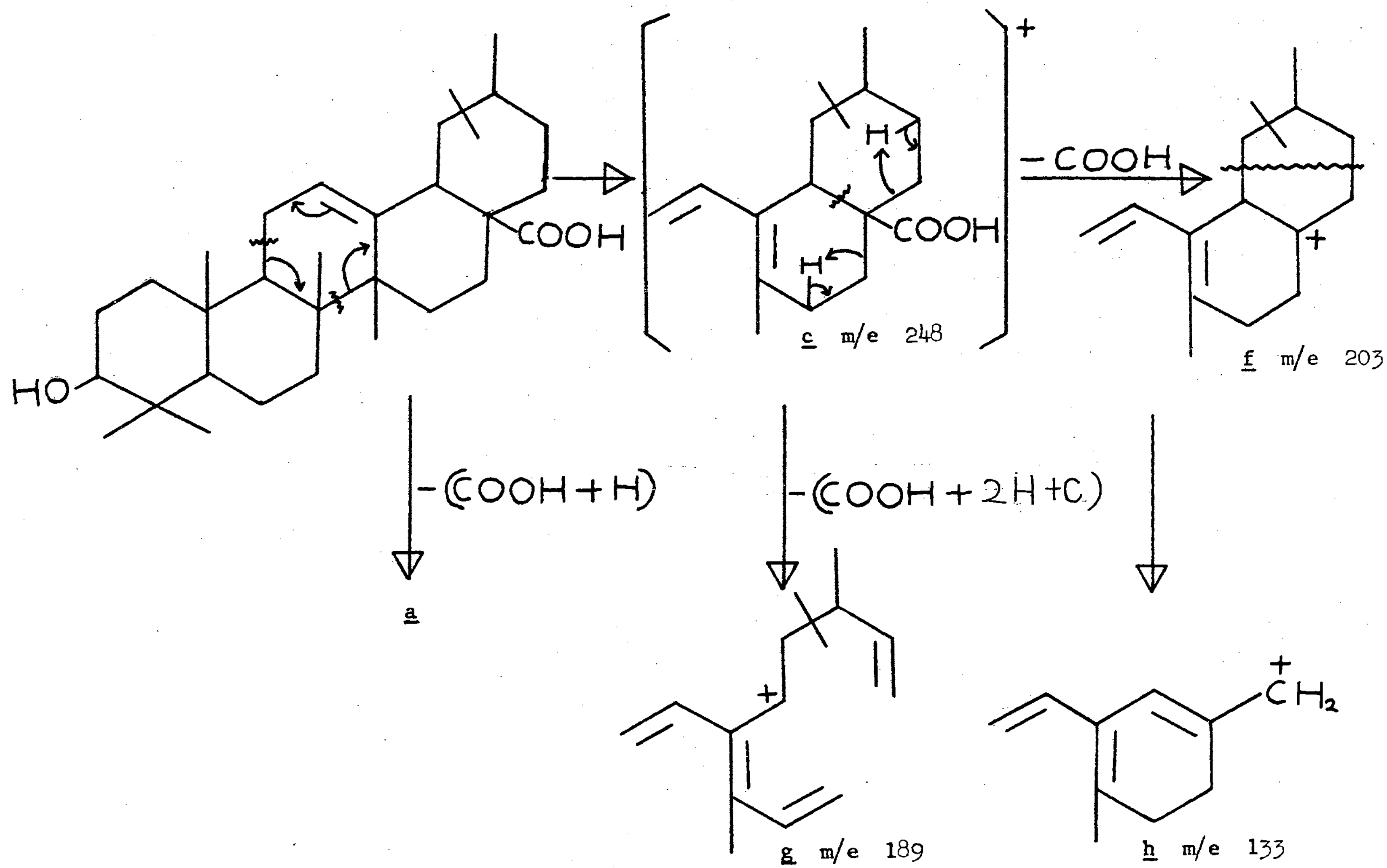
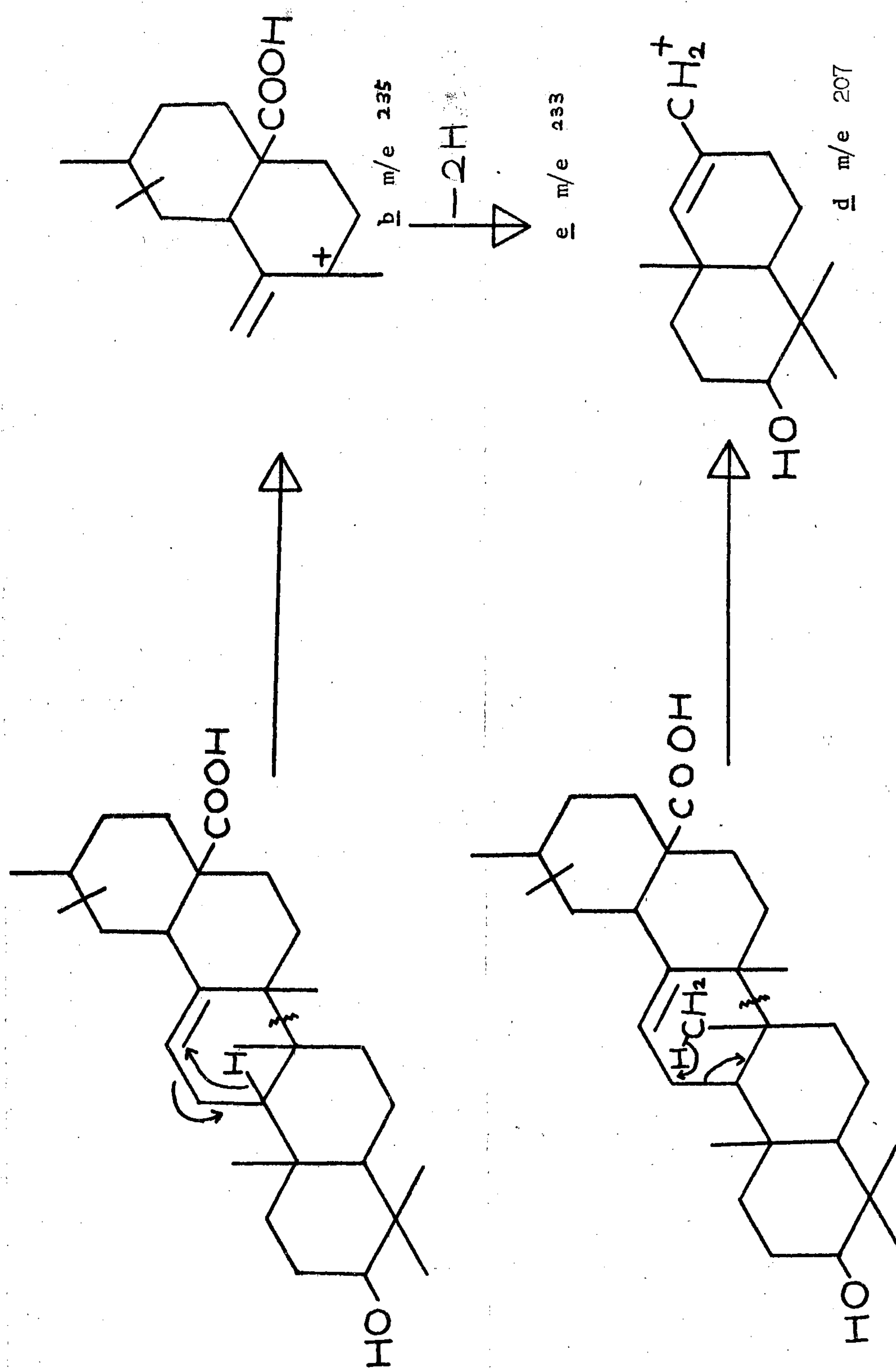

HO
COOH
H
H
COOH
c m/e 248
−COOH
f m/e 203
−(COOH + H)
a
−(COOH + 2H + C)
g m/e 189
CH2
h m/e 133

Figure 16. (Continued)



COOH
HO
b m/e 235
−2H
e m/e 233
COOH
HO
CH_2^+
d m/e 207

a. Glottidigenin A gave a molecular peak at 456 which, when considered together with the infrared spectrum data, indicates that this compound is a monohydroxy monocarboxylic acid. Similarly, glottidigenin B, which gave a molecular peak at 486, was characterized as a monohydroxy dicarboxylic acid.

b. Both glottidigenin A and B are members of the amyrin group since the presence of a 12 - 13 double bond was indicated by the characteristic retro-Diels-Alder fragment c (Fig. 16).

c. The structural feature at rings C, D, and E is identical for both compounds, as all of the identificable peaks corresponding to this portion of the molecular fragments (a, b, c, e, f, g, h, in Fig. 16) are present in both spectra, with the same intensity.

d. A carboxyl group is present at the C-17 position. This is indicated by the ready loss of this group from molecular ion to give a, and from ion c to give f. (Fig. 16).

e. The only difference between glottidigenin A and B is that one of the four methyl groups attached to the A and B ring in glottidigenin A is replaced by a carboxyl group to give glottidigenin B. This latter conclusion is supported by two facts; 1) glottidigenin B has a molecular weight 30 units higher than glottidigenin A which is equivalent to the difference of a carboxyl and a methyl group ($COOH - CH_3 = 30$), and 2) the most characteristic fragment derived from this portion of glottidigenin A (d, Fig. 16) is almost unperceivable in the spectrum of glottidigenin B.

Two other points remain to be established before the complete elucidation of these two structures can be made. 1) The location of the methyl group in ring E can not be deduced from present data due to the

fact (109) that the mass spectra of analogous derivatives of alpha- and beta- amyrin are very similar, differing in most cases only slightly in the relative intensity of some fragments. 2) The location of the second carboxylic group of glottidigenin B has to be established.

Characterization of Glottidinin I, Glottidinin II, and Glottidinin III from *Glottidium vesicarium*

Experimental procedures based on the information gathered above have led to the fractionation and characterization of three saponin components which will be referred to as glottidinin I, glottidinin II, and glottidinin III, for the purpose of recognition. Data obtained on the constituents of these three saponins are summarized in Table III.

The fractionation of individual saponins was achieved by thin-layer chromatography on silica gel using a basic solvent system n-butanol - ethanol - 25% aqueous ammonia (10 : 2 : 5). This is in accordance with the finding of Khorlin *et al*. (121) that the best results for separating acidic saponins may be obtained by the use of solvent systems having a pH greater than 7, since the two major sapogenins from *Glottidium vesicarium* were characterized as acidic in nature. After developing the thin-layer chromatogram in the above mentioned solvent system, the chromatogram was visualized by exposure to iodine vapor. The appropriate portion of the plate was scraped clean of silica gel and the saponin was then leached into 50% aqueous ethanol. The major compounds isolated were subject to complete acid hydrolysis as described in the experimental section. Modifications were made due to the small sample quantity: the volume of the hydrolyzing solution was reduced to 10 ml, also, the reaction mixture was evaporated to dryness

TABLE III

CONSTITUENTS OF SAPONINS FROM
GLOTTIDIUM VESICARIUM

Saponin	R_f[1]	Sapogenin[2]	Sugars[3]
Glottidinin I	0.277	Glottidigenin B	Glucuronic acid, Rhamnose, Galactose, Glucose
Glottidinin II	0.314	Glottidigenin A	Glucuronic acid, Deoxyribose, Rhamnose, Arabinose, Galactose, Glucose, Sorbose
Glottidinin III	0.396	Glottidigenin A	Rhamnose

1 Thin-layer chromatography on Eastman chromagram sheet (silica gel) at room temperature. Solvent system: n-butanol - ethanol - 25% aqueous ammonium hydroxide (10 : 2 : 5, v/v). Detected with iodine vapor.

2 Identified by mass spectrometry.

3 Glucuronic acid was identified by thin-layer chromatography. Other sugars were detected by gas-liquid chromatography of their alditol acetate derivatives.

after hydrolysis, the carbohydrate fraction was extracted into distilled water, and the sapogenin fraction was extracted into absolute ethanol, without further purification. The characterization of these two fractions followed exactly the same approach applied to the characterization of unfractionated saponins. The identification of monosaccharides in each saponin was verified by direct comparison with the carbohydrate fraction obtained from the hydrolysate of unfractionated saponin isolates, both by thin-layer and gas-liquid chromatography. Without such direct comparisons, an unambiguous identification of the sugar components would not be possible. This was also the case in the characterization of the sapogenin portion.

The mass spectra of the sapogenins from these three saponins were checked for the peaks with m/e values of: 189, 203, 248, and 207 (fragments g, f, c, and d respectively in Fig. 16). The first three were major peaks in the spectra of both glottidigenin A and B, whereas the peak with a m/e of 207 was characteristic for glottidigenin A only. The sapogenin portion of both glottidinin II and III was identified as glottidigenin A since their mass spectra contained all of the four above mentioned peaks as major peaks. The sapogenin portion of glottidinin I was identified as glottidigenin B since in its mass spectrum only m/e 189, 203, and 248 appeared as major peaks.

The Biological Effects of Glottidium vesicarium Saponin Isolates

Effect on Rats

2000 grams of dry beans were extracted with 70% ethanol. The volume was reduced to give a final concentration corresponding to 20

grams starting material per millimeter. This preparation was not toxic to rats when administered orally or intermuscularly. Similar experiments using fresh bean extracts were also unsuccessful.

Effect on Chicks

The feeding experiments with chicks are summarized in Table IV. The results indicate that the dry beans are toxic to chicks and that the toxic principle is extractable into 70% ethanol. Schoenheimer and Sperry (113) have shown that saponins have the property of forming stable complexes with cholesterol; therefore, cholesterol might be expected to function as an antidote. In a study performed on the effect of alfalfa meals on the performance of chicks, Peterson (27) reported that the growth depression effect produced by feeding alfalfa extracts was overcome by simultaneous feeding of cholesterol. We were unable to demonstrate the same phenomenon, as the addition of cholesterol showed no suppressing effect (Table IV).

During attempts to fractionate the poisonous principle, a loss of toxicity was observed as fractionation proceeded. The final saponin isolate was harmless when fed orally to two chicks at a total dose of 250 mg per chick, over a period of 10 days. Possible reasons for this are: 1) Saponins are not the only toxic components of *Glottidium vesicarium*. 2) Saponins are not readily absorbed into the bloodstream through the uninjured digestive tract. To be toxic, therefore, saponins must be accompanied by some plant substances which have irritant properties sufficient to injure the wall of the digestive tract and permit absorption (1). 3) The saponins are unstable and underwent a chemical change either during the fractionation process or during storage. A

TABLE IV

EFFECT OF GLOTTIDIUM VESICARIUM ON CHICKS[1]

Addition to Basal Diet	Level (%)	Mean Survival (days)
Ground dry beans	10	3
Ground dry beans + Cholesterol	10 10	3
70% Ethanol extracts of dry beans	50[2]	5
Alcohol extracted bean residue	10	+

1 Single groups of five chicks were used in each experiment. The experiments were for 10 days duration.

2 Based on the amount of starting material.

\+ No effect was observed.

comparison of this observation with those obtained by earlier workers, suggests that 3) is the most plausible explanation. In 1942 feeding experiments undertaken in this campus (5) also gave an inconsistent result. In one experiment a total of four grams of ground *Glottidium vesicarium* bean was fed to a white Leghorn hen, it died in six days. Postmortem examination showed that the intestines, especially the duodenal loop, were greatly inflamed. Later, in an effort to concentrate the toxic principle of the beans, ether-extracts and alcohol-extracts were prepared and evaporated. The resulting material produced no symptoms when fed to Leghorn hens. Residues from these extracts also produced no symptoms when fed to Leghorn hens. One month after the first experiment, some of the original beans which had been used to kill the animals previously were ground and fed to a white Leghorn hen and no toxic effects were observed. Thus the dry beans must have lost toxicity during storage. Similar toxicity variation has been reported for other types of saponins. Working with holothurin, a saponin from sea cucumber, Nigrelli (26) observed that one sample of processed holothurin showed a ten-fold loss in toxicity when compared with the crude samples.

Effect on Fish

The piscicidal property of *Glottidium vesicarium* saponins is shown by the data in Table V. The reactions of fish were characterized by great irritability and gasping movements followed by loss of equilibrium and the power of locomotion. The toxicity of saponins to fish appears to result directly from their detergent (surface-active) properties.

TABLE V

EFFECT OF GLOTTIDIUM VESICARIUM SAPONINS ON FISHES[1]

Compounds	Level (%)	Mean Survival (Minutes) Experiment[2]	
		1	2
Glottidium vesicarium Saponin isolate (root)	0.01	34	-
Digitonin	0.01	20	-
Glottidium vesicarium Saponin isolate (root)	0.002	-	54
Digitonin	0.002	-	22

1 *Glottidium vesicarium* saponin was obtained from root tissue. Digitonin was from commercial source. The experiments were carried out at room temperature.

2 Single groups of 3 guppies were used in experiment 1; single groups of 3 minnows were used in experiment 2.

Hemolytic Effects

In animals experimentally poisoned by feeding with Glottidium vesicarium dry beans, inflammation is one of the symptoms elicited (5). Inflammation probably reflects the cytotoxicity of this plant, and since hemolysis is one expression of cellular toxicity, the hemolytic effects of the Glottidium vesicarium saponins were investigated in some detail.

In keeping with the studies on other hemolytic biological compounds (vitamin A, steroids, bile acids) (138,139,140), a sharp concentration dependence, marked loss of activity as temperature decreases, and an inhibition by the presence of serum were observed. On the other hand, in contrast to vitamin A or neutral steroids, these saponin isolates showed no detectable effect on lysosome membranes. The experimental data as well as their implications are given below.

(1) Effect of saponin concentration on hemolysis: In Fig. 17 a sharp concentration effect was demonstrated. Hemolysis did not occur unless the saponin concentration exceeded a certain value. For concentrations in excess of this limiting concentration the rate of hemolysis was rapid and was virtually completed in 50 minutes. After this rapid initial hemolysis the extent of hemolysis increased only slightly. Such concentration dependence of hemolysis can be viewed more clearly when the percent hemolysis at the 50 minutes period in Fig. 17 is plotted as a function of the saponin concentration as given in Fig. 18. The degree of hemolysis was then shown to be dependent on saponin concentration, but only over a narrow range. Thus, under the conditions of the experiment shown in Fig. 17, the saponins were hemolytically inactive at concentrations below 11.25 µg/ml and the hemolytic capacity increased

Figure 17. Effect of Saponin Concentration on Rate of Hemolysis

Saponins in appropriate amounts were added to 8 ml of 0.9% NaCl solution to give the indicated concentrations and incubated at 37°C for at least 15 min. 1.5 ml of a 10% (v/v) suspension of washed bovine erythrocytes were then added, mixed gently, and incubated at 37°C. At given time intervals an aliquot of 1.5 ml was taken from the test suspension, centrifuged for 10 min. and the supernate removed. A 0.85 ml portion of supernate was then added to 4 ml of 0.6% ammonium hydroxide and the quantity of hemoglobin determined by measurement of the absorbance in a Klett-Summerson photometer using a No.54 green filter. The test suspensions were prepared in duplicate, the value shown is the average value. Control tubes for assessment of 100% hemolysis contained 1.5 ml of erythrocyte suspension in 8 ml distilled water. The bovine erythrocytes employed were obtained on the day of the experiment, washed three times with 0.9% NaCl solution, and suspended in this solution to a 10% (v/v) concentration.

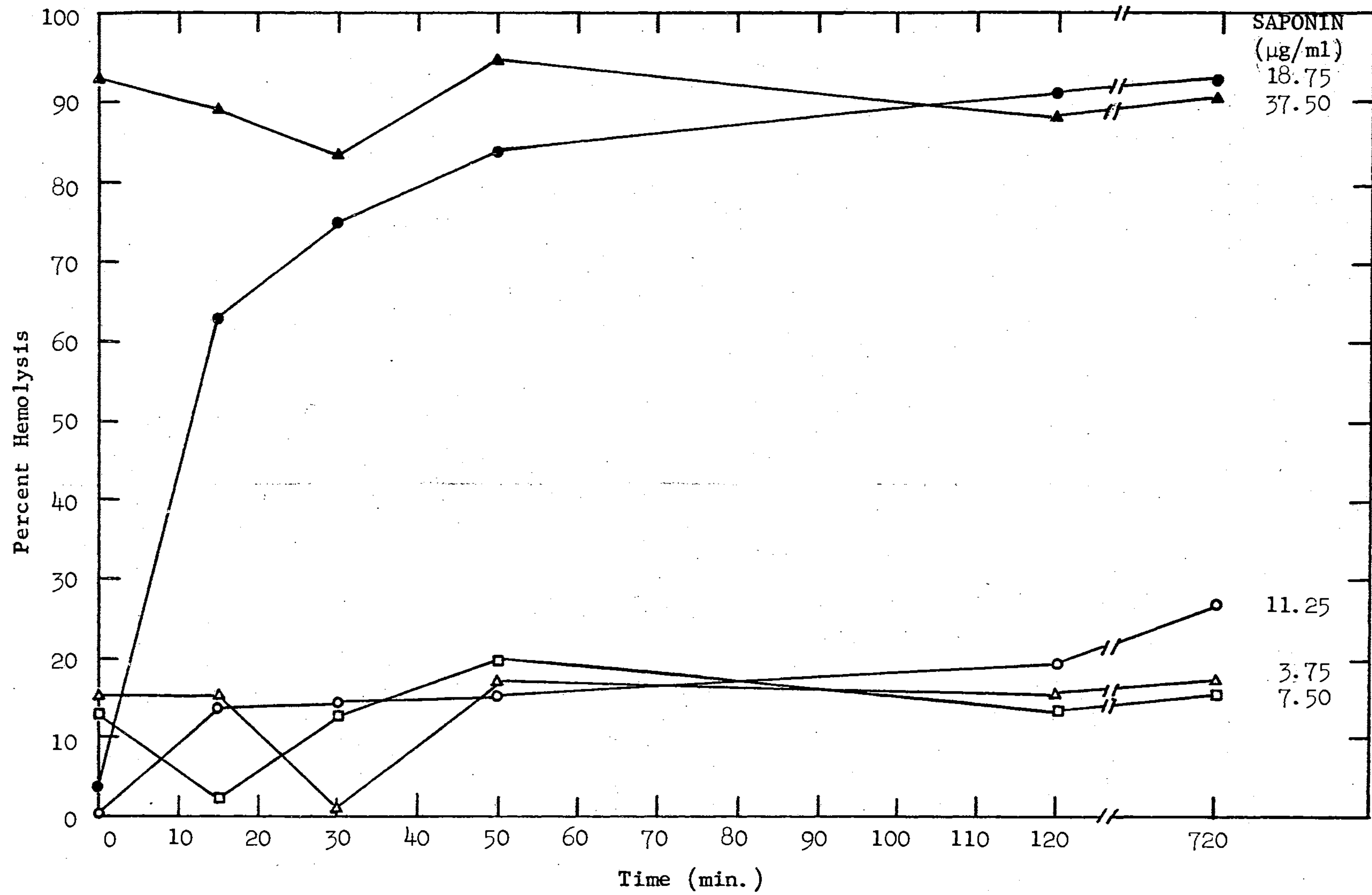
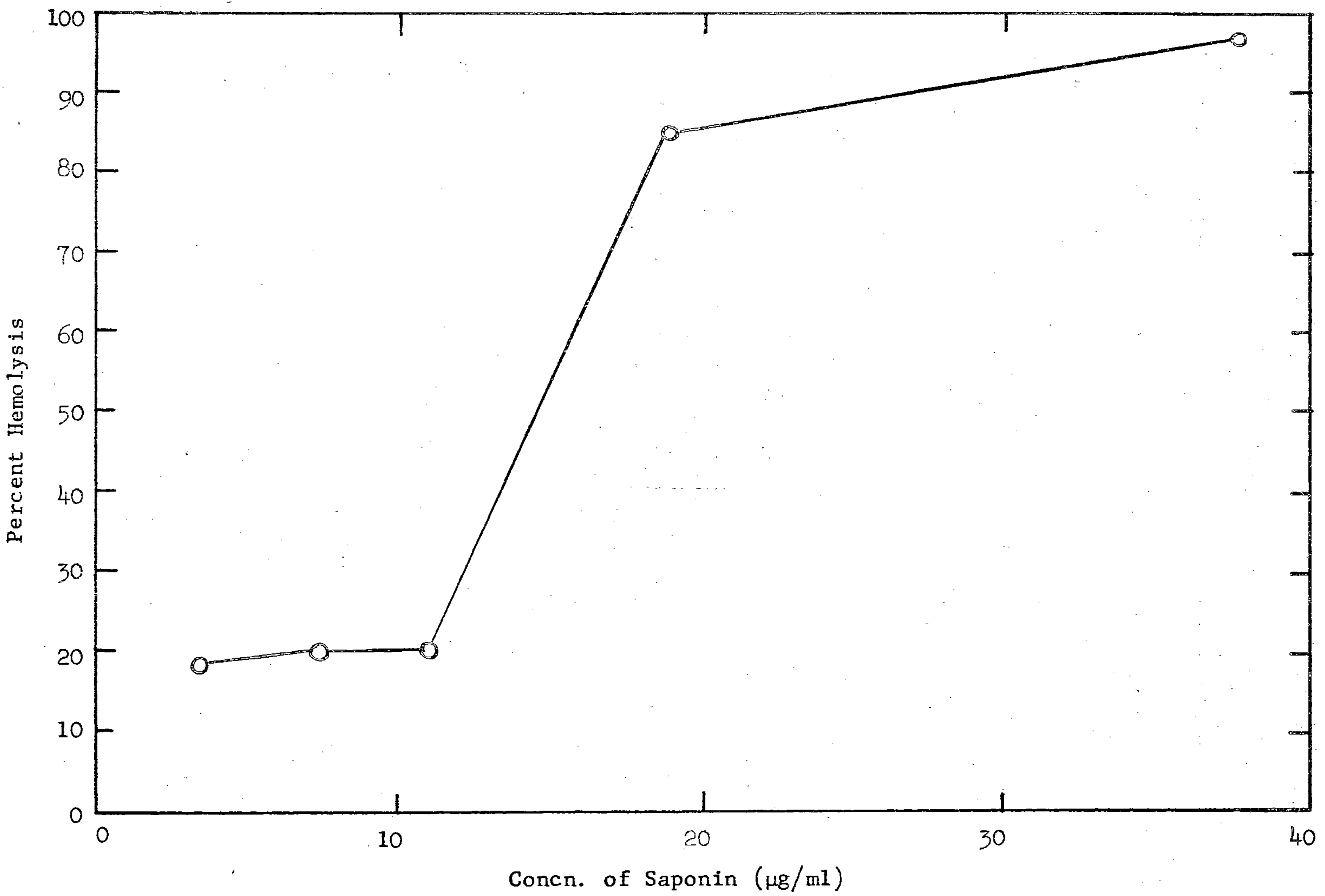

SAPONIN
(μg/ml)
18.75
37.50
11.25
3.75
7.50
Percent Hemolysis
100
90
80
70
60
50
40
30
20
10
0
Time (min.)
0
10
20
30
40
50
60
70
80
90
100
110
120
720

Figure 18. Effect of Saponin Concentration on Hemolysis

The experiment was the same as described in Fig. 17


100
90
80
70
60
50
40
30
20
10
0
Percent Hemolysis
0
10
20
30
40
Concn. of Saponin (μg/ml)

sharply as the saponin concentration went to 18.75 μg/ml. One possible implication from such sharp concentration dependence is that an adsorbed film of lysin is formed on the cell surface before hemolysis can occur.

(2) Effect of temperature on hemolysis: The extent of hemolysis induced by saponin isolates was found to be markedly temperature dependent (Fig. 19). The hemolytic activity was not obvious at $6^{o}C$, increased sharply when temperature increased to $24^{o}C$, and reached a maxium at $37^{o}C$. The pronounced temperature dependence of hemolysis might be expected to involve considerable activation energy, which then suggests that physical rather than enzymatic processes are involved.

(3) Inhibition of hemolysis by serum: The inhibitory effects of serum are indicated in Fig. 20. The presence of 0.2%, 1%, and 2% of serum in the final volume of 5 ml completely inhibits the release of hemoglobin from erythrocyte cells treated with 66 μg/ml of saponin preparation. In some reports the hemolytic effect of saponins was proposed to account for their physiological reactions (1). Our data do not support this point, as it is clear from Fig. 20 that the hemolytic activity of saponins can be abolished effectively in the presence of blood serum at physiological concentrations.

Figure 19. Effect of Temperature on Saponin-induced Hemolysis

One ml of a 10% (v/v) suspension of washed bovine erythrocytes was added to 6 ml of saponins (in 0.9% NaCl solution) to a final concentration of 43 µg/ml. The test suspensions were incubated at the given temperatures for 60 min. At the end of the incubation period the suspension was centrifuged for 5 min. and the supernatant removed. The absorbance of the supernatant was read in a Klett-Summerson photometer (using a No. 54 green filter) as a measure of the release of hemoglobin.

Control tubes for assessment of 100% hemolysis contained 1 ml of erythrocyte suspension in 6 ml distilled water. The bovine erythrocytes employed were prepared as described in Fig. 17.

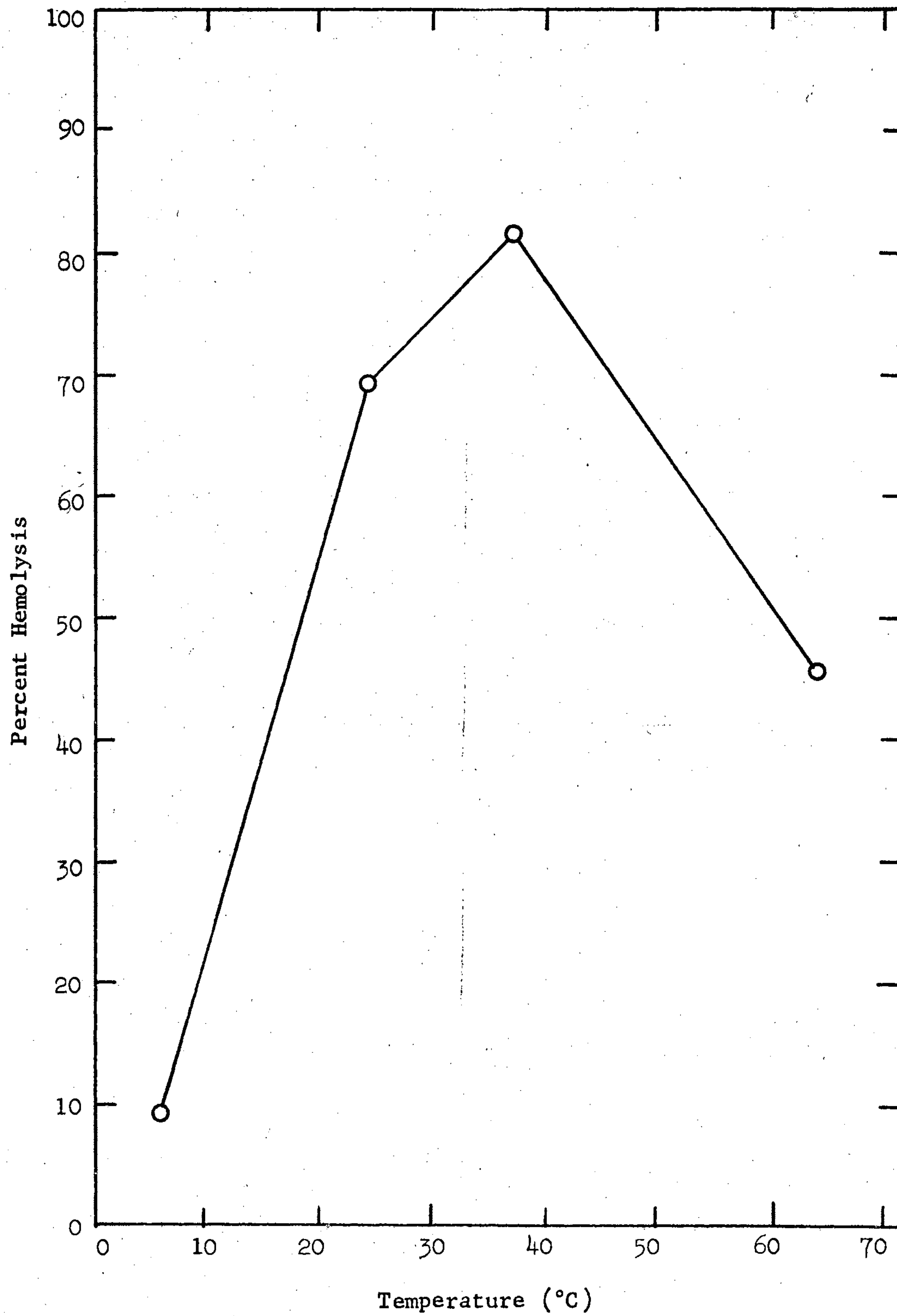
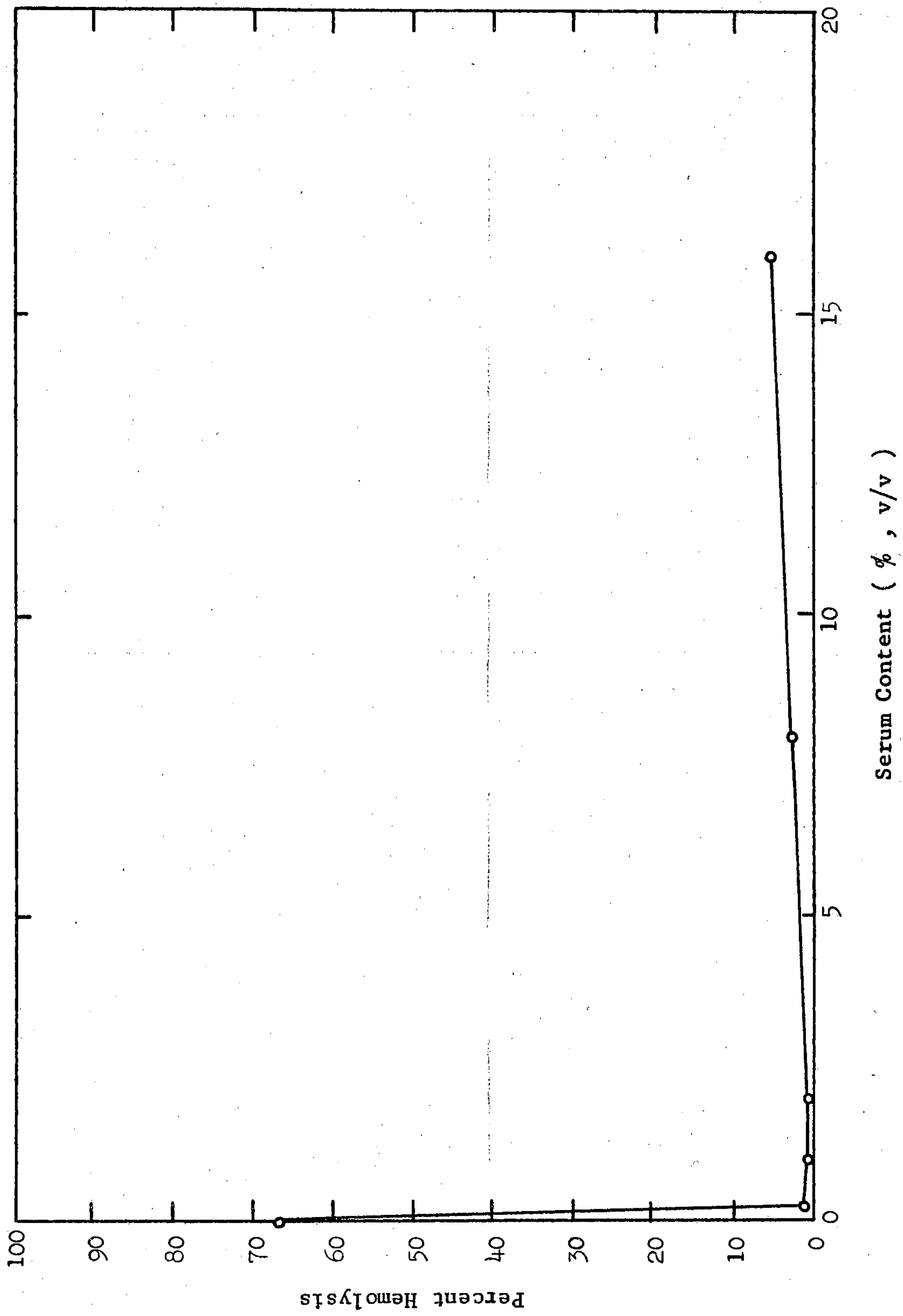

100
90
80
70
60
50
40
30
20
10
0
Percent Hemolysis
0
10
20
30
40
50
60
70
Temperature (°C)

Figure 20. Inhibition of Saponin-induced Hemolysis by Serum

One ml of saponins (66 μg/ml) was added to a series of dilutions of bovine serum in 0.9% NaCl immediately bdfore the addition of 0.5 ml of a 10% (v/v) suspension of erythrocytes from the same animal. The suspension in a total volume of 5 ml was then incubated at 37°C for 60 min. The extent of hemolysis was determined as described in Fig. 19.



Percent Hemolysis
0
10
20
30
40
50
60
70
80
90
100
Serum Content (% , v/v)
0
5
10
15
20

CHAPTER V

SUMMARY

Studies performed on the complete acid hydrolysis products of unfractionated saponin isolates led to the identification of eight sugars, glucuronic acid, deoxyribose, rhamnose, arabinose, xylose, galactose, glucose, and sorbose, and the characterization of two major sapogenins, glottidigenin A and B. Glucuronic acid was identified by thin-layer chromatrgraphy on silica gel layers impregnated with 0.3 M sodium monohydrogen phosphate in 0.1 M phosphoric acid (pH = 6.8). Other sugars were identified by gas-liquid chromatography of their alditol acetates on a 3% QF-1 column at 215°C. Infrared and mass spectrometric studies indicated that glottidigenin A is a monohydroxy monocarboxylic acid with a molecular weight of 456 and the molecular formula $C_{30}H_{48}O_3$, whereas glottidigenin B is a monohydroxy dicarboxylic acid with a molecular weight of 486 and the molecular formula $C_{30}H_{46}O_5$. Both sapogenins are composed of an amyrin type skeleton with a carboxyl group attached to the C-17 position. The spectrometric features of these two compounds strongly suggest that the only structural difference is that one of the four methyl groups attached to rings A and B in glottidigenin A is replaced by a carboxyl group to yield glottidigenin B.

Three saponin components, glottidinin I, II, and III, were isolated, fractionated, and characterized using experimental procedures developed in studies of unfractionated saponin isolates. Glottidinin I was

found to contain glottidigenin B as well as glucuronic acid, rhamnose, galactose, and glucose. Glottidinin II was found to contain glottidigenin A as well as glucuronic acid, deoxyribose, rhamnose, arabinose, galactose, glucose and sorbose. Glottidinin III was found to contain glottidigenin A and rhamnose.

The 70% ethanol extracts of both dry and green beans showed no toxic effect on rats when applied orally or intermuscularly. The mature beans were toxic to chicks when fed orally and this toxic effect was not suppressed by the addition of cholesterol. The toxic principle was extractable into 70% ethanol, but further fractionation was found to result in the loss of toxicity. The piscicidal property of saponin preparations was demonstrated at levels below 0.005%.

The hemolytic effects of saponin isolates were investigated. In keeping with the studies on other hemolytic biological compounds (vitamin A, steroids, bile acids), a sharp concentration dependence, marked loss of activity as temperature decreases, and an inhibition by the presence of serum were observed.

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