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# CHEMICAL AND NUTRITIONAL STUDIES ON ALKALOIDS OF LUPINE (*Lupinus termis*)

By

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CHEMICAL AND NUTRITIONAL
STUDIES ON ALKALOIDS OF LUPINE
(Lupinus termis)

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LUPINE ALKALOIDS

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## AN ABSTRACT OF THE THESIS OF

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Title: Chemical and Nutritional Studies on Alkaloids of lupine (*Lupinus termis*).

Lupine (Lupinus termis) is grown in the Middle East. The seeds are consumed to a limited extent after removing the bitter taste by boiling and then soaking in running water for several days.

The high protein content (35-40%) of this legume, makes it a potential source of protein for human and animal consumption. However, its bitter taste, when untreated, and the toxic effects observed when it is fed to various animals have restricted its use. There is limited information on the toxic effects of alkaloids and the nutritive value of lupine. Considering the shortage of protein in the Middle East, this information may be of value, since lupine may provide an inexpensive source of protein for consumption.

In the present study, chemical investigations were carried out to determine the composition of lupine seeds and their alkaloid content. The formation of an iron-alkaloid complex *in vitro* and *in vivo* was attempted.

Nutritional experiments, using rats, were carried out to investigate the effects of administering lupine alkaloids to rats on rat growth and mortality, reproductive ability, intestinal alkaline phosphatase activity and hemoglobin levels.

The results show that, protein constitutes 37.8%, and alkaloids 1.22% of the weight of the untreated lupine seeds. The crude alkaloid extract had lupanine as its major component. An iron-alkaloid complex is formed *in vitro*, but has not been shown to occur *in vivo*.

Nutritional experiments indicated that untreated lupine diets inhibited growth of rats due to the alkaloid content and to the poor quality of lupine protein. Also it was found that an intraperitoneally injected dose of 20 to 25 mg of alkaloid was lethal to rats.

Impairment of reproductive ability, and lowering of intestinal alkaline phosphatase activity and hemoglobin levels were observed in rats fed lupine diets compared to rats fed 10% casein diets. It is believed that these changes are due to the poor growth of rats fed the lupine diets.

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# I. INTRODUCTION

Lupine (Lupinus termis) is commonly grown in the Middle East. The seeds of this legume are consumed as a pastime food after removing the bitter taste by boiling and then soaking them in running water for several days.

The high protein content (35-40%) of this leguminous seed makes it a potential source of protein for human and animal consumption. However, its bitter taste, when untreated, and the toxic effects observed when it is fed to various animal species, have restricted its use.

There is little information about the toxic effects of lupine alkaloids and the nutritive value of lupine protein. And considering the shortage of protein in the world for human consumption, such information may be of value, since lupine will provide an inexpensive source of dietary protein.

The present study was carried out to investigate primarily the toxic effects of feeding untreated lupine seeds to rats and the effects of debittering the seeds using the same parameters. This seems essential before lupine seeds can be extensively used as a protein source for human consumption.

In this study, chemical and nutritional investigations were carried out to determine the proximate composition of lupine seeds, their alkaloid content and the chemical composition of their crude alkaloid extract. It was also intended to investigate the toxic effects of

feeding lupine seeds or injecting the crude alkaloid extract on rat growth, reproductive ability and other biochemical effects. Determination of the lethal dose of injecting alkaloids into rats was also attempted.

## II. REVIEW OF LITERATURE

Various lupine species, as many as 80 in North America alone, have been described. Some are wild, but a number of them are cultivated for forage, being valued for their high content of nitrogenous nutrients. Even the Greeks and Romans have used the seeds of certain lupines for making flour, after loosening the hulls by cooking and then soaking in running water to remove the bitter and poisonous principles.

In certain seasons and certain fields the commonly cultivated lupines are quite poisonous for all species of animals consuming them (Smith and Jones, 1958, pp. 621-622).

Lupine seeds can be classified into two major categories; the sweet lupine seeds or the low alkaloid containing seeds, and the bitter lupine seeds or the high alkaloid containing seeds. The percentage of alkaloid in the different species of sweet lupine seeds varies between 0.01 and 0.4%, and from 0.9 to 4.26% in the different species of bitter lupine seeds (Godnev and Mironenko, 1953; Mironenko, 1962; Tannous and Cowan, 1966; Maisuryan and Edel'shtein, 1960).

The alkaloid content depends upon the presence of genes promoting low or high alkaloid content, and can be determined by comparing thé intensity of the orange color developed, when Dragendorff's reagent is put on a sample of powdered seeds; an intense brick red color is obtained with seeds of bitter lupine, and a yellow color with the seeds of sweet lupine (Nowacki, 1959).

## Chemical Studies

### Quantitative Isolation and Determination of Alkaloids in Lupine

The total alkaloids, their composition and distribution within the various organs vary depending on the stage of ontogenic development of the plant (Mironenko, 1962). The growing plants have a higher alkaloid content than the aged ones (Mironenko, 1961; Mironenko and Rykovskaya, 1958), and the decrease in alkaloid content in the old leaves can be considered an indication that alkaloids are not an end product of metabolism, but rather an active participant (Godnev and Mironenko, 1953), synthesized exclusively in the green parts of the plant, and in the presence of light (Kamimierski and Nowacki, 1960).

The alkaloid content of plants increases with maturity. This increase accelerates sharply after flowering, and is accompanied by a migration of the alkaloids from the vegetative to the reproductive organs of the plant. It is influenced by the meteorological conditions during vegetation (Maisuryan and Edel'shtein, 1962). The alkaloid content increases on moving from the roots to the seeds of the plant; the roots and the seed covers containing none (Byszewski, 1950).

Examination of the seeds of 18 lupine species for their qualitative alkaloid composition, indicated that most species contained two to six alkaloids, few more than seven, and only one species, Lupinus venustus had one alkaloid. The major alkaloids identified were lupanine, sparteine, hydroxylupanine, lupinine and angustifoline, listed in the order of their predominance in the plant (Birecka *et al*. 1959; Mironenko, 1961; Maisuryan and Edel'shtein, 1960).

The number of alkaloids present depends upon the species, variety, part of the plant examined and the maturity of the plant (Mironenko, 1962).

Beal and Brady (1916), isolated and determined quantitatively the alkaloids of lupine seeds. They extracted the alkaloids with boiling diethyl ether, and precipitated them as the hydrochloride salts, which were then weighed, dissolved in water, and titrated with a standardized alkali solution using phenolphtalein as indicator.

Bozzo (1944), extracted the alkaloids by treating the powdered seeds successively with a dilute hydrochloric acid solution, an alkali solution, drying, then adding a known amount of 0.01N hydrochloric acid, the excess of which was back titrated with 0.01N sodium hydroxide.

The iodometric determination of alkaloids was introduced by Boiko (1950). The alkaloid was extracted from a powdered seed sample with a mixture of diethyl ether and chloroform, sodium hydroxide added to the extract and left for sixteen hours with periodic shaking. The mixture was then filtered, the filtrate treated with one percent hydrochloric acid in a separatory funnel, and the lower colorless layer, containing the alkaloids collected. To a known amount of this solution, a mixture of iodine and potassium iodide in solution was added. Then, the solution was acidified and titrated with 0.01N sodium thiosulfate.

Further improvements on the determination of alkaloids were introduced by Gordon and Henderson (1951). They extracted the

alkaloid from a sample of seed powder with 95% ethanol, which was then evaporated and the extract dissolved in boiling water. Fats and proteins were then precipitated with lead acetate, excess lead removed by precipitation with sulfuric acid, the whole made basic with sodium hydroxide and extracted with chloroform in a separatory funnel. The alkaloids were obtained by evaporating the chloroform, and then were weighed.

The nephelometric determination of the crystalline salts of the alkaloids was introduced by Graham and Spencer (1964). The alkaloids were extracted with water, precipitated in a phosphoric acid cation exchange column, eluted with an acid, then made basic with sodium hydroxide and extracted with chloroform. The crystalline salts obtained after addition of acetic acid and evaporation of the solvent could be determined nephelometrically.

## Separation and Identification of Component Alkaloids in a Crude Extract

For the separation and identification of component alkaloids of the crude extracts various methods were devised.

For the microchemical identification of the alkaloids, reagents and alkaloid solutions were treated together on glass plates, and precipitates formed were examined microscopically. Identification of the alkaloids was made by comparison with control solutions having comparable concentrations of known alkaloid samples (Clarke and Williams, 1955). Bromoauric acid gave quick precipitates with alkaloids which could be identified thus (Sandri, 1958).

Various methods for the colorimetric determination of alkaloids

have been reported. When an alkaloid solution was added to a solution of Reinecke salt, a precipitate formed. The filtrate could be collected and compared colorimetrically with the filtrate of a solution of a known concentration of Reinecke salt. The amount of Reinecke salt precipitated by the alkaloid is used as an indication of the quantity of alkaloid present in the solution (Del Pozzo, 1949).

The reaction of alkaloid bases with methyl orange in chloroform, followed by acidification (Riefer and Niziolok, 1957), or with bromthymol blue (Wiewiorowski and Skolik, 1959), or with molybdenum blue (Latawiec, 1958), also allowed the quantitative determination of the alkaloids, by measuring the amount of unused indicator colorimetrically.

Studies with the polarizing microscope have been suggested for the qualitative analysis of lupine alkaloid extracts (Keenan, 1927).

Chromatographic separation and identification of alkaloids has been extensively used. Gas chromatography (Lloyd _et al_. 1960), column chromatography (Andreeva, 1959; Birecka and Nalborczyk, 1961; Brutko and Utken, 1962; Dzhalilov _et al_. 1963), and paper chromatography, which proved more advantageous than the previous two (Macek _et al_. 1956; Bonnishsen _et al_. 1957; Grims, 1957; Waldi, 1959; Wiewiorowski and Bratek, 1957; Mironenko _et al_. 1959), have been used.

For better separation of the alkaloids on the paper chromatogram, electrolytes, such as sodium sulfate, were used in the solvent system (Resplandy, 1954), while for the best identification of the alkaloids spraying with Dragendorff's reagent was recommended

(Nowacki, 1963; Lewandowski and Wittowski, 1959; Wiewiorowski et al. 1958; Rengei, 1961).

Reifer (1959), suggested the use of circular paper chromatography for the separation of mixtures of lupanine, hydroxylupanine and sparteine.

Electrophoresis was reported as a quick and easy method for the separation and identification of alkaloids in general (Tatsuo et al. 1953), and of lupine alkaloids in particular (Nehring and Brandhoff, 1961). Although good separation and identification of alkaloids could be attained with paper chromatography, comparative experiments with electrophoresis showed that electrophoretic mobility was more reproducible than Rf values, and that electrophoresis was quicker and more reliable (Buff et al. 1959). The alkaloids separated by paper electrophoresis were best identified when stained with a spray of Dragendorff's reagent, and the color developed examined photometrically (Miessner, 1963).

A more recent development in the technique of separation and identification of alkaloids has been thin layer chromatography (Waldi et al. 1961; Paris and Paris, 1963; Korzun et al. 1962). According to Ramaut (1963), the best conditions for the thin layer chromatographic separation of the leguminosae alkaloids were using silica gel as adsorbant, a mixture of cyclohexanol, cyclohexane, hexane and diethylamine as the solvent, and a modified Dragendorff's reagent as the color developing spray. The alkaloids were dissolved in chloroform for application.

Acid or alkali addition to the adsorbant gel, produced better

separation of the alkaloids by increasing the Rf values (Shu and Chang, 1965; Doepke, 1962), and lupine alkaloids were best separated on silica gel plates prepared with 0.1N sodium hydroxide (Abu Chaar, 1963).

The ultra violet fluorescence of alkaloids has also been used for their identification (Wartman-Hafner, 1966).

## Nutritional Studies

### Nutritive Value of Lupine Seeds

Lupine seeds are rich in protein. The protein content of the various species varies between 16 to 45% (Ripa and Geidans, 1964; Brauer, 1922; Tannous and Cowan, 1966).

Mangold and Lintzel (1935), suggested feeding swine with sweet lupine seeds, because of their high protein content of 39.60%, and their high digestibility coefficient of 92.6%.

The protein in the seeds of sweet yellow lupines, when fed to swine, gave a biological value of 57.16 (Lintzel and Mangold, 1935); and when fed to ruminants an average value of 34.22% was obtained for digestible proteins; the average digestibility coefficient being 90.18% for crude protein, 93.37% for crude fat, 75.31% for crude fiber and 79.58% for crude nitrogen free extract (Mangold and Stotz, 1935).

With sheep, the digestible crude protein content was 11.8%, and substitution of half the basal ration with dried sweet lupine seeds did not affect the health of cows and lambs (Nehring _et al._ 1939). The digestibility coefficient of the seed tested on sheep

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varied between 79 and 82% (Ritcher and Becker, 1951), and digestibility decreased with maturity, dropping from 80 to 64% when in full bloom, due to the increase of fiber content (Fissmer, 1941).

Three day balance tests, with bitter lupine seeds, performed on human subjects, showed the digestibility of lupine protein to be 86.18 to 89.52%, of lupine fat 38.01 to 62.85% and of lupine carbohydrate 80.79 to 97.47%. In spite of their high digestibility values, lupine seeds could not be used for human consumption because of their bitter taste and unpalatability (Heupke and Buttenhoff, 1938).

The nutritive value of sweet lupine seeds of Lupinus albus and Lupinus luteus compared favourably with soybean. The high lecithin value, the high biological value and the good binding properties of sweet lupine made its use in the confectionary industry instead of milk and eggs a possibility (Fekete and Korpaczy, 1955).

Animal Nutritional Studies with Lupines

When sweet lupine was used as a diet for rats, older rats showed favourable growth and produced healthy litters when fed a 60% lupine diet, while growth was arrested and some litters died when fed 80% lupine diets. Growth of rats was impaired and breeding impossible when 100% lupine diets were used. In all cases the animals showed no injury in any of the organs (Columbus, 1935).

Four week old, weaned albino rats, lost weight and showed liver damage when fed a 50% lupine diet. After removal of the alkaloids, the toxicity was reduced, but liver function was slightly impaired as confirmed by histological studies (Tarjan and Laszlo, 1957).

According to Wittenberg and Nehring (1965), no toxic manifestations appeared upon adding lupanine to the fodder of rats. On feeding sweet lupine, Lupinus albus with 0.0006% lupanine and 0.008% hydroxylupanine, and bitter lupine, with 1.21% lupanine and 0.608% hydroxylupanine, the animals remained normal and their livers showed no changes. About 70 to 80% of the lupanine ingested was excreted, 50 to 70% in the urine and 10 to 14% in the feces. Generally, the alkaloid excretion in the urine from a single feeding was complete in two days, while it continued in the feces for 27 days. The organism was observed to change some lupanine to hydroxylupanine.

The crude alkaloid extract of Lupinus termis seeds, when added to a 10% casein diet, had a growth depressing effect on rats. At the same protein levels, rats fed debittered lupine gained more weight than rats fed the untreated bitter seeds, and the gain in weight increased in both cases with an increase in dietary protein, irrespective of the concurrent increase in alkaloid content. It was concluded that the presence of alkaloids was not the only cause for the poor growth of rats fed lupine diets. The poor protein quality, as shown by low protein efficiency ratio values, is an important factor as well. Amino acid analysis showed that methionine was the limiting amino acid, followed by lysine and threonine. Amino acid supplementation had a more pronounced effect on improving rat growth than debittering (Tannous and Cowan, 1966; Tannous _et al_. 1968).

Bitter lupine, as such, is unsuitable for feeding cattle, while sweet lupine, as Lupinus angustifolius with an alkaloid content of 0.08% has been safely used (Zewicz, 1917).

Blue lupine alkaloids have a highly toxic effect on various pests, specially on the gooseberry sawfly. A 0.02 to 0.05% solution of the alkaloids kills 75 to 100% of these animals within one or two days after spraying (Isaev, 1939).

When sheep were fed toxic lupines, such as Lupinus angustifolius and Lupinus varius, the total iron binding capacity of serum increased in all animals affected with lupinosis. Serum and liver iron increased in all cases, but liver cobalt levels decreased below normal (Gardiner, 1965). Also concentrations of liver copper increased, while total liver solids decreased (Gardiner, 1966). Sheep with high liver copper levels, were more easily affected by lupinosis, and showed more liver damage than sheep with low copper levels, grazing on the same lupine. Thus, the main pathological changes of the disease were directly correlated with liver coppor concentrations (Gardiner, 1967).

Acute poisoning in the animals ingesting lupine, becomes evident from one to 24 hours after ingestion, with symptoms usually of depression, weakness, prostration and coma. Excitement and convulsions have also been reported. In horses impairment of the proprioreceptive sense is shown by a tendency to lift the feet unusually high at each step. Other symptoms are those of gastrointestinal irritation. Often the poisoned animal lies for days unconscious, the urine is voided frequently, is darkened by bile pigments and contains high levels of albumin. The glucose of the blood drops to very low levels. Upon autopsy, hepatic and renal injury, jaundice,

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hydropericardium, and in chronic cases, hydroperitoneum, pulmonary congestion and edema are observed. Fatty degeneration is specially prominant in the liver, which is often deep yellow in color. Also, fat is often present in the renal epithelium and myocardium. Congestion of the menengial and cerebral vessels may occur, as well as terminal hemorhages beneath the epicardium (Smith and Jones, 1958, pp 621-622). In certain cases glucose was observed to revert the toxic symptoms of lupanine (Abozzo, 1952).

Toxic Effects of Lupine Alkaloids

The alkaloid extract from the seeds of Lupinus argentius was found to be lethal to rabbits when injected intravenously in doses of 28 mg per kg body weight (Beath, 1917). Subcutaneous or intraperitoneal injections to guinea pigs of dl-lupanine, lupinine, spathulatine and hydroxylupanine, produced depressant and paralyzing effects; while muscular tremors were observed in the case of sparteine injections. The minimum lethal doses of toxicity for the alkaloids per kg body weight were: d-lupanine, 22 to 25 mg, sparteine 23 to 30 mg, lupinine 28 to 30 mg, and hydroxylupanine 22 mg. The lethal dose of each of the alkaloids mentioned above, was twice higher when injected subcutaneously as compared to when injected intraperitoneally (Couch, 1926).

D-lupanine, extracted from blue lupine (Lupinus angustifolius) seeds was lethal to guinea pigs, mice and rats when injected intraperitoneally. The minimum lethal doses per kg body weight were, 225 mg for guinea pigs, 180 to 192 mg for rats and 75 to 81 mg for mice (Gordon and Henderson, 1951).

The crude alkaloid extract from Lupinus termis seeds showed no estrogenic activity when injected subcutaneously to rats, apparently because the high toxicity of the extract prevented the use of sufficiently high doses. The total alkaloids, in daily subcutaneous injections of one mg, produced estrogenic effects and increased uterine weight by 65% (Elghamry and Shihata, 1964).

Ligon (1941), found that lupanine increased the amplitude and rate of the mobility of isolated rabbit uterus. Very high concentrations however, had the opposite effect. On the isolated uterus of rabbits and guinea pigs, an aqueous extract of Lupinus albus, $8x10^{-3}$ dilution, caused an elevation of tone and regular rythmic contractions. Lower doses were ineffective, while a higher dose paralyzed the action (Longo, 1948).

Removal of Bitter Substances from Lupine Seeds

The toxic effects of lupine alkaloids, which are associated with the bitter substances in the seed, on the animal organism, necessitate the debittering of lupine seeds before consumption. Boiling the seeds for two hours in water, and then keeping them for three days in cold running water, removed 84% of the bitter taste (Dietrich and Jankon, 1920). Bitterness could also be removed by treating the seeds with water at 65°C for an hour, with slow stirring, and finally treating with 0.5 or 0.25% hydrochloric acid solution to remove all traces of the bitter taste (Rewald, 1921).

Heating lupines at 130 to 135°C under pressure for a short time, and bursting the cell walls by a momentary release of pressure, releases the poisons and the bitter principles (Kinthof, 1925). Also, heating whole seeds in water containing a small amount of hydrochloric acid, and subsequent extraction with 50% denatured alcohol (Gerlach, 1930), or with alkali (Prakhin, 1938), removed the bitter principles.

Electrodialysis has been used by Makaro (1938) to remove bitterness. An aqueous suspension of lupine meal was dialized in a two cell apparatus. The anode was carbon and the cathode was iron. After a three hour treatment, with a direct current of 0.5 amperes, the alkaloid content was reduced from 2.39 to 0.43%, and to 0.35% after an eight hour treatment. The alkaloids travelled to the anode. However, along with the loss of the alkaloids, there was a loss of protein content and value, probably due to the high temperature occuring because of the current.

Liu (1955), found that maceration of lupine seeds for three hours with five times their volume of a 2.5% hydrochloric acid solution, eliminated the alkaloids and 10% of the proteins.

The effect of trace elements added to the soil, on the alkaloid content of the lupine plants is of interest in producing low alkaloid lupine plants. Manganese and magnesium added to the soil, were found to decrease the alkaloid content, while boron had the opposite effect, in the leaves (Baryshpol, 1960). In the seed, boron decreased the alkaloid content and increased the protein content (Mironenko, 1955; Zabalotmy and Mironenko, 1967). Inoculation of green sweet lupine also decreased the alkaloid content and increased

the protein content in the leaves, but had the opposite effect on the seeds (Zeimecka and Golebiowska, 1943).

Effect of Diet on Alkaline Phosphatase Activity and Hemoglobin Level

Decreasing the level of protein in the diet of rats increased the alkaline phosphatase activity in the kidneys (Barrows et al. 1965), and the liver (Rosenthal et al. 1952; Muramatsu and Ashida, 1962; Dinu, 1965). A three day repletion diet restored the activity back to normal in the liver (Ely and Ross, 1954). Kfoury et al. (1968) reported a decrease in alkaline phosphatase activity in the kidney of rats, with a decrease of the level of dietary protein.

Meier and Simon (1961) reported that an 80% protein diet increased the alkaline phosphatase activity in the liver parenchyma of rats, while Ross and Batt (1957) reported no change in alkaline phosphatase activity in the liver of rats with an increase in the protein level of the diet. An increase in alkaline phosphatase activity was reported in the liver and intestinal mucosa of rats after oral administration or intraperitoneal injections of amino acids into rats (Girelli, 1956; Pasquinelli and D'Alessandro, 1952).

A decrease in the protein level of the diet was observed to cause a decrease in the level of hemoglobin in rats (Aschkenasy et al. 1959; Bencze et al. 1966). A four week repletion diet returned levels back to normal (Weimer et al. 1959).

Sakamoto and Yokoyama (1967), however, reported that rats fed 7,13 and 26% protein diets, for seven generations, showed no significant difference in their hemoglobin levels.

A decrease in hemoglobin level with a decrease of the level of dietary protein has also been reported in bulls (Meacham *et al.* 1964), and in sheep (Rys and Sokol, 1963).

## III. MATERIALS AND METHODS

### Chemical Determinations

Proximate composition of Lupinus termis seeds was determined according to the Handbook of the Association of Official Agricultural Chemists (AOAC, 1960).

### Determination of Alkaloids

Crude alkaloid content was determined according to the method described by Gordon and Henderson (1951). In this method, the untreated bitter lupine seeds were ground to a powder, extracted with 95% ethanol until a few drops of the extract showed no cloudiness upon the addition of a drop of Mayer's reagent (prepared by dissolving 1.35 g of mercuric chloride in 60 ml of water and adding it to a solution of 5 g of potassium iodide in 10 ml of water, and diluting the mixture to 100 ml), the alcohol evaporated and the remaining extract dissolved in boiling water. Proteins, fats and organic acids were precipitated and removed from the aqueous solution with a solution made of a mixture of basic and neutral lead acetate (1 g of basic lead acetate in 16 ml of water added to 1 g of neutral lead acetate in 1.6 ml of water). Excess lead was precipitated with 1N sulfuric acid as lead sulfate, and then the solution was made alkaline with 50% sodium hydroxide solution. The free alkaloid bases liberated were then extracted with chloroform in a separatory funnel. Evaporation of the chloroformic solution then yielded the crude alkaloids.

For further purification, the alkaloidal mass was dissolved in dilute hydrochloric acid, the solution shaken with chloroform to remove any adhering lipids, and the aqueous solution boiled with charcoal, filtered, evaporated to a convenient volume, made basic with sodium hydroxide, and extracted with chloroform which was then evaporated. To remove the last traces of chloroform from the mixture, some methanol was added to form an azeotropic mixture which boils at 35.6°C. The solution was distilled and the alkaloids thus obtained were weighed.

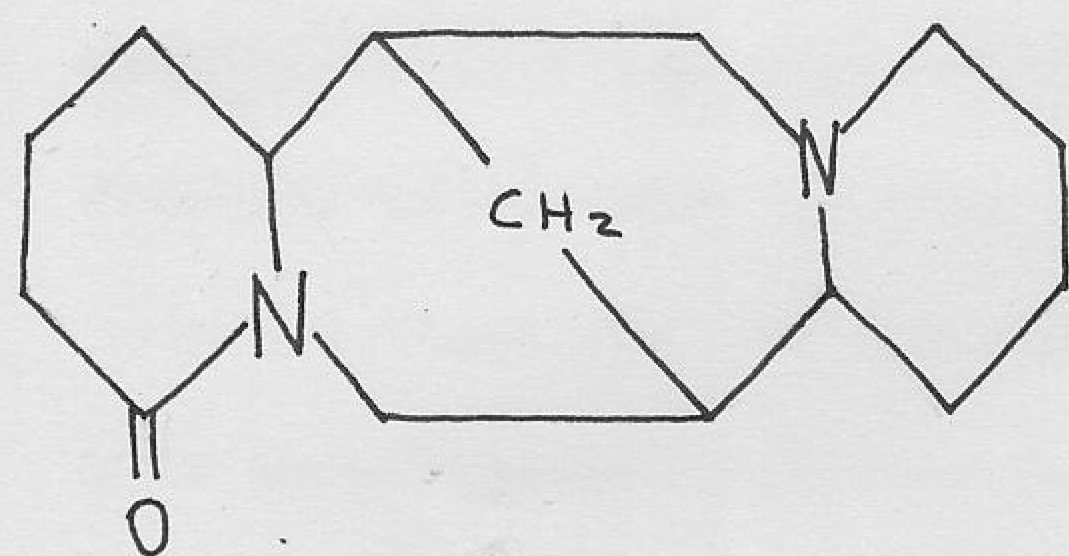
Identification of Alkaloids

Separation and identification of the component alkaloids in the crude alkaloid extract was carried out by thin layer chromatography as described by Abu Chaar (1963). The plates were covered with a mixture of 25 g of silica gel and 50 ml of 0.1N sodium hydroxide, 0.25 mm in thickness, and dried in an oven at 115°C for 30 minutes. The alkaloid extract in chloroform was spotted using a microsyringe. Twenty five microliters of the solution, containing 1 mg alkaloid per ml, was applied at each spot. The solvent system used was a 17:3, volume/volume mixture of chloroform and methanol. The spray used to develop the color of the alkaloid spots was the modified Dragendorff's reagent as described by Grant and Kennedy (1955). The alkaloids used as standards for comparison were lupanine, hydroxylupanine, cytisin (purchased from Fluka A.G., Buchs, Switzerland), and sparteine (purchased from Sigma Chemical Company, St. Louis, Missouri). The alkaloids used as standards have the following structural formulas:

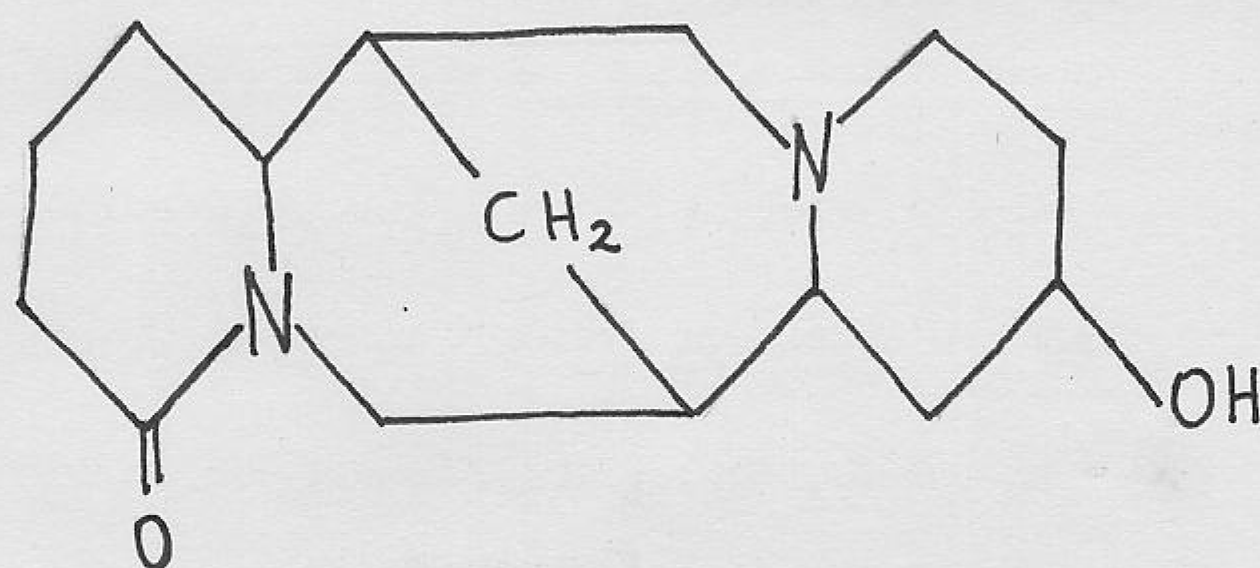
For further purification, the alkaloidal mass was dissolved in dilute hydrochloric acid, the solution shaken with chloroform to remove any adhering lipids, and the aqueous solution boiled with charcoal, filtered, evaporated to a convenient volume, made basic with sodium hydroxide, and extracted with chloroform which was then evaporated. To remove the last traces of chloroform from the mixture, some methanol was added to form an azeotropic mixture which boils at 35.6°C. The solution was distilled and the alkaloids thus obtained were weighed.

## Identification of Alkaloids

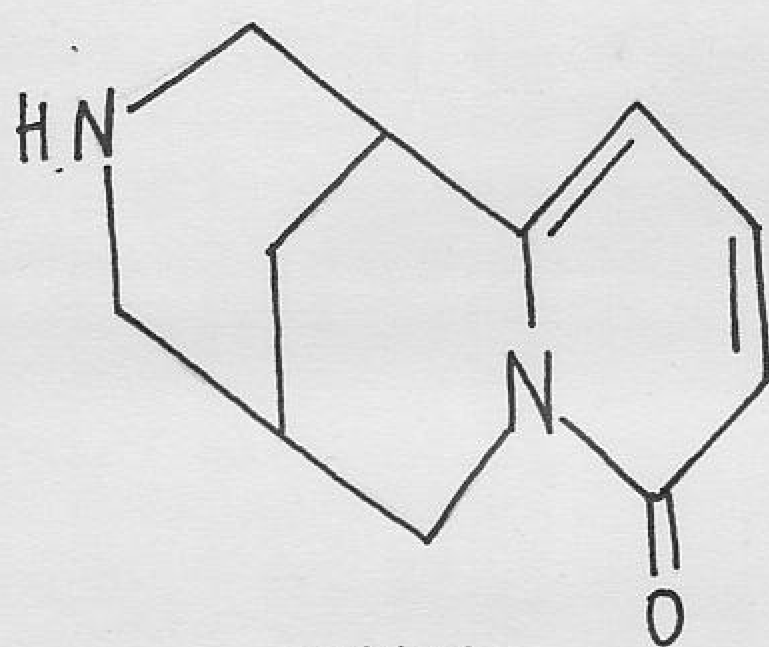
Separation and identification of the component alkaloids in the crude alkaloid extract was carried out by thin layer chromatography as described by Abu Chaar (1963). The plates were covered with a mixture of 25 g of silica gel and 50 ml of 0.1N sodium hydroxide, 0.25 mm in thickness, and dried in an oven at 115°C for 30 minutes. The alkaloid extract in chloroform was spotted using a microsyringe. Twenty five microliters of the solution, containing 1 mg alkaloid per ml, was applied at each spot. The solvent system used was a 17:3, volume/volume mixture of chloroform and methanol. The spray used to develop the color of the alkaloid spots was the modified Dragendorff's reagent as described by Grant and Kennedy (1955). The alkaloids used as standards for comparison were lupanine, hydroxylupanine, cytisin (purchased from Fluka A.G., Buchs, Switzerland), and sparteine (purchased from Sigma Chemical Company, St. Louis, Missouri). The alkaloids used as standards have the following structural formulas:



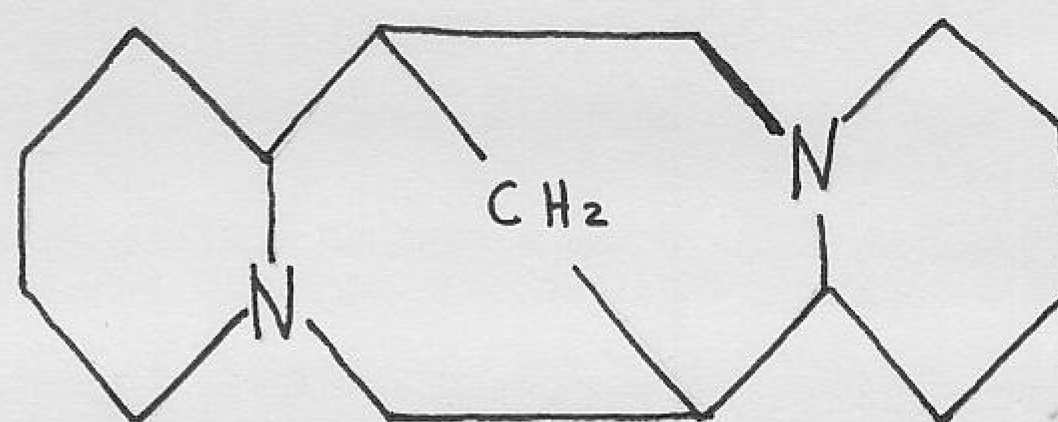

lupanine




hydroxylupanine




cytisin




sparteine

<u>Tests for Alkaloid-Iron Complex Formation</u>

The formation of an iron-alkaloid complex was performed by mixing a 5% methanolic solution of ferric chloride with a methanolic solution of crude alkaloid extract and of lupanine. The brown precipitate obtained in both cases was left in contact with the solution for 12 hours and then collected.

Solubility, in various laboratory solvents, of pure lupanine and the lupanine-iron complex was tested as described by Shriner <u>et al</u> (1964, pp 67-72). The iron-alkaloid complex was washed several times with chloroform to remove any adhering free lupanine; complete removal of lupanine was tested by showing the absence of lupanine in the final chloroform washing by thin layer chromatography.

The complex was broken by pyridine into free iron and free alkaloid, the pyridine evaporated under a nitrogen flow, the remaining precipitate dissolved in chloroform and tested for the presence of lupanine by thin layer chromatography.

To test for the formation of an alkaloid-iron complex *in vivo*, rats were injected intraperitoneally, sublethal doses of crude alkaloid extract, dissolved in an isotonic (1.15%) solution of potassium chloride, killed after 15 minutes, and their hearts, kidneys, livers, spleens, brains, blood and ovaries or testees removed. Each organ was washed with chloroform to remove any adhering alkaloid, homogenized in chloroform, centrifuged and the clear supernatent chloroformic solution analyzed for the presence of lupanine by thin layer chromatography. The precipitate of cell mass was then washed with chloroform several times to remove any free lupanine adhering to it, and then treated with pyridine to break any alkaloid-iron complex present and liberate the free alkaloid. The pyridine was then evaporated under nitrogen, the remaining cell mass washed with chloroform, centrifuged, and the clear chloroformic solution tested for the presence of alkaloids by thin layer chromatography.

## Determination of Intestinal Alkaline Phosphatase and Hemoglobin

Intestinal alkaline phosphatase activity in rats was determined as described by Bergmeyer (1963, pp 783-785), using p-nitrophenylphosphate as substrate. Hemoglobin level in rats was determined according to the method described by Cannan (1958), but,

using an extinction coefficient of 44 (Assendelft et al. 1967). Results were analyzed statistically, using the Student's t-distribution test.

## Nutritional Experiments

Male and female albino rats of the Sprague-Dawley strain were used. The animals were housed in mesh bottom cages in an air conditioned room maintained at a temperature of $23 \pm 2^{\circ}C$. Food and water were supplied ad libitum.

The experimental animals were fed three experimental diets; namely, untreated lupine, debittered lupine, and casein at various protein levels. Protein levels were adjusted at the expense of starch in the diet. Composition of the experimental diets is shown in table one. Each kilogram of vitamin mixture contained the following vitamins, triturated in dextrose: (in grams) vitamin A conc (200,000 units / g), 4.5; vitamin D conc (400,000 units / g), 0.25; $\alpha$-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; riboflavine, 1.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; pyridoxine hydrochloride, 1.0; thiamine hydrochloride, 1.0; calcium pantothenate, 3.0; and (in micrograms) biotin, 20; folic acid, 90; and vitamin $B_{12}$, 1.35. The mineral mixture contained the following: (g / 100 g) ammonium alum, 0.009; calcium biphosphate, 11.28; calcium carbonate, 6.86; calcium citrate, 30.83; cupric sulfate, 0.008; ferric ammonium citrate, 1.526; magnesium sulfate, anhydrous, 3.83; manganese sulfate, 0.02; potassium chloride, 12,47; potassium iodide, 0.004; potassium phosphate, dibasic, 21.88; sodium chloride, 7.71; sodium fluoride, 0.05.

Table 1. Composition of Experimental Diets Fed to Rats

| Ingredients | Untreated lupine diet, g / kg | | | Debittered lupine diet, g / kg | | | Casein diet g / kg | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Percent Protein in the Diet | | | | | | | | | |
| | 10 | 15 | 20 | 10 | 15 | 20 | 3 | 10 | 15 | 20 |
| Casein | | | | | | | 30 | 100 | 150 | 200 |
| Untreated lupine | 250 | 375 | 500 | | | | | | | |
| Debittered lupine | | | | 250 | 375 | 500 | | | | |
| Corn starch | 600 | 475 | 350 | 600 | 475 | 350 | 820 | 750 | 700 | 650 |
| Corn oil | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Vitamin mixture[1] | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Mineral mixture[2] | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |

1. Vitamin Diet Fortification Mixture; Product of Nutritional Biochemicals Corporation. Cleveland, Ohio.

2. Mineral Mixture U.S.P. XIV; Product of General Biochemicals Incorporation. Chagrin Falls, Ohio.

The lupine seeds were debittered by boiling them in water for 30 minutes, followed by soaking in running water for several days till no bitterness could be tasted.

Weight gain and food intake of the animals was recorded weekly.

For the breeding experiments the male and female rats were caged together for a period of 10 days, after which they were separated, the males sacrificed and the females kept for an additional month. The number of puppies, if any, was recorded.

For the toxicity experiments, the crude alkaloid extract was dissolved in an isotonic (1.15%) potassium chloride solution, and injected intraperitoneally into the animals by means of sterile, plastic, disposable syringes.

Rats, when not on an experimental diet, were fed the laboratory stock diet.[1]

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1. VITASNI, cow chow, produced by the National Industrial and Commercial Company, Beirut.

## IV. RESULTS AND DISCUSSION

### Chemical Studies

The proximate composition of the untreated lupine seeds is shown in table 2. It can be seen that the protein content of lupine seeds of 37.8% is higher than most other legume seeds, thus making lupine seeds a potentially rich source of dietary protein. Also, the fiber content of the seeds seems relatively high, although most legume seeds contain higher amounts of fiber than other foods. Analysis indicated that debittering the seeds did not incur any changes in their protein content.

Table 2. Proximate composition of untreated lupine seeds

| Constituents | Percentage |
|---|---|
| Water | 7.3 |
| Protein | 37.8 |
| Fat | 8.9 |
| Ash | 3.8 |
| Fiber | 10.9 |
| Nitrogen free extract | 31.3 |

The crude alkaloid content of local lupine is shown in table 3. The average value of 12 determinations indicates that local lupine seeds

Table 3. Crude alkaloid content of lupine seeds

| Weight of sample g | Weight of crude alkaloid extract g | Percent crude alkaloid extract in seeds |
|---|---|---|
| 25.0 | 0.25 | 1.00 |
| 25.0 | 0.30 | 1.20 |
| 25.0 | 0.32 | 1.28 |
| 25.0 | 0.26 | 1.04 |
| 25.0 | 0.26 | 1.04 |
| 25.0 | 0.30 | 1.20 |
| 25.0 | 0.31 | 1.24 |
| 25.0 | 0.33 | 1.32 |
| 25.0 | 0.33 | 1.32 |
| 25.0 | 0.37 | 1.42 |
| 32.9 | 0.40 | 1.22 |
| 29.0 | 0.40 | 1.37 |
| Average | | 1.22 |

contain 1.22% of alkaloids. Values reported in the literature for the alkaloid content of the seeds vary between 0.01 and 4.26%, while Abu Chaar (1964) reported that Lupinus termis contain 2.075-2.34% of total alkaloids calculated as lupanine. The difference in the results observed might be due to different varieties used.

Results of the separation and identification of the alkaloids in lupine seeds by thin layer chromatography are shown in table 4.

Table 4. Identification of alkaloids in lupine seeds by thin layer chromatography

| Substance | Rf value x100 | | Identified alkaloid |
|---|---|---|---|
| **Standards** | Run 1 | Run 2 | |
| Lupanine | 57 | 52 | |
| Hydroxylupanine | 32 | 30 | |
| Cytisin | 28 | 27 | |
| Spartein | 23 | 23 | |
| **Alkaloid Extract** | | | |
| Spot A | 57 | 51 | Lupanine |
| Spot B | 32 | 29 | Hydroxylupanine |

The alkaloids identified in the crude extract were lupanine and hydroxylupanine. Since lupanine showed a more intense spot on the chromatogram, it can be said that lupanine is the major component in the alkaloid extract. Hydroxylupanine appears to be present, but in lower concentrations, since it gives a faint spot on the chromatogram. Also, overlaping of the spots of hydroxylupanine and cytisin standards was observed on the thin layer chromatogram. This suggests that cytisin is a probable component of the extract. This can be established when a better separation of the two alkaloids can be achieved. Abu Chaar (1967) has reported that the alkaloid extract from Lupinus termis seeds contains lupanine in highest concentrations and hydroxylupanine in smaller concentrations. The presence of other alkaloids in concentrations still smaller than that of hydroxylupanine are also suspected.

The solubilities of pure lupanine and *in vitro* formed lupanine-iron complex in various laboratory solvents were determined in an attempt to find a solvent in which the complex would be insoluble and thus could be separated from pure lupanine. The results are shown in table 5. It can be seen that lupanine was soluble in chloroform while the lupanine-iron complex was insoluble. Thus before its identification, the lupanine-iron complex was thoroughly washed with chloroform to remove any adhering lupanine. In the test for the formation of an alkaloid-iron complex *in vitro*, a methanolic solution of crude alkaloid extract or lupanine was mixed with a 5% methanolic solution of ferric chloride. Identification of the brown precipitate formed was done by thin layer chromatography, after adequate washings with chloroform. The results, shown in table 6, indeed suggest the formation of an alkaloid-iron complex.

The formation of an alkaloid-iron complex *in vivo* following intraperitoneal injections into rats of crude alkaloid extract could not be established using the same identification procedure used for the *in vitro* experiment. However, penetration of the alkaloids into the tissues of various organs of the rat, within 30 minutes after injection, was observed. The results are shown in table 7. It can be seen from these results that lupanine was present in all the internal organs tested, except the brains, where transportation of lupanine was probably prevented by the blood-brain barrier.

Nutritional Studies

The high alkaloid content of 1.22% of the seeds of lupine, was thought to have a detrimental effect on rat growth. Accordingly,

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Table 5. Solubility[1] of lupanine and *in vitro* formed lupanine-iron complex in various laboratory solvents.

| Solvent | Solubility of lupanine | Solubility of lupanine-iron complex |
|---|---|---|
| Acetone | S | S |
| Benzene | I | I |
| Butanol | S | S |
| Carbontetrachloride | sS | sS |
| Chloroform | S | I |
| Dioxane | S | S |
| Ethyl ether | I | I |
| Ethylene glycol | S | S |
| Glycerol | S | S |
| Isoamyl alcohol | S | S |
| Methanol | S | S |
| Methyl cellusolve | S | S |
| Octanol | S | S |
| Pyridine | S | S |
| Toluene | S | S |
| 5% Trichloro acetic acid | S | S |
| Water | S | S |
| Xylene | S | I |

1. S = soluble; I = insoluble; sS = slightly soluble

Table 6. In vitro formation of lupanine-iron complex; identification by thin layer chromatography.

| Substance | Rf value x100 | | Identified alkaloid |
|---|---|---|---|
| | Run 1 | Run 2 | |
| Standards | | | |
| Lupanine | 52 | 42 | |
| Hydroxylupanine | 29 | 15 | |
| Cytisin | 27 | | |
| Spartein | 23 | | |
| Crude alkaloid extract | | | |
| Spot A | 51 | 41 | Lupanine |
| Spot B | 29 | 15 | Hydroxylupanine |
| Lupanine-iron complex | | | |
| Spot A | 51 | 38 | Lupanine |
| Alkaloid-iron complex | | | |
| Spot A | 50 | 39 | Lupanine |
| Spot B | 29 | 15 | Hydroxylupanine |

feeding experiments were carried out to investigate the effects of feeding untreated and debittered lupine diets on the health and growth of rats. The results of these experiments are shown in tables 8 to 10, and figure 1.

In all experiments, rats offered the untreated lupine diets lost weight, while those fed the debittered lupine diets gained some weight during the experimental period. Rats fed the control casein

diet, at similar protein levels, gained considerably more weight than the debittered lupine fed group, and showed normal growth, as shown in table 8.

Table 7. Presence of lupanine in rat tissues following intraperitoneal injections of crude alkaloid extract[1].

| Tissue analyzed | Number of rats in which lupanine was identified[2]. |
|---|---|
| Heart | 4 |
| Kidney | 10 |
| Spleen | 9 |
| Liver | 11 |
| Lung | 5 |
| Brains | None |
| Blood[3] | None |
| Ovaries or testes | 3 |

1. The alkaloid extract injected was dissolved in 0.15M potassium chloride.

2. Total number of rats examined was 13; identification was done by thin layer chromatography.

3. Blood was collected from the animals by the heart puncture technique.

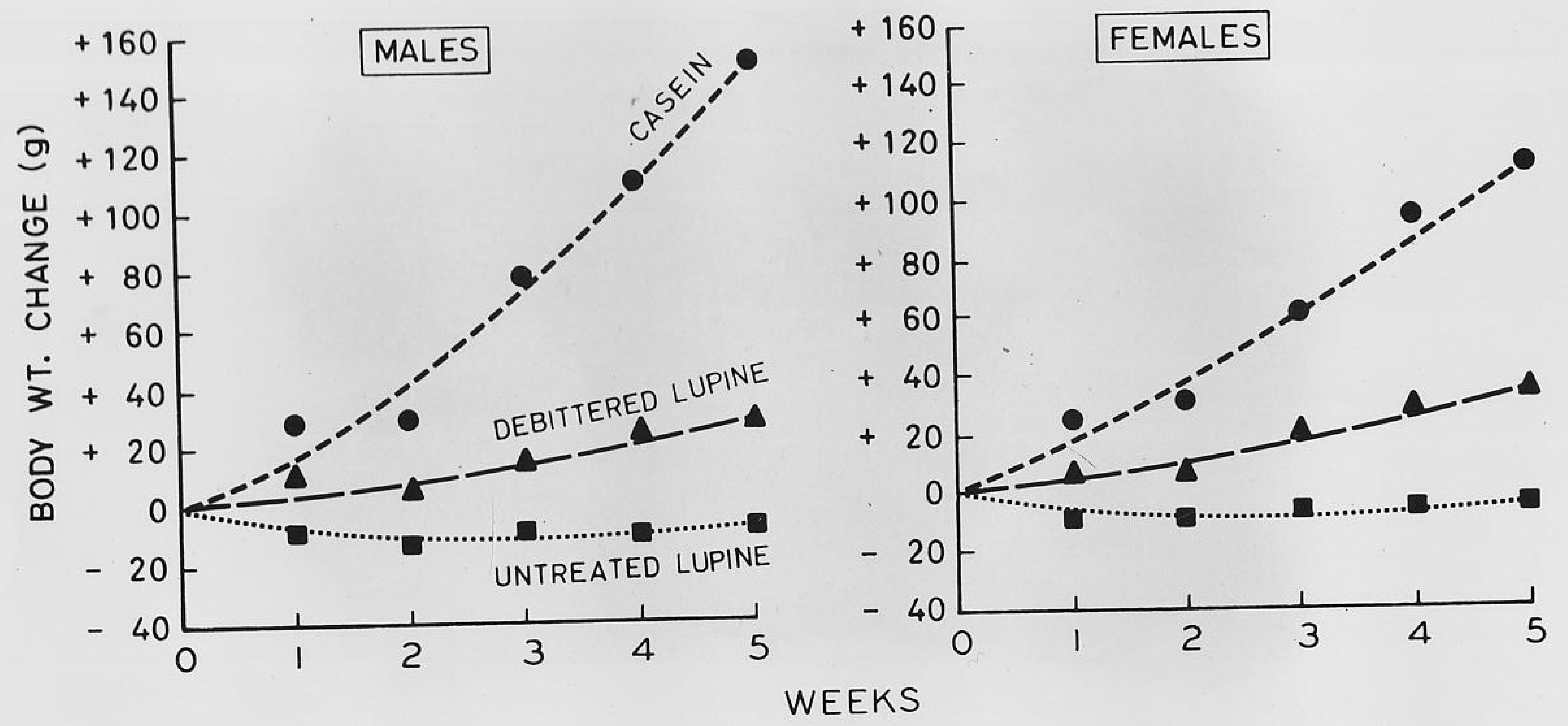


Fig. 1. EFFECT OF FEEDING 15 % PROTEIN LUPINE DIETS ON THE GROWTH OF RATS

Table 8. Effect of feeding 10% protein lupine diets on the growth of rats.

| Diet | Average[1] initial body weight, g | Average body weight change, g | | | | |
|---|---|---|---|---|---|---|
| | | First Week | Second Week | Third Week | Fourth Week | Fifth Week |
| Untreated lupine | 76 | -8 | -3 | -8 | -5 | -9 |
| Debittered lupine | 69 | 8 | 12 | 14 | 15 | 17 |
| Casein | 84 | -1 | 7 | 13 | 42 | 60 |

1. Average of four male rats per group; rats were six weeks old when started on the experimental diet.

In the second feeding experiment, the effect of feeding diets containing 15% protein from untreated lupine, debittered lupine and casein on the growth of male and female rats was investigated. Results, shown in table 9 and figure 1, indicate no appreciable difference in growth between male and female rats fed the same diets. The pattern in growth response of both sexes to the different diets at the 15% protein level was similar to that of rats fed the 10% protein diets. Rats fed the untreated lupine diet lost weight, while those fed the debittered lupine diet gained little weight, and the control casein fed groups gained weight considerably more than the debittered lupine fed groups.

The growth of rats fed a diet containing 15% protein from untreated lupine was comparable to the growth of rats fed a 3% casein diet, as shown in table 9.

In a third feeding experiment, the effect on rat growth observed

Table 9. Effect of feeding 15% protein lupine diets on the growth of rats.

| Diet | Sex | Average initial body weight g | Average body weight change, g | | | | |
|---|---|---|---|---|---|---|---|
| | | | First Week | Second Week | Third Week | Fourth Week | Fifth Week |
| 15% protein untreated lupine | Male | 80 | -9 | -14 | -10 | -11 | -9 |
| 15% protein debittered lupine | Male | 80 | 10 | 4 | 14 | 24 | 28 |
| 15% casein | Male | 80 | 29 | 29 | 78 | 109 | 149 |
| 3% casein | Male | 80 | -15 | -20 | -13 | -15 | -18 |
| 15% protein untreated lupine | Female | 73 | -9 | -9 | -7 | -6 | -6 |
| 15% protein debittered lupine | Female | 73 | 7 | 7 | 20 | 28 | 34 |
| 15% casein | Female | 73 | 25 | 31 | 61 | 94 | 111 |
| 3% casein | Female | 73 | -7 | -15 | -13 | -11 | -16 |

1. Average of 8 rats per group; rats were four weeks old when started on experimental diets.

by increasing the protein level in the diets from 10 to 20% did not improve growth of rats fed the untreated lupine diets, as shown in table 10, since the increase in protein was accompanied by an increase in alkaloid content. Rats fed the debittered lupine diets, however, showed some improvement in growth when the protein level was raised from 10 to 20%, while rats fed the control casein diet showed considerably better growth, upon increasing the protein level in the diet.

Table 10. Effect of feeding 10 and 20% protein lupine diets on the growth of rats.

| Diet | Average[1] initial body weight g | Average body weight, change g | | | |
|---|---|---|---|---|---|
| | | First Week | Second Week | Third Week | Fourth Week |
| 10% casein | 38 | +12 | +34 | +44 | +59 |
| 20% casein | 47 | +35 | +80 | +113 | +161 |
| 10% protein untreated lupine | 51 | -2 | -10 | -9 | -15 |
| 20% protein untreated lupine | 54 | -3 | -7 | -7 | -14 |
| 10% protein debittered lupine | 55 | 0 | 0 | +8 | +2 |
| 20% protein debittered lupine | 74 | +8 | +18 | +32 | +17 |

1. Average of 5 weanling rats per group.

Thus, it is observed that feeding untreated lupine inhibits the growth of rats. Tannous et al. (1968) attributed this impairment in growth to the poor protein quality and high alkaloid content of the lupine seeds.

Since the growth inhibiting effect of untreated lupine diets was partially attributed to the high alkaloid content of the seeds, the toxic effects of injecting crude alkaloid extracts into rats, on the growth and health of rats was investigated.

Results of the effect of injecting sublethal doses of crude alkaloid extract on the growth of rats are shown in table 11. The results indicate that one sublethal injection, had no apparent growth depressing effect.

Results of the determination of the lethal dose of crude alkaloid extract injections to rats is shown in table 12. A dose of 20 mg alkaloid per 100 g body weight is apparently lethal to rats below 200 g in body weight, while 25 mg alkaloid per 100 g body weight is required to kill rats above 200 g in body weight. This would indicate perhaps that the toxic effect of the alkaloids decreases with an increase in the body weight of the rat. In all cases, however, mortality of the rats occurred within the 15 minutes following the injection and was preceeded by strong convulsions and extreme irritablity.

To investigate whether the diet fed to rats before the injections has an effect on the toxicity observed, a similar experiment was conducted in which rats were fed lupine diets before being injected with the alkaloid extract. The results are shown in table 13. It can be observed that the diet had no effect on the reaction of the rats to

the toxic dose, since rats in all groups died within 15 minutes following injections. It was noted, however, that female rats died quicker than male rats when injected with the same dose.

Table 11. Effect of injecting sublethal doses of crude alkaloid extract on the growth of rats[1].

| Amount of crude alkaloid extract injected mg[2] | Initial body weight of rat g | Alkaloid injected per 100 g body weight mg | Body weight gain in 8 days, g |
|---|---|---|---|
| None | 50 | None | 9 |
| 0.01 | 46 | 0.02 | 11 |
| 0.1 | 46 | 0.22 | 10 |
| 1.0 | 47 | 2.1 | 2 |
| 2.0 | 77 | 2.6 | 31 |
| 4.0 | 79 | 5.1 | 8 |
| 6.0 | 59 | 10.1 | 24 |
| 6.0 | 126 | 4.8 | 6 |
| 8.0 | 68 | 11.8 | 31 |
| 8.0 | 106 | 7.5 | 6 |
| 9.0 | 135 | 6.7 | 13 |
| 1.0 | 180 | 0.55 | 19 |
| 5.0 | 184 | 2.72 | 0 |
| 10.0 | 226 | 4.43 | 27 |

1. Female rats kept on stock diet after weaning.

2. Administered by intraperitoneal injection, in 1 ml of .154M potassium chloride.

Table 12. Determination of lethal dose of injecting crude alkaloid extract in rats[1].

| Dosage mg alkaloid extract per body weight g | Sex | Body Weight of rat g | Observations |
|---|---|---|---|
| 2/100 | Male | 152 | No apparent effect |
| 2/100 | Male | 194 | No apparent effect |
| 2/100 | Female | 167 | No apparent effect |
| 2/100 | Female | 258 | No apparent effect |
| 10/100 | Male | 174 | No apparent effect |
| 10/100 | Male | 177 | No apparent effect |
| 10/100 | Male | 191 | Convulsions, death in 3 minutes |
| 10/100 | Female | 175 | No apparent effect |
| 10/100 | Female | 187 | Convulsions, no death |
| 10/100 | Female | 211 | No apparent effect |
| 20/100 | Male | 60 | Convulsions, death in 15 minutes |
| 20/100 | Male | 98 | Convulsions, death in 10 minutes |
| 20/100 | Male | 163 | Convulsions, death in 6 minutes |
| 20/100 | Male | 181 | Convulsions, no death |
| 20/100 | Male | 187 | Convulsions, death in 7 minutes |
| 20/100 | Male | 200 | Convulsions, no death |
| 20/100 | Male | 205 | Convulsions, no death |
| 20/100 | Male | 225 | Convulsions, no death |
| 20/100 | Female | 198 | Convulsions, death in 4 minutes |
| 20/100 | Female | 208 | Convulsions, no death |
| 20/100 | Female | 213 | Convulsions, death in 3 minutes |
| 25/100 | Male | 210 | Convulsions, death in 9 minutes |
| 25/100 | Male | 213 | Convulsions, death in 6 minutes |
| 25/100 | Female | 159 | Convulsions, death in 5 minutes |
| 25/100 | Female | 180 | Convulsions, death in 4 minutes |
| 25/100 | Female | 183 | Convulsions, death in 3 minutes |
| 25/100 | Female | 187 | Convulsions, death in 7 minutes |

1. Alkaloid extract injected intraperitoneally in 0.154M potassium chloride.

Table 13. Toxicity of crude alkaloid extract injections on rats prefed lupine diets[1].

| Diets[2] | Sex | Body weight range[3], g | Observations |
|---|---|---|---|
| Untreated lupine | Male | 114-298 | Convulsions and death in 5 to 10 minutes |
| Debittered lupine | Male | 146-319 | Convulsions and death in 4 to 14 minutes |
| Casein | Male | 282-329 | Convulsions and death in 5 to 8 minutes |
| Untreated lupine | Female | 133-272 | Convulsions and death in 3 to 5 minutes |
| Debittered lupine | Female | 90-184 | Convulsions and death in 2 to 5 minutes |
| Casein | Female | 275-287 | Convulsions and death in 3 to 5 minutes |

1. Each rat was injected introperitoneally at the end of the 5 week feeding period with 25 mg of crude alkaloid extract per 100 g body weight.

2. All diets contained 10% protein supplied by lupine or casein.

3. Range of weights of 5 rats per group.

Preliminary experiments in this laboratory indicate that an impairment in spermatogenesis of male rats fed untreated lupine diets may be observed. Thus as experiment to investigate the effects of feeding diets on the reproductive ability of male and female rats was carried out. The results are shown in tables 14 to 16. It was observed

Table 14. Effect of feeding lupine diets on the reproductive ability of male and female rats[1].

| No. of pair | Diet of male before mating | Diet of female before mating | Diet during mating | No. of puppies |
|---|---|---|---|---|
| 1 | 15% protein, untreated lupine | 15% protein, untreated lupine | 15% protein, untreated lupine | None |
| 2 | 15% protein, untreated lupine | 15% protein, untreated lupine | 15% protein, untreated lupine | None |
| 3 | 15% protein, untreated lupine | 15% protein, untreated lupine | 15% protein, untreated lupine | None |
| 4 | 15% protein, untreated lupine | 15% protein, untreated lupine | 15% protein, untreated lupine | None |
| 5 | 15% protein, debittered lupine | 15% protein, debittered lupine | 15% protein, debittered lupine | None |
| 6 | 15% protein, debittered lupine | 15% protein, debittered lupine | 15% protein, debittered lupine | None |
| 7 | 15% protein, debittered lupine | 15% protein, debittered lupine | 15% protein, debittered lupine | None |
| 8 | 15% protein, debittered lupine | 15% protein, debittered lupine | 15% protein, debittered lupine | None |
| 9 | 15% Casein | 15% casein | 15% casein | None |
| 10 | 15% Casein | 15% casein | 15% casein | 8 |
| 11 | 15% casein | 15% casein | 15% casein | 9 |
| 12 | 15% casein | 15% casein | 15% casein | 9 |
| 13 | 3% casein | 3% casein | 3% casein | None |
| 14 | 3% casein | 3% casein | 3% casein | None |

1. Weanling rats were kept on experimental diets for 2 months; rat weight varied between 62 to 229 g.

Table 15. Effect of feeding lupine diets on the reproductive ability of male rats[1].

| No. of pair | Diet of male before mating | Diet of female before mating | Diet during mating | No. of puppies |
|---|---|---|---|---|
| 1 | 15% protein untreated lupine | 15% casein | stock diet | none |
| 2 | 15% protein untreated lupine | 15% casein | stock diet | none |
| 3. | 15% protein untreated lupine | 15% casein | stock diet | none |
| 4 | 15% protein untreated lupine | 15% casein | stock diet | none |
| 5 | 15% protein debittered lupine | stock diet[2] | stock diet | none |
| 6 | 15% protein debittered lupine | stock diet[2] | stock diet | none |
| 7 | 15% protein debittered lupine | stock diet[2] | stock diet | none |
| 8 | 15% protein debittered lupine | stock diet[2] | stock diet | none |

1. Weanling rats were kept on experimental diets for 2 months; rat weight between 62 and 229 g.

2. Cow chow, Vitasni, produced by the National Industrial and Commercial Company, Beirut.

Table 16. Effect of feeding lupine diets on the reproductive ability of female rats[1].

| No. of pair | Diet of male before mating | Diet of female before mating | Diet during mating | No. of puppies |
|---|---|---|---|---|
| 1 | 15% casein | 15% protein untreated lupine | stock diet | none |
| 2 | 15% casein | 15% protein untreated lupine | stock diet | 5 |
| 3 | 15% casein | 15% protein untreated lupine | stock diet | 10 |
| 4 | 15% casein | 15% protein untreated lupine | stock diet | none |
| 5 | stock diet[2] | 15% protein debittered lupine | stock diet | none |
| 6 | stock diet[2] | 15% protein debittered lupine | stock diet | none |
| 7 | stock diet[2] | 15% protein debittered lupine | stock diet | 10 |
| 8 | stock diet[2] | 15% protein debittered lupine | stock diet | none |

1. Weanling rats were kept on experimental diets for 2 months; rat weight varied between 67 and 184 g.

2. Cow chow, Vitasni, produced by the National Industrial and Commercial Company, Beirut.

that reproduction was impaired when both males and females or when only males were fed the untreated or debittered lupine diets, as shown in tables 14 and 15. The impairment, however, was only partial, when the same diets were fed only to female rats as shown in table 16. It should be noted that complete impairment was also observed when 3% casein diets are fed to male and female rats. The average body weights, just before mating, of the rats fed the experimental diets were as follows: untreated lupine fed rats, 69 g, debittered lupine fed rats, 100 g, 15% casein fed rats, 207 g, and the 3% casein fed rats, 60 g.

It can be said then, that the detrimental effect on reproduction was more pronounced on males than on females, and can not be attributed to the alkaloid content of the lupine seeds, since debittering the seeds did not alleviate the effect. The effect might be attributed to the poor growth of rats observed with the experimental diets, since rats fed 3% casein diets also showed similar effects. Columbus (1935) reported that reproduction was impossible when rats were fed 100% lupine diets.

Chemical studies with lupine alkaloids showed the possible formation of an alkaloid-iron complex _in vitro_. The _in vivo_ formation of the complex, however, could not be established. It was postulated, that if an alkaloid-metal complex is formed _in vivo_ in rats fed untreated lupine diets, containing alkaloids, then the rats would show a decrease in the activity and concentration of the metal linked enzymes and proteins such as alkaline phosphatase and hemoglobin, since the metal may be chelated to the alkaloid and thus become unavailable.

To test the validity of this postulate, the effect of feeding

lupine diets on the intestinal alkaline phosphatase activity and serum hemoglobin levels of rats was investigated. Results of the intestinal alkaline phosphatase activity are shown in table 17. Analysis, using the Student's t-test, indicated that at both the 10 and 20% protein levels in the diets, rats on the untreated and debittered lupine diets had, at the 0.1 level of significance, lower intestinal alkaline phosphatase activity than the casein fed control groups. However, no significant difference, at the 0.1 level, was observed between rats fed the untreated and debittered lupine diets. Further, the results were analyzed after combining values for the 10 and 20% protein diets, so that instead of six groups, three groups were compared, namely, the untreated lupine group, the debittered lupine group and the control casein group. Analysis indicated that, at the 0.05 level of significance, the alkaline phosphatase activity of the groups fed the untreated lupine diet and the debittered lupine diet was significantly lower than that of the control group. However, there was no significant difference, even at the 0.1 level, between the rats fed untreated and debittered lupine diets.

It is concluded, that the decrease in intestinal alkaline phosphatase activity was not due to the alkaloid content of the seeds, since debittering did not change the results, but probably due to the poor growth of the rats fed lupine diets.

Results of the effect of feeding lupine diets on the serum hemoglobin levels of rats are shown in table 18. Analysis, using the Student's t-test, indicated that at the 10% protein level, hemoglobin levels in rats fed the untreated and debittered lupine diets was, at the 0.1 level of significance, lower than the level in rats fed the

Table 17. Effect of feeding lupine diets on intestinal alkaline phosphatase activity of rats.

| Diet | Average[1] body weight g | Average intestinal alkaline phosphatase activity measured in Micromoles p-nitrophenol produced per minute per g wet cell weight |
|---|---|---|
| 10% casein | 94 | 33.7 |
| 10% protein untreated lupine | 36 | 13.4 |
| 10% protein debittered lupine | 45 | 15.4 |
| 20% casein | 201 | 27.4 |
| 20% protein untreated lupine | 47 | 13.4 |
| 20% protein debittered lupine | 101 | 12.4 |

1. Average of 4 rats per group.

Table 18. Effect of feeding lupine diets on hemoglobin level in rats.

| Diet | Average[1] body weight g | Average hemoglobin percent |
|---|---|---|
| 10% Casein | 94 | 15.6 |
| 10% protein untreated lupine | 36 | 13.6 |
| 10% protein debittered lupine | 45 | 13.1 |
| 20% casein | 201 | 14.6 |
| 20% protein untreated lupine | 47 | 13.9 |
| 20% protein debittered lupine | 101 | 14.8 |

1. Average of 4 rats per group.

control casein diet. However, the difference between the hemoglobin levels of the untreated and debittered lupine fed rats was not significant. At the 20% protein level, the hemoglobin level of rats fed the experimental diets was not significantly different, even at the 0.1 level, from that of the casein fed control group. Again results of combining groups fed the same diets, namely, untreated lupine, debittered lupine and casein, indicated a significant difference, at the 0.05 level, between the untreated lupine fed group and the casein fed control group, while no significant difference was observed, even at the 0.1 level, between the untreated and debittered lupine fed groups, and between the debittered lupine and casein fed groups.

These results suggest again that, similar to the previous results, the decrease in hemoglobin level in the blood of rats fed lupine diets is probably not due to the alkaloids since no significant difference was observed between the untreated and debittered lupine fed groups.

In general, it can be observed from the results of the experiments with rats, that the detrimental effects observed on reproduction, alkaline phosphatase activity and hemoglobin level appear to be due to the poor growth of rats fed lupine or low protein diets, rather than to the alkaloid content of untreated lupine seeds. However, the lupine alkaloids appear to have had an inhibiting effect on growth of rats as well as be lethal when injected in certain doses.

## V. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS


### Summary

Protein constituted 37.8%, and alkaloids 1.22% of the total weight of untreated lupine seeds. The crude alkaloid extract had lupanine as its major component. The formation of an iron-alkaloid complex *in vitro* was observed but was not shown to occur *in vivo*.

Feeding untreated and debittered lupine diets to rats showed that untreated lupine diets inhibit growth of rats. Debittering the seeds alleviated the effect to a limited extent. The inhibition of growth apparantly was due not only to the high alkaloid content, but also to the poor protein quality of lupine seeds.

Intraperitoneal injections of crude alkaloid extract were found to be lethal to rats. The resistance of rats to this toxic effect appears to increase with an increase in rat weight, and does not change by prefeeding the animals with lupine diets. It was found that 20 mg of alkaloid per 100 g of body weight were lethal to rats below 200 g in weight, while 25 mg per 100 g body weight were lethal to rats above 200 g in weight. Injections of crude alkaloid extract, when not lethal, did not affect rat growth. Death of rats occurred in all cases within the 15 minutes following the injections.

The reproductive ability of rats, particularly males, fed untreated and debittered lupine diets was below normal. The impairment of reproduction does not appear to be due to the alkaloid content of the seeds, since debittering did not alleviate the effect, but due to the poor growth of rats fed the lupine diets.

Intestinal alkaline phosphatase activity and hemoglobin levels in rats fed untreated and debittered lupine diets were lower than the controls. The decrease, again, does not seem to be due to the high alkaloid content, since debittering the seeds does not eliminate it, but to the poor growth of rats fed the lupine diets.

## Conclusion

Debittering of lupine seeds to remove their alkaloids, and supplementing with other proteins or the proper amino acids to improve their protein quality, are necessary processes, before lupine seeds can be safely used as a protein source for animal or human consumption.

## Recommendations

Further investigations are needed to elucidate the physiological effects of consumed lupine alkaloids in animals.

Studies on the absorption and excretion of lupine alkaloids by animals will help provide more information on the toxicity of alkaloids as effected by the method of administration.

The technique of pair feeding control animals with animals fed lupine and low protein diets should be useful to clarify further the cause of the detrimental physiological effects observed in this study. It may provide information on whether lupine alkaloids, or the poor growth of animals fed the lupine and low protein diets, are responsible for the observed effects.

It is also recommended that debittered lupine seeds be tested nutritionally on animals as a source of protein in food mixtures containing other dietary proteins.

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