Characterization of non-polar compounds from Pinus ponderosa needles causing reproductive failure in mice during early gestation
by Yolanta Miroslawa Kubik

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Biochemistry
Montana State University

Abstract:
Ingestion of hexane extracts of Pinus ponderosa needles causes reproductive failure in mice during early stages of gestation. Virgin mice of ICR strain were mated with a male of proven fertility. The day the copulatory plug was observed was designated day 1 of gestation. The hexane extracts were administered daily via stomach tube on day 1-5 of gestation. Implantation sites were stained by injection of pontamine sky blue dye on day 8 of gestation, 15 minutes before sacrifice. Interrupted implantation and embryonic resorption were observed in the uterus. The extract was found to be most effective around the time of implantation (day 5 of gestation). Through chromatographic analysis the active compounds were identified as non-conjugated diterpene resin acids: pimaric--0.8%, isopimaric--58.2%, and sandaracopimaric--3.3%. Ingestion of the above mixture of non-conjugated diterpene resin acids of ponder-osa pine needles during early gestation result in failure to maintain implantation and subsequent embryonic resorption in mice.

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CHARACTERIZATION OF NON-POLAR COMPOUNDS FROM *PINUS PONDEROSA* NEEDLES CAUSING REPRODUCTIVE FAILURE IN MICE DURING EARLY GESTATION

by

Yolanta Miroslawa Kubik

A thesis submitted in partial fulfillment
of the requirements for the degree

of

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in

Biochemistry

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ABSTRACT

Ingestion of hexane extracts of Pinus ponderosa needles causes reproductive failure in mice during early stages of gestation. Virgin mice of ICR strain were mated with a male of proven fertility. The day the copulatory plug was observed was designated day 1 of gestation. The hexane extracts were administered daily via stomach tube on day 1-5 of gestation. Implantation sites were stained by injection of pontamine sky blue dye on day 8 of gestation, 15 minutes before sacrifice. Interrupted implantation and embryonic resorption were observed in the uterus. The extract was found to be most effective around the time of implantation (day 5 of gestation). Through chromatographic analysis the active compounds were identified as non-conjugated diterpene resin acids: pimaric--0.8%, isopimaric--58.2%, and sandaracopimaric--3.3%. Ingestion of the above mixture of non-conjugated diterpene resin acids of ponderosa pine needles during early gestation result in failure to maintain implantation and subsequent embryonic resorption in mice.

INTRODUCTION

Pinus ponderosa (Western yellow pine) is a conifer that grows in the Western coastal states east through the Rocky Mountains, north to British Columbia, and south to Mexico. The needles are 5-11 inches long and grow in bundles of three or two and three.

Ponderosa pine needles have been reported to be poisonous to cattle, causing abortion during the late stages of gestation. "Pine needle abortion" as this phenomena is termed, has plagued ranchers for many years. In 1950, the Canadian Range Experimental Station in British Columbia was requested to determine if Pinus ponderosa needles were the causative agent in abortion in range beef cattle (MacDonald, 1950). Booth (1950) implies that non-infectious abortion was common in mountain areas abounding in ponderosa pine. This observation was substantiated by Halver (1953) when he observed that 31 out of 192 head of range heifers aborted when they had ingested ponderosa pine needles. Jacobsen (1952) reported that the "pine needle abortion" problem was responsible for numerous calf losses in nineteen, mostly mountainous, counties in Montana.

In 1959, Nicholson reported that consumption of ponderosa pine needles consistently caused abortion among range heifers. He also concluded that it was impossible to induce abortion with other species of conifers and confirmed that certain quantities of ponderosa pine needles would induce bovine abortion and/or the birth of stillborn or weak calves. Cows were found to be suffering from symptoms of starvation and were generally in poor physical condition.

It has been established experimentally that ponderosa pine needles do indeed cause bovine abortions, but more frequently the birth of weak calves that die shortly after birth (Gunn, 1949; Nicholson, 1957). At present, it is not known to what extent ponderosa pine needles are involved in the etiology of the"weak calf syndrome". Neither is it understood why cattle preferentially turn to ponderosa pine needles when other sources of forage are available.

In an attempt to elucidate the cause of pine needle induced abortion many investigators have turned to laboratory animals (rodents) as a biological assay system. Allen and Kitts (1961) examined several extracts (aqueous, ether and acetone) of ponderosa pine needles for estrogenic or anti-estrogenic effects on the uterus of the female

labortory mouse. They found the aqueous extract to contain anti-estrogenic compounds. The ether fraction had the greatest toxicity to adult mice and the acetone fraction resulted in the highest embryo mortality. In assessing the reproductive failure, it was observed that in all cases the mice showed signs of starvation. McClure (1959) reports that starvation alone can affect reproduction by inhibiting implantation.

Cook (1962) examined the antifertility aspects of "pine needle abortion", using acetone and ethyl acetate fractions of an aqueous extract of pine needles. The acetone fraction decreased uterine weight in immature females, but neither fraction caused reproductive failure.

In recent years, Chow, et al. (1972) have considered reproductive dysfunction in the female mouse due to ingestion of various extracts (volatile, acetone, and aqueous) of ponderosa pine needles. The acetone and volatile fractions have no effect on uterine weight or embryonic development. The aqueous extract, however, has a very dramatic effect (uterine wt./body wt. - aqueous .72 mg/g; control 1.00mg/g) on the uterine weight and also causes the greatest number of fetal deaths (11 when compared to two in the controls). This activity could be

destroyed by heating. The active compound(s) in ponderosa pine needles was concluded to be thermo-labile and water soluble.

Weideman (1973), in an attempt to further determine the active compound, extracted ponderosa pine needles by the method of Chow (1972) and administered the extract to immature rats supplemented with subcutaneous injections of estradiol in anticipation of uterine atrophy. Perplexing results were observed. Immature rats exhibited uterine and ovarian weights that were only slightly below that of controls. Some adult rats had functional corpora lutea while others did not. None of the necropsied animals exhibited signs of reproductive failure as described by other investigators. On the basis of this data Weideman concluded that he had not assayed the rats for a sufficient period of time. Weideman's inability to obtain similar results suggests to me that different types of compounds (ie. lipophilic) should be evaluated for their role in "pine needle abortion".

Cogswell (1974) evaluated the effects of ponderosa pine needles and extracts of pine needles on implantation, offspring viability and average litter size. Both aqueous and acetone extracts reduce average litter size. To

determine the critical period for disrupting embryonic development, Cogswell fed these same extracts on day 5 of gestation and observed that both extracts reduced the number of viable offspring and at a high concentration (2:1 ratio of extract to Purina Mouse Chow) both extracts disrupted embryonic development on day 6 or 7 of gestation. Mice lost weight, showed signs of gastric ulcerations and had blood filled implantation sites, suggesting embryonic resorption.

In addition to the reproductive failure aspects of "pine needle abortion", four other features have been considered: vitamin A deficiency, phosphorus deficiency, external parasites and toxic agents.

It was suggested that cattle turn to ponderosa pine neeldes as a source of vitamin A. After Muenscher (1945) concluded that range cattle did not find conifer needles palatable unless driven by starvation to eat them, Oh, et al. (1967) showed that monoterpene alcohols such as abinene, B-phellandrene, cis-acimene, and 1,8-cineole were unpalatable to browsing animals and had an inhibitory effect on the functioning of rumen microorganisms. Nicholson (1959) designed an experiment to address the hypothesis that pine needles are a source of vitamin A.

Using leptospirosis and brucellosis free heifers administered a ponderosa pine needle ration, he observed that one heifer aborted nine days after initiation of the feeding period. The other two animals gave birth to immature, weak calves which died shortly after birth. Blood vitamin A levels were analyzed to determine the effects of ponderosa pine needles on intestinal absorption. Results indicate a normal level of vitamin A in both experimental and control heifers.

During winter months range beef cattle maintained on near starvation rations frequently suffer from phosphorus deficiency which Rombouts and Guegen (1961) suggest may decrease fertility. The correlation between phosphorus deficiency and a pine needle craving was examined by Toner (1971). Results indicate that phosphorus deficient rats preferentially turn to pine needles as a source of phosphorus.

At this time it was suggested that external parasites (ie. fungi in the pine needles) could be responsible for the reproductive failure observed. Using an aqueous fungi, administered to mice on day 1 of gestation, Chow, et al. (1974) discovered that the extract of the fungi disrupted pregnancy, whereas the extract of the pine needles did not.

They concluded that the fungus in some manner converts the compounds in the needles to fetal-lethal materials. Utilizing "rejuvenated fungi", they demonstrated that fetal resorption occurred with ingestion of incubates of fungi, whereas the aqueous extract of the pine needles allowed for embryonic development. On the basis of this data, it was concluded that mycotoxins were the agents responsible for the reproductive dysfunction observed as opposed to compounds located in the needles.

In 1977, Anderson and Lozano investigated the toxicity characteristics associated with "pine needle abortion". Their results indicate that a mycotoxin is present in the aqueous incubates of pine needles. Necropsied mice showed no unusual organ cytology and therefore it was suggested that the greatest effect of ponderosa pine needle ingestion was on implantation. The extracted needles were thought to contain a water insoluble toxin that could be more toxic than the water soluble toxin.

In an attempt to return to the natural condition observed with range beef cattle, Neff, et al. (1979) administered a pine needle ration to mice from the ninth day of gestation until parturition. Results indicate a wasting syndrome, abortion and maternal death.

Adams, et al. (1979) in administering a pine needle ration to mice during late gestation (day 9-18) induced abortion and maternal death. In addition, a bacteria, Listeria monocytogenes, was isolated from the blood, uterus and cecum of the mice fed pine needle ration. In light of these results it was suggested that ponderosa pine needles in some manner enhance the growth of Listeria monocytogenes, a naturally occurring bacteria in the mouse.

Despite almost thirty years of investigations, the "pine needle abortion" problem is still not well understood. Although a number of aspects of this problem have been examined, the solution to this economic loss for the rancher remains unsolved. Some investigators feel that the compound responsible for the reproductive failure is located in the needles; others feel that it is a by-product of a pine needle fungus. Extensive experimentation utilizing an aqueous extract of pine needles suggests that an active compound is water soluble and thermo-labile.

Results have also suggested the possibility of lipophilic compounds being involved (Allen and Kitts, 1961 and Anderson and Lozano, 1977); however, until this study little work has been done in this area. A precedent has been established for lipophilic compounds being implicated in

reproductive problems (Farnsworth, et al. 1975), though not always in interruption of pregnancy during early gestation.

In lieu of this knowledge, the objectives of this thesis are to examine lipophilic molecules of ponderosa pine needles and evaluate their involvement in pine needle induced reproductive failure in laboratory mice during early gestation.

RESEARCH OBJECTIVES

This research study is divided into three areas of investigation:

1. Isolation of lipophilic material from ponderosa pine needles.

2. Demonstration of reproductive failure in mice due to ingestion of lipophilic compound(s) of ponderosa pine needles.

3. Purification and chemical identification of lipophilic compounds that are biologically active.

The purpose of this work is to isolate and identify the lipophilic compound(s) responsible for pine needle induced reproductive failure during early gestation in mice. With the knowledge of the identity of the active compound(s) responsible for pine needle induced reproductive failure during early gestation in mice, it might be possible in later studies to elucidate the mechanism by which this biological phenomena occurs. Once this was established, a method to counteract this affect could be developed thus solving the problem of economic loss for the rancher. Also, by understanding the mechanism involved, the active compound(s) could be utilized as a contra-

ceptive method during the early stages of gestation.

MATERIALS AND METHODS

Extraction of Pine Needles

Lipophilic compounds of the conifer Pinus ponderosa (collected from Grey Cliff, Montana) were obtained by cutting the needles into 1-2 cm lengths and extracting in hexane (3 ml/gm) for six hours on a mechanical shaker. This scheme (Figure 1) was repeated twice and the solvent was filtered and then cooled at 4°C for six hours. The resulting white precipitate that formed was filtered off utilizing a Buchner suction apparatus and the hexane was evaporated on a Brinkman/Buchi rotoevaporator (model Rotavapor-R) at 40°C. The extract, dissolved in hexane, was passed over a charcoal (Nuchar M.C.B.) column 2.2 cm in diameter and 5.1 cm in length. The solvent was evaporated and the extract stored in vials at 4°C until use.

Animals

Virgin female ICR mice (originally from Flow Laboratories, but bred and reared locally) weighing 28-32 grams, were placed two to a cage with a male of proven fertility and were examined daily for the presence of a copulatory plug (Parkes, 1926). The day the plug was observed was designated day 1 of gestation and treatments were initiated.

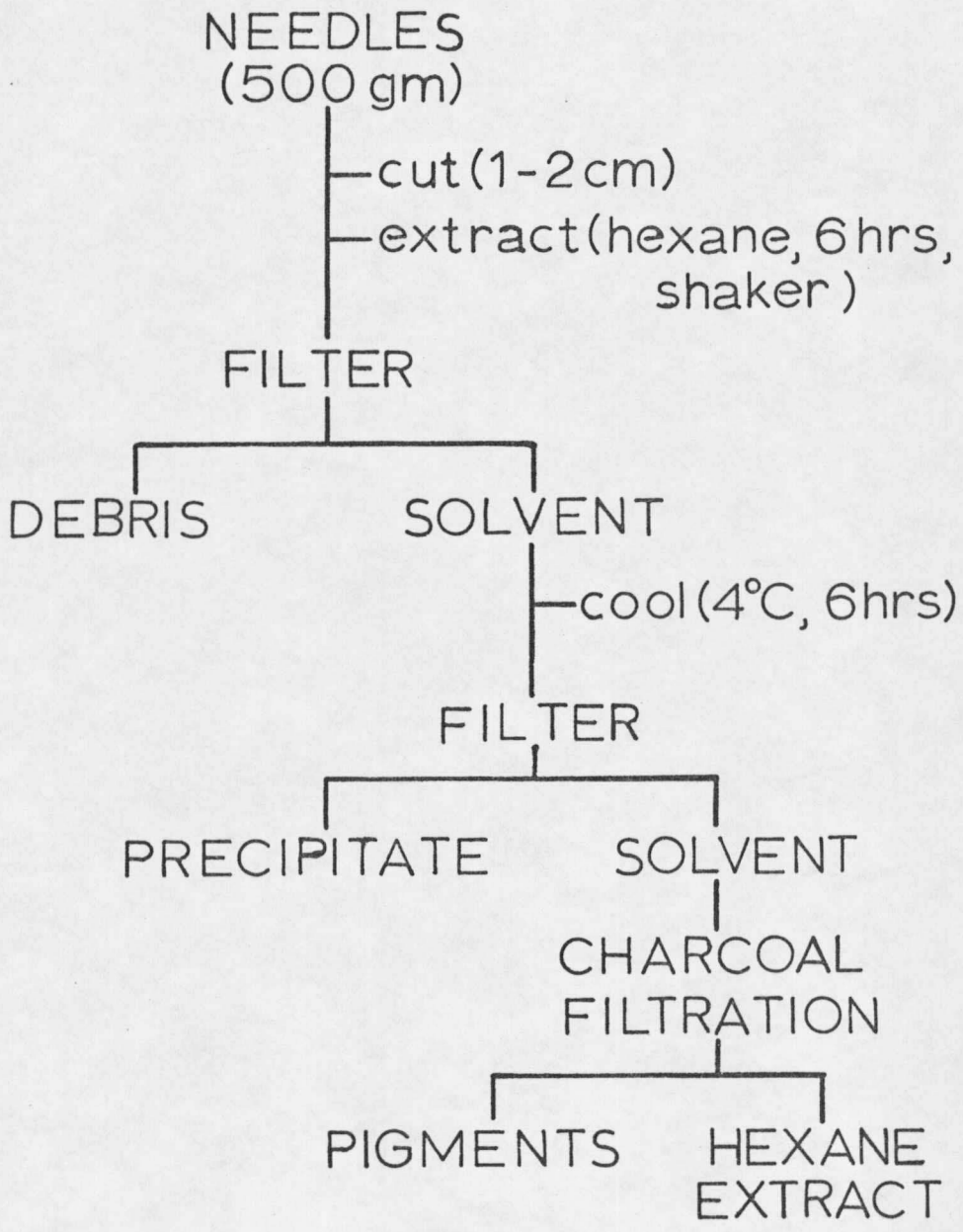


Fig.1 EXTRACTION SCHEME

On day 8 of gestation mice were sacrificed by cervical dislocation and uteri were excised and evaluated, by visual comparison to controls, for increased capillary permeability (positive pontamine blue reaction) and embryonic resorption. In this evaluation embryos smaller in size and weight and exhibiting no definition of form were considered to be in the process of resorption.

Pontamine Sky Blue Assay

Mice were injected with a 0.1 ml solution of 0.5% pontamine sky blue dye in 10% NaCl in the tail vein (Psychyos, 1961) to facilitate examination of the implantation site as displayed by increased capillary permeability. Fifteen minutes later mice were sacrificed and uteri were examined for the presence of implantation sites and resorptions as previously described.

Hexane Extract Ration

Hexane extract (500 grams of needles equivalent) was incorporated into lab chow at a 1:1 (weight equivalent of needles:weight of chow) ratio by dissolving the extract in 100 ml of diethyl ether and adding it to 300 grams of ground lab chow (Wayne Lab Blox). To this mixture was added 100 ml of distilled water and 100 ml of molasses

(unsulphured). The hexane extract ration was allowed to dry overnight, cut into pellets and administered ad lib on day 1-8 of gestation. Mice were allowed free access to water during the test period. Control mice had free access to lab chow and water.

Stomach Tube Administration

After establishing a bioassay system, the extract samples were dissolved in olive oil as a vehicle and administered via stomach tube at the concentrations and for the durations mentioned in results. Control mice received 0.2 ml per day of olive oil by stomach tube in addition to lab chow and water ad lib. The stomach tube apparatus consisted of a 1 cc Tuberculin syringe with a leur-lock tip, a B-D Yale 25G x 5/8" needle and a 4 cm length of 1 mm polyethylene tubing (Clay-Adams Intramedic).

Column Chromatography

Aliquots (3.0 grams) of hexane extract were eluted through an activated silica gel column having the following dimensions: total length 36.0 cm; with the top 9.0 cm having a diameter of 5.2 cm and the bottom 27.0 cm having a diameter of 3.0 cm. A slurry was prepared in hexane using 130 grams of Bio-Sil A (BioRad), 100-200 mesh.

Solvents utilized and fractions obtained are listed in results (Table 6).

Preparative thin layer chromatography was used to further purify the fractions obtained by column separation. The developing solvents were hexane:diethyl ether:acetic acid(75:25:1, v:v:v) or toluene:ethyl acetate (90:10, v:v).

Purified fractions were individually assayed for physiological activity.

Argentation Chromatography

The column fraction shown to interrupt implantation was further separated into two constituents by argentation thin layer chromatography. The 15% $AgNO_3$ plates were prepared by dissolving 5.6 gm of $AgNO_3$ crystals (J.T. Baker Chemical Company) in 85 ml of distilled water and adding this solution to 50 grams of silica (Adsorbosil-5, no binder, Applied Science Laboratories, Inc.). The slurry was applied to five glass plates(20 cm x 20 cm); when dry the silica was activated at 120°C for two hours.

The active column fraction (100-150 mg in hexane/plate) was applied to the silica and the plate was developed in hexane:diethyl ether:acetic acid (75:25:1, v:v:v). Bands were separated and eluted from the silica with toluene.

An aliquot (100 mg) of each of the constituents was removed for further chemical analysis. The remainder was assayed for biological activity.

Chemical Modification

The two constituents obtained by argentation chromatography were esterified with diazomethane as described by Schlenk and Gellerman (1960), with slight modification. Generation of diazomethane (yellow gas) was initiated by adding 0.1-0.5 grams of Diazald (Aldrich Chemical Company) to the generation flask. When the solution in the reaction flask turned yellow, the reaction was considered complete and the solvent was evaporated under N_2 gas. The esterified constituents were analyzed by TLC and GLC.

Gas-Liquid Chromatography

The esterified constituents were analyzed on a Varian (model 3700) gas chromatograph equipped with a flame ionization detector (FID) and a Varian (model 9176) recorder. Two different liquid phases were used for analysis. These were 0.9 meters of 15% DEGS on Gas Chrom-Z, 80-100 mesh, at 200°C with a flow rate of 50 ml/min and 2.0 meters of 3% Dexil-300 on Chromosorb-W, 80-100 mesh, at 225°C with a flow rate of 70 ml/min.

Preparative Gas-Liquid Chromatography

Constituents were purified in a Varian (model 3700) gas chromatograph, equipped with a thermal conductivity detector (TCD) and a Varian (model 9176) recorder. The column was 11 mm x 0.9 meters of 15% DEGS on Gas Chrom-Z, 80-100 mesh, operating at 200°C and a flow rate of 50 ml/min. Peaks were collected in glass capillaries, 93 mm in length and 3 mm in diameter, containing glass wool. Peaks were analyzed for completeness of separation by gas-liquid chromatography (FID).

Ultraviolet Spectroscopy

The ultraviolet spectra were obtained from solutions of unknowns in cyclohexane (spectroquality; Matheson, Colemen and Bell Company) using 1.0 cm cuvettes in a Varian Techtron (model 635) dual-beam scanning UV-VIS spectrophotometer equipped with a recorder. Slit width 1.0 nm; scan speed 50 nm/min: optical density range 0.0-1.0.

Mass Spectroscopy

The purified compounds were characterized by their mass spectra. The instrument used was a Varian (model CH-5) single focus mass spectrometer. The compounds were dissolved in ether and carefully applied to gold/brass crucibles,

which were introduced by direct insertion probe at a source temperature of 75°-100°C. Unit resolution spectra were recorded on light sensitive paper.

RESULTS

Extraction Capability of Non-Polar Solvents

Hexane, diethyl ether and acetone were evaluated for their extraction ability to determine which solvent would best extract the lipophilic compounds of ponderosa pine needles (Table 1). Ether was found to be more effective than acetone and equal to hexane in ability to extract lipophilic compounds. Hexane, however, extracted less of the more polar compounds than did ether and subsequently was utilized for pine needle extraction. After many extractions, it was determined that 19.6 ± 0.4 mg of extract are obtained per gram of needles extracted in this manner.

Reproductive Failure As Influenced By Hexane Extract

Hexane extract was incorporated into lab chow at a 1:1 (weight equivalent of needles:weight of chow) ratio and administered *ad lib* to mice on day 1-8 of gestation to determine if a hexane extract of ponderosa pine needles contained a compound(s) that caused reproductive failure in mice. Mice sacrificed on day 8 showed signs of normal capillary permeability, as evidenced by blue dye sites, marking the onset of decidualization (Glasser and Clark,

Table 1. Comparison of Extraction Capability of Solvents

Solvent	Treatment	gm extract/ gm needles
Hexane	whole; 2 x 15 min	.012
Ether	whole; 2 x 15 min	.011
Acetone	whole; 2 x 15 min	.006
Hexane	cut; 2 hours	.020
Ether	cut; 2 hours	.020
Acetone	cut; 2 hours	.018

1975). Implantation, however, had been interrupted and embryos were in the process of resorbing when compared to the control (Table 2). A few embryos had completely resorbed. This conclusion was reached on the basis of barren uteri containing dye sites indicative of implantation sites (Psychyos, 1973).

In addition, adult mice showed a 5.9 ± 0.9 gm loss of body weight while on the hexane extract ration.

Reproductive Failure As Influenced By Starvation

Mice were given 1.5 grams (established average chow consumption of mice receiving hexane extract ration) of lab chow/mouse/day on day 1-8 of gestation to determine if starvation (as suggested by McClure, 1959) was a factor in the interruption of implantation observed in mice receiving hexane extract ration. When sacrificed on day 8, these mice also showed signs of interrupted implantation and embryonic resorption when compared to controls. Table 3 shows a 58 ± 3% embryo loss and a weight gain by adult mice of 4.5 ± 1.1 grams during the treatment period. This may indicate that restriction of caloric intake can result in reproductive failure.

Table 2. Reproductive Failure Due to Hexane Extract Ration Ingestion

Treatment	Avg. Wt. of Mouse (gm) (Initial)	(Final)	Avg. Consumption per day (gm) *	% Embryo Loss**
Pine Needle Ration Day 1-8	27.1 ± 1.1	21.2 ± 1.0	1.5 ± 0.5	100 ± 3
Control (lab chow)	28.3 ± 1.0	36.3 ± 1.4	5.2 ± 0.6	3 ± 1

n = 10 mice in each group

* 20 mg of extract/1 gram of ration

** Embryo loss includes interrupted implantation and embryos in the process of resorbing.

Table 3. Reproductive Failure as Influenced by Starvation

Treatment	Avg. Wt. of Mouse (gm) (Initial)	(Final)	% Embryo Loss*
Lab Chow 1.5 gm/day Day 1-8	28.9 ± 1.3	33.4 ± 1.0	58 ± 3
Lab Chow 5.1 gm/day Day 1-8	28.7 ± 1.1	37.4 ± 1.1	4 ± 1

n = 10 mice in each group

* Embryo loss includes embryos whose implantation has been interrupted and which are in the process of resorbing.

Stomach Tube Administration Of Hexane Extract

The following study was designed to determine if administration of hexane extract by stomach tube could eliminate starvation as a source of the reproductive failure observed and to ensure that equivalent dosages of extract are ingested at the time of feeding by the test animals. The extract, dissolved in olive oil, was administered to mice at a dose of 4 gm/Kg/day on day 1-8 of gestation. The mice gained weight (Table 4) and did not display signs of starvation. Nevertheless, on day 8 of gestation, when sacrificed, mice exhibited a 97% embryo loss characterized by interrupted implantation and embryonic resorption.

From this it was concluded that despite normal caloric intake, mice administered hexane extract via stomach tube showed significant signs of interrupted implantation and embryonic resorption. This suggests that the hexane extract of ponderosa pine needles does contain lipophilic compound(s) that have a deleterious effect on early gestation in mice.

Dose vs. Response

After demonstration that ingestion of hexane extract

Table 4. Reproductive Failure As Influenced By Stomach Tube Administration of Hexane Extract

Treatment	Avg. Wt. of Mouse (gm) (Initial)	(Final)	% Embryo Loss*
Hexane Extract S.T.** Day 1-8	30.0 ± 1.3	39.4 ± 1.1	97 ± 2
Control Olive Oil S.T. Day 1-8	30.3 ± 1.0	38.9 ± 1.0	3 ± 1

n = 10 mice in each group

* % Embryo loss includes interrupted implantation, and resorbing embryos

** S.T. = administration via stomach tube (4 gm/Kg/day)

of ponderosa pine needles resulted in reproductive failure in mice, the dose vs. response relationship was determined. When the hexane extract is dissolved in an olive oil vehicle at various concentrations and administered on day 1-5 of gestation, the effect on embryo loss is minimal through 1.6 gm/Kg/day and then increases to a plateau at about 2.4 gm/Kg/day (Figure 2). The effective dose (ED_{50}), calculated statistically by the Reed-Meunch (1938) method, is 2.5 gm/Kg/day. However the data strongly suggest the presence of a threshold dosage.

The type of embryo loss, characterized by uneven spacing of blastocysts, interrupted implantation, and embryonic resorption is illustrated in figure 3b and in each case was compared to controls of which an example is shown in Figure 3a.

Duration of the Feeding Period vs. Response

The critical period for interrupting implantation in the mouse was determined by administration of the hexane extract at a concentration of 4 gm/Kg/day for several different durations (Table 5a and 5b). The two studies discussed below did not utilize a hexane extract from the same batch of needles.

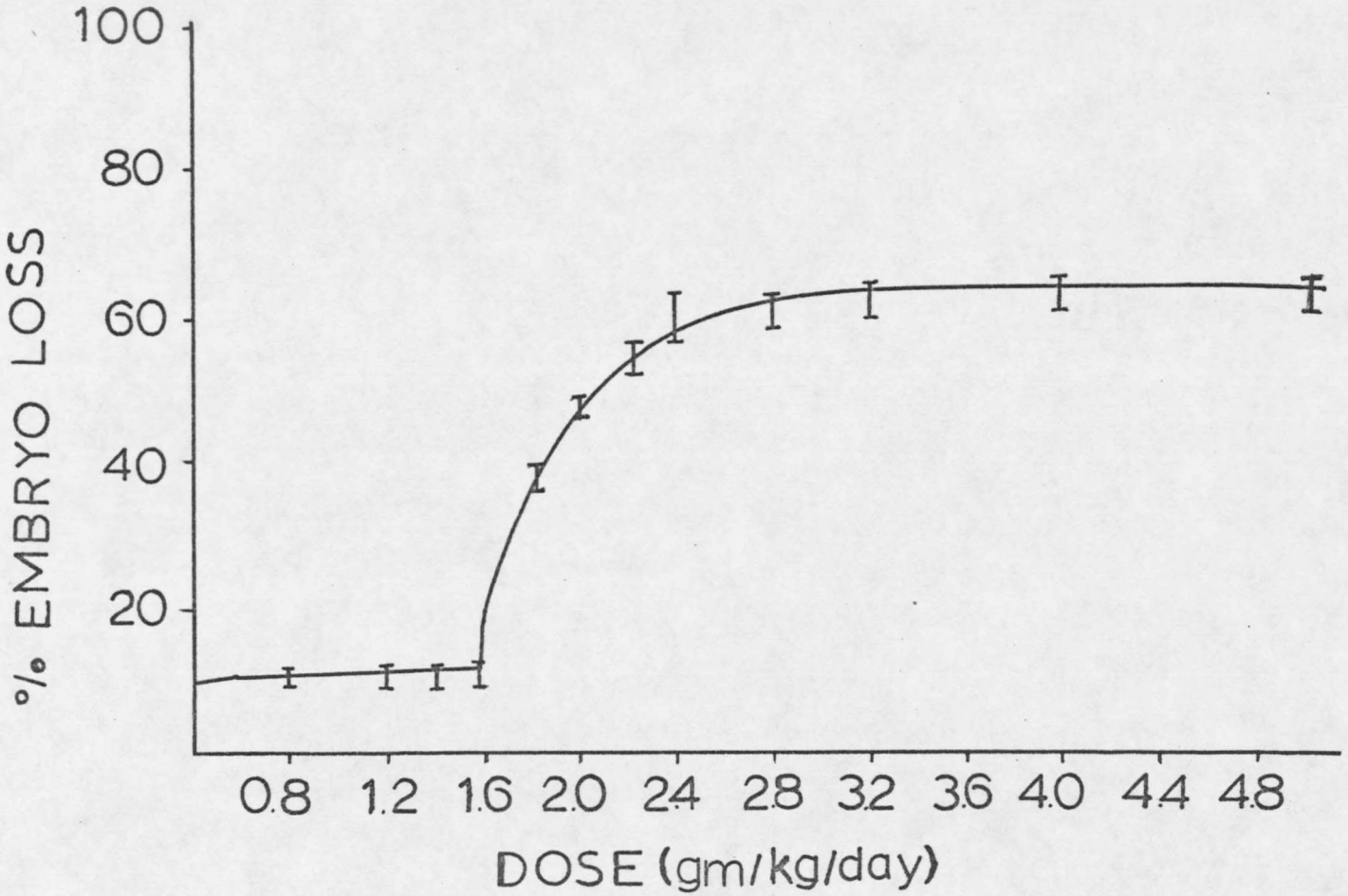


Fig.2 Dose vs Response Relationship (Day 1-5)

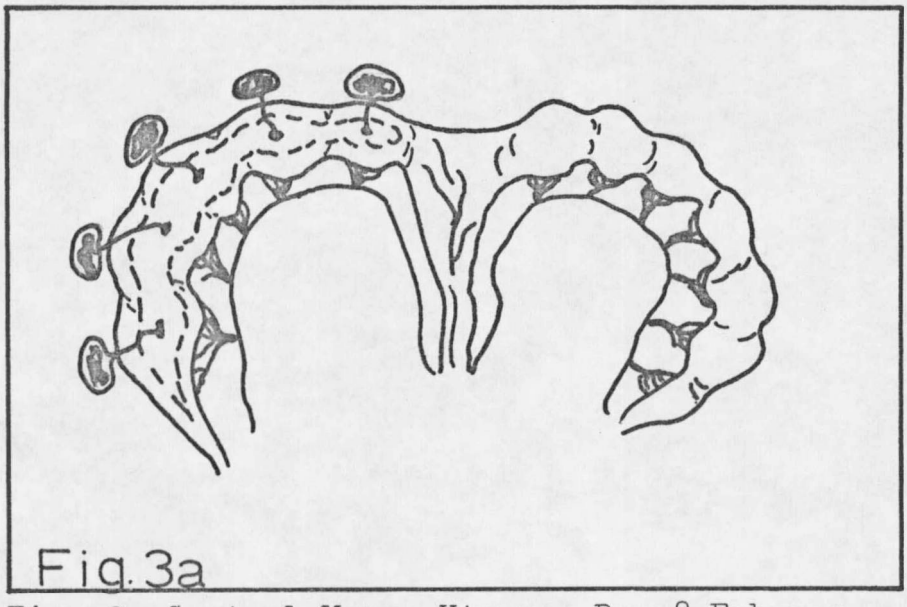


Fig. 3a.Control Mouse Uterus; Day 8 Embryos

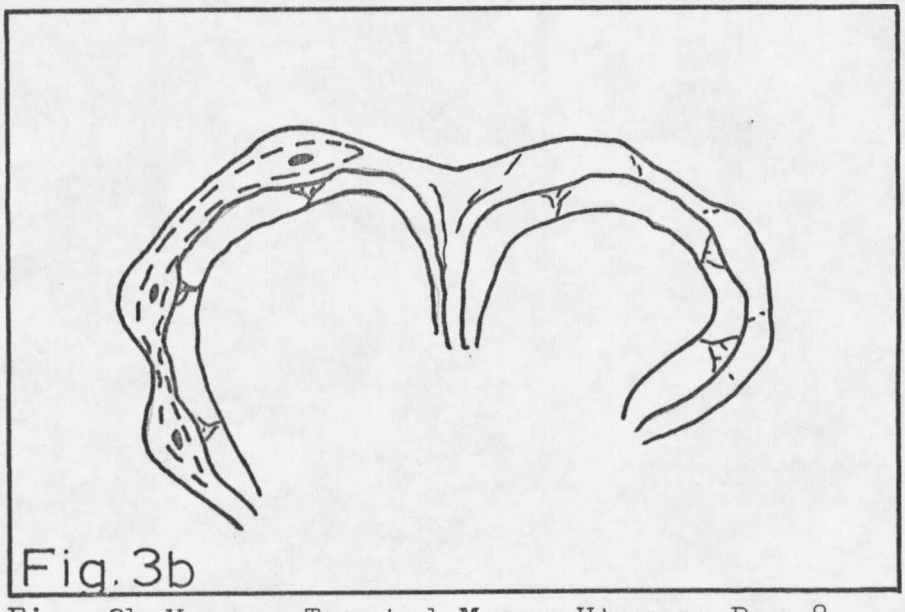


Fig. 3b.Hexane-Treated Mouse Uterus; Day 8 Embryos in Resorption Process

Table 5. Reproductive Failure as Affected by Duration of Administration of Hexane Extract

	Day of Gestation	% Embryo Loss
a.	1 - 3	56 ± 2
	1 - 4	74 ± 2
	1 - 5	81 ± 2
	1 - 6	88 ± 2
	1 - 7	92 ± 2
	1 - 8	98 ± 3
b.	1 - 5	59 ± 1
	2 - 5	62 ± 1
	3 - 5	87 ± 2
	4 - 5	92 ± 3
	5	89 ± 3

n = 10 mice per group

Mice were administered 4 gm/Kg/day of hexane extract via stomach tube

Results of the first study (Table 5a) suggest that as the duration of the feeding period becomes greater, the percent embryo loss increases with the greatest losses occurring with feeding periods involving the day of implantation (day 5 of gestation; Noyes, et al. 1963).

The data collected in the second study (Table 5b) also support the time of implantation as being a critical period. The losses observed with the first two durations are relatively low when compared to 90% embryo losses in Table 5b, but are significant when compared to controls. This may be due to the maternal system's ability to partially detoxify the active compound(s) before the critical time period of implantation. Also, the pre-implantation embryo may have a better repair mechanism and a lesser dependence on an external food supply (Gebhart, 1969). The last three feeding schedules result in embryo losses that plateau at about 90%. Here again time may be a very important factor. The maternal system may not have a sufficient chance to detoxify the active substance(s) before the day of implantation arrives. In addition, the nutrient requirements for the embryo may be changing.

From this data it was concluded that the critical time for interrupting implantation occurs around the day of

implantation (day 5 of gestation; Noyes, et al. 1963) and that increasing the length of time of ingestion of the hexane extract increases the chances of embryonic loss during early gestation.

Separation of Extract by Column Chromatography

The extract was separated by column chromatography (Table 6) to facilitate identification of the lipophilic compounds responsible for reproductive failure in mice observed upon ingestion of ponderosa pine needle extract. Any additional purification of fractions was accomplished by preparative thin layer chromatography. The hexane extract was separated into seven fractions which were individually assayed for physiological activity.

Bioassay of Column Fractions

The fractions were individually dissolved in an olive oil vehicle and administered at a dose of 4 gm/Kg/day on day 1-5 of gestation to determine which fraction of the hexane extract contained the physiologically active compound. Column fraction four is active (Figure 4) displaying 75% embryo loss. The other six fractions were not considered active when compared to the controls.

Table 6. Column Chromatography of Hexane Extract

Fraction	Solvent % Hexane: % Diethyl Ether	Volume of Eluent (ml)	% of Total Extract
1	100:0	100	1.0 ± 0.1
2	95:5	150	3.0 ± 0.1
3	95:5	150	6.0 ± 0.2
4	90:10	200	39.0 ± 0.8
5	85:15	200	44.0 ± 0.7
6	50:50	100	2.0 ± 0.1
7	0:100	100	5.0 ± 0.2

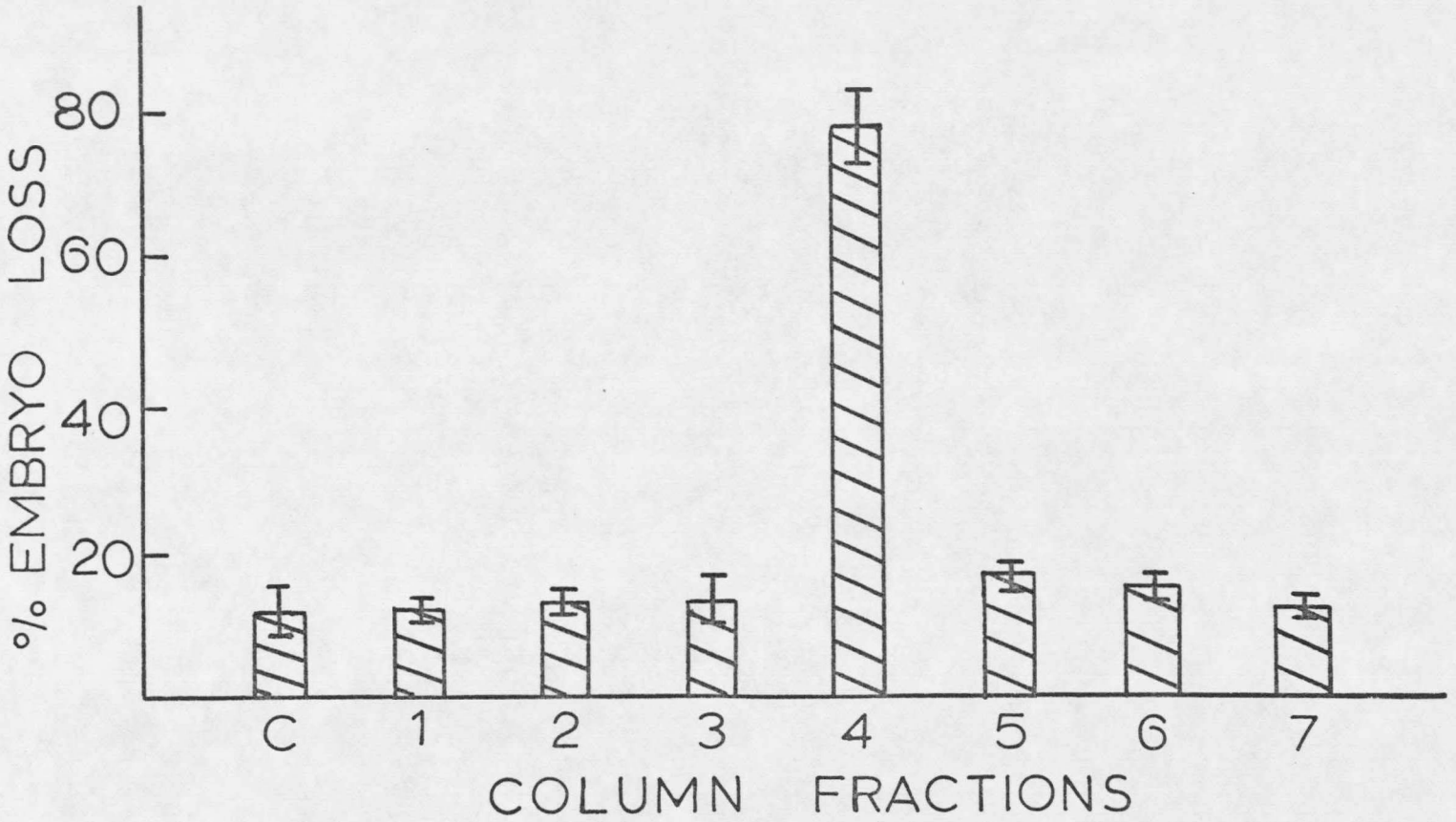


Fig.4 REPRODUCTIVE FAILURE AS INFLUENCED BY COLUMN FRACTIONS (Day1-5; 4gm/Kg/day n=10mice/group)

Argentation Chromatography

The active column fraction (number 4) was further separated on the basis of unsaturation by argentation chromatography utilizing the combined methods of Barrett, et al. (1962), Norin and Westfelt (1964) and Zinkel and Rowe (1964). Two constituents were observed on $AgNO_3$-silica thin layer plates, having R_f values of .27(A) and .19(B) in the following solvent system: hexane:diethyl ether:acetic acid (75:25:1:, v:v:v). Constituent A corresponds, on the basis of R_f value, to abietic and dehydroabietic acid (resin acids) standards.

An aliquot (100 mg) of both constituents was saved for further chemical analysis; the remainder being individually assayed for biological activity.

Gas-Liquid Chromatography

Gas-liquid chromatography was utilized to determine the chemical identity of the methylated samples. Seven peaks were resolved and were labeled A-G (Figure 5).

Peaks (A-G) were separated and purified for mass spectrometric analysis by preparative gas-liquid chromatography. The purified peaks were checked by gas-liquid chromatography for completeness of separation and were found to be 95% pure.

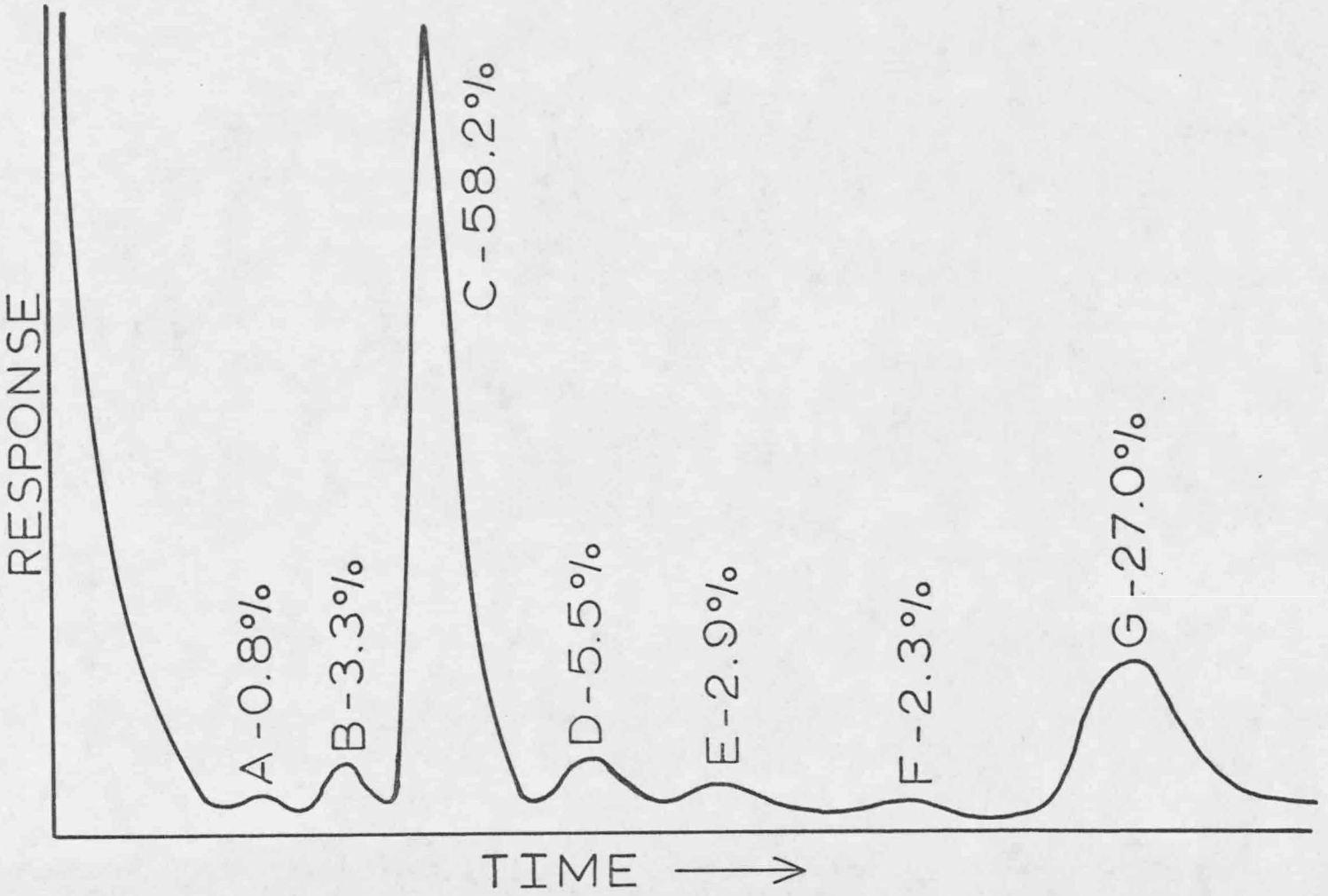


Fig.5 Chromatogram Of Peaks Resolved By GLC

Ultraviolet Spectroscopy

Ultraviolet spectroscopy of peaks A_G was used to delineate the nature of the ultraviolet absorbing systems. The absorption characteristics (Table 7) for the peak samples indicate that peaks A,B, and C are non-conjugated dienes (Figure 6); peaks D,F,and G are conjugated dienes and that peak E is a conjugated triene and therefore aromatic (Figure 7).

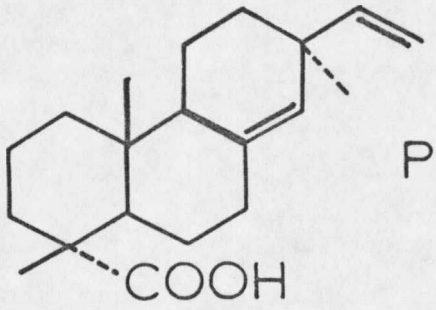
Mass Spectroscopy

The mass spectra of the samples were analyzed and compared to those obtained by Zinkel, et al. (1971) to determine the chemical identity of the purified peaks. The peaks were identified as follows: peak A-- 8(14), 15-pimaradien-18-oate (Pimarate); peak B -- 8(14), 15-iso-pimaradien-18-oate (Sandaracopimarate); peak C-- 7,15-iso-pimaradien-18-oate (Isopimarate); peak D-- 8,13- abietadien-18-oate (Palustrate); peak E-- 8,11,13- abietatrien-18-oate (Dehydroabietate); peak F-- 7,13-abietadien-18-oate (Abietate); peak G-- 8(14),13(15)-abietadien-18-oate (Neo-abietate).

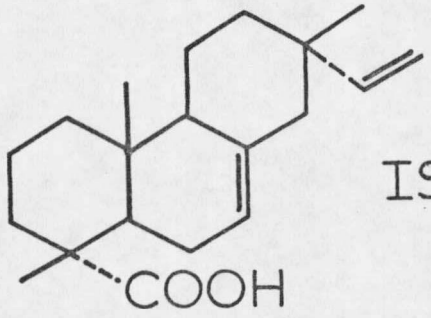
The purified compounds are all diterpene resin acid methyl esters of the aromatic, conjugated, and non-conju-

Table 7. Ultraviolet Spectra of Resin Acid Methyl Esters

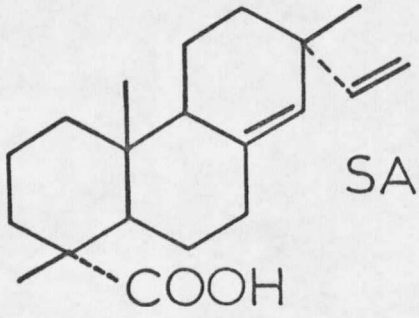
Peak	Chemical Identification	Maxima (nm)
A	Pimarate	205
B	Sandaracopimarate	203
C	Isopimarate	205
D	Palustrate	265
E	Dehydroabietate	275; 210
F	Abietate	242
G	Neoabietate	252



PIMARIC

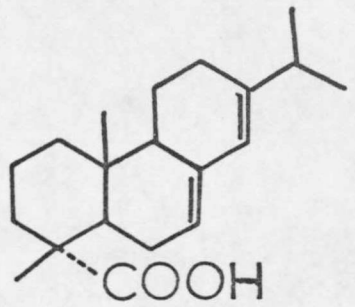


ISOPIMARIC

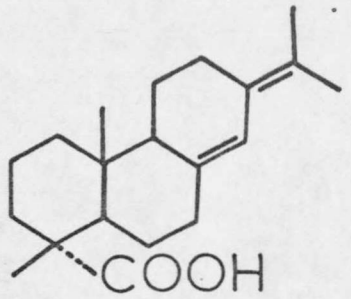


SANDARACOPIMARIC

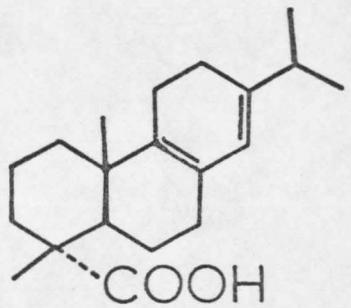
Fig.6 Types of Non-conjugated Resin Acids



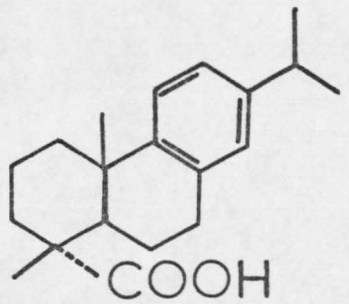
ABIETIC



NEOABIETIC



PALUSTRIC



DEHYDROABIETIC

Fig. 7 Types Of Conjugated Resin Acids

gated types.

Reproductive Failure As Influenced By Conjugated and Non-Conjugated Compounds of Ponderosa Pine Needles

The constituents obtained by argentation chromatography were dissolved in an olive oil vehicle and administered at a concentration of 2.5 gm/Kg/day on day 3-5 of gestation to determine if they were biologically active. The conjugated compounds elicited an embryo loss only slightly above that of the control mice (Figure 8). The non-conjugated compounds exhibited a 57% embryo loss, indicating that the active substance located in the hexane extract of ponderosa pine needles may be composed of non-conjugated resin acids.

Resin Acid Bioassay

Commercial preparations of abietic acid and dehydroabietic acid were dissolved in olive oil and administered at a concentration of .8 and 1.6 gm/Kg/day on day 1-5 of gestation to determine if reproductive failure similar to that of hexane extract treated mice could be induced. Abietic acid, at these levels (Figure 9), does not cause reproductive failure in mice when compared to controls. At a concentration of .8 gm/Kg/day, dehydroabietic acid does

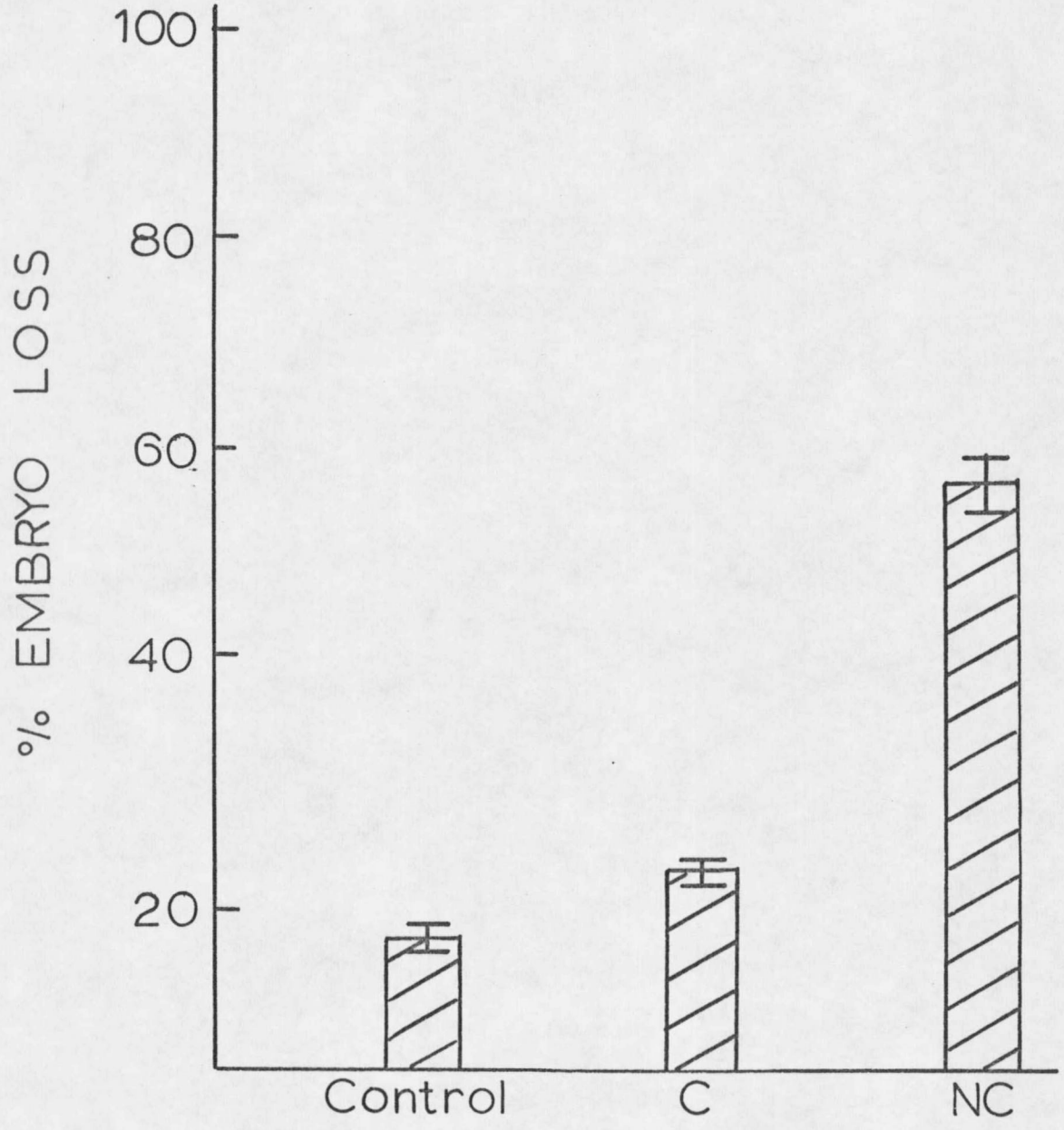


Fig. 8 Conjugated vs Non-conjugated Cpds., Of Hexane Extract Bioassay (Day 3-5; 2.5gm/Kg; n=6mice/group)

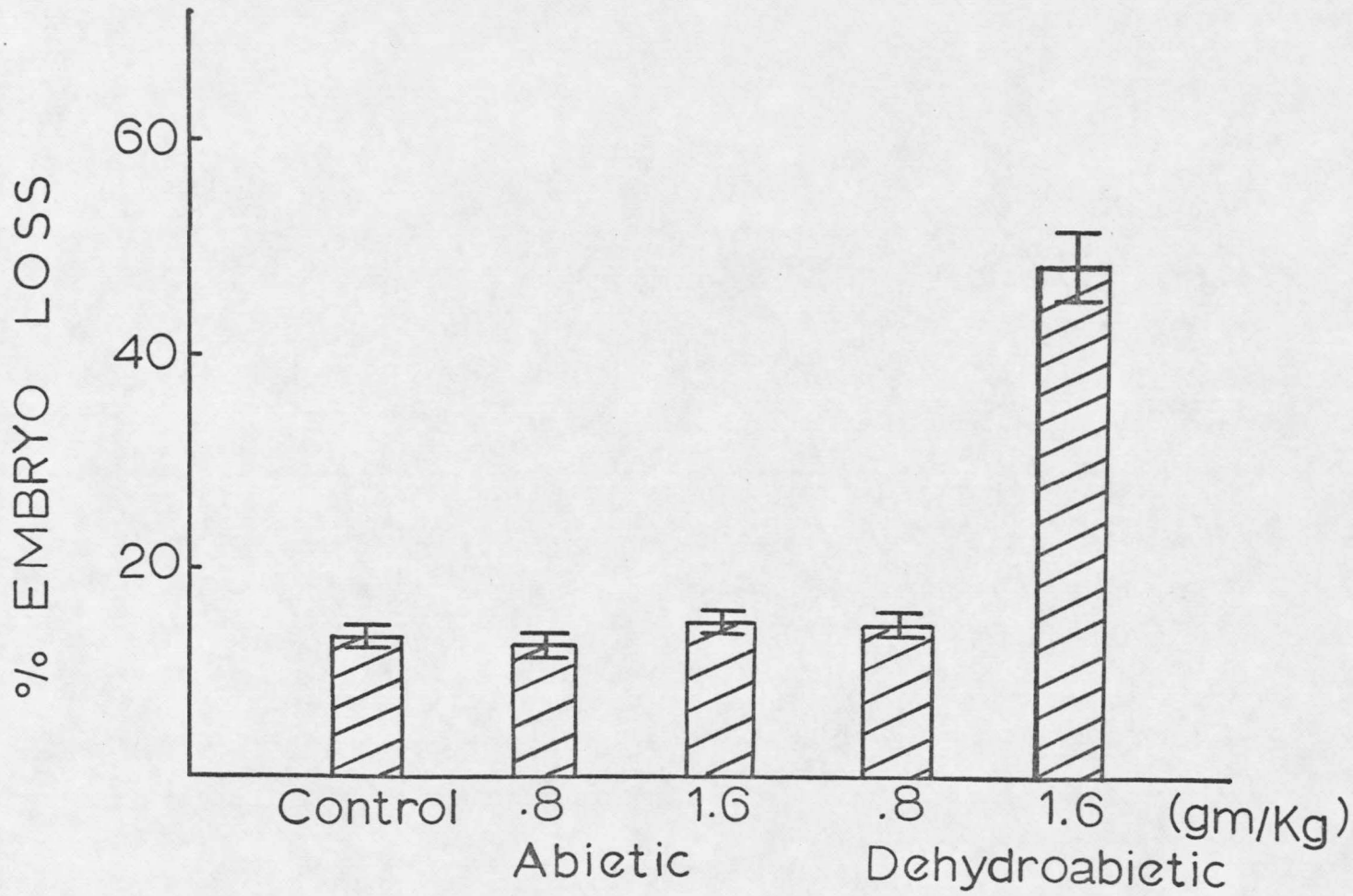


Fig.9 Reproductive Failure As Influenced By Conjugated Resin Acids (Day 1-5; n=5mice/group)

not elicit an embryo loss significantly above the control; however, at 1.6 gm/Kg/day, the embryo loss observed is 49% and is similar to that observed in mice upon ingestion of hexane extract of ponderosa pine needles. Since non-conjugated resin acids are not commercially available for bioassay, it is unknown at this time what degree of effect they have on interruption of implantation.

DISCUSSION

Determination of Reproductive Failure

Diterpene resin acids located in the hexane extract of ponderosa pine needles when ingested by gestating mice lead to reproductive failure during early gestation. The dysfunction observed can not be ascribed to starvation (McClure, 1959) since the percent embryo loss recorded in mice treated with hexane extract via stomach tube was similar to that observed in mice fed hexane extract ration.

The ingestion of diterpene resin acids does not seem to affect fertilization or decidualization. A positive local reaction to pontamine blue dye is observed on day 8 of gestation. It usually occurs as early as day 4 of gestation and indicates localized permeability changes at implantation sites which mark the initiation of the ovum-endometrial relationship (Glasser and Clark, 1975); a response which is enhanced by the onset of decidualization. The decidual cell reaction is stimulated by the presence of a blastocyst in the uterus (McLaren, 1970). The presence of a blastocyst in the uterus indicates that the ovum has been fertilized by the sperm and has undergone cleavage which results in the division of the egg to the 32-cell

blastocyst stage by day 4 of gestation (Dickerman and Noyes, 1960; Eaton and Green, 1963).

It is well established that implantation in the mouse occurs on day 4 of gestation (Noyes, et al. 1963). This timing of implantation is adjusted to the embryological designation of day 0 as the day the vaginal plug is first observed (Shelesnyak, 1957). This thesis utilizes the popular literature designation in which the day the vaginal plug is observed is termed day 1 of gestation. Therefore, for the purpose of this work, day 5 of gestation will be considered the day of implantation.

Implantation can be separated into three stages (Schlafke and Enders, 1975): a) appositional— initial positioning of the blastocyst in the uterus; b) adhesion — adhesion of trophoblast to uterine epithelium; c) attachment, which Boving (1965) equates with implantation — epithelial penetration by trophoblast where maternal and embryonic tissues touch.

Diterpene resin acids of ponderosa pine needles in some manner interrupt implantation. The appositional stage is not affected since a positive blue dye reaction is observed indicating a "pre-sensitization" change in the uterus in response to the presence of a blastocyst (McLaren

1970)

The adhesion stage is considered by some (Enders and Schlafke, 1969; Young, 1956) to involve increased resorptive activity and adhesiveness of the uterine epithelium. This type of change in the luminal epithelium surface is accelerated by the presence of blastocysts in the uterus (Pollard and Finn, 1974). These changes occur at the same time as the pontamine blue response and are regarded as part of implantation. Since uterine dye sites are observed, even at day 8 of gestation, the diterpene resin acids of ponderosa pine needles do not affect the adhesion stage of implantation.

Penetration by trophoblast may be active or passive depending on structural organization at the site of implantation, tendency of epithelium to dislodge, and proliferation of decidual reaction (Schlafke and Enders, 1975). In mice, the type of trophoblast penetration observed is termed displacement implantation in which the uterine epithelium is released from the basal lamina, thus allowing the trophoblast to spread through and beneath the epithelium. The individual uterine cells that dislodge are phagocytized by the trophoblast before it descends beyond the level of the basal lamina. This occurs after

the decidua had begun to form around the epithelium of the implantation chamber day 4 of gestation (Finn and Lawn, 1968) As of yet there is no established rate of penetration, however, it is felt that implantation is accomplished by day 6 of gestation (Glasser and Clark, 1975).

The inclusion of the day of implantation (day 5) in the feeding of diterpene resin acids results in a relatively high embryo loss, suggesting that implantation is a vulnerable and critical time period. At this time (with the data available) it is unclear if the diterpene resin acids in some manner interrupt implantation at this stage of penetration or if the effect occurs after implantation has been accomplished. However, by day 8 of gestation, embryos are in the process of resorbing. At a duration of 1-3 or 1-4 days, mice exhibit a lower embryo loss. This may be due to a) the ability of the maternal system to partially detoxify the active substance(s) prior to implantation or b) delayed implantation.

The vertical region of the dose-response relationship suggests that a threshold dose occurs above which the variation of response with increasing dosage is negligible. This plateau region occurs at about 60% embryo loss and not higher. This may be due to the concentration of the

diterpene resin acids in the particular hexane extract used in this experiment.

The purified individual diterpene resin acids, rather than a mixture of lipophilic compounds from the hexane extract, should be administered at various dosages to accurately examine the dose-response relationship. Due to the difficulty of purifying large quantities (necessary for bioassay) of lipophilic compounds by preparative gas-liquid chromatography this study was not undertaken.

Chemical Identification of the Active Compound

The hexane extract was separated into seven fractions. The active fraction was found to have an R_f value similar to the resin acids, abietic and dehydroabietic. This suggests that the active fraction may contain diterpene acids.

Argentation chromatography separated the active fraction into two constituents based on their unsaturation. In comparing the two constituents of the active fraction to resin acid standards, it was observed that constituent A had an R_f value (.27) similar to abietic acid, thus suggesting that this constituent was made up of conjugated dienes. No non-conjugated resin acid laboratory standard was available thus no definite conclusion could be made concerning

constituent B (R_f = .19). However, on the basis of $AgNO_3$ separation, it was assumed that constituent B may be composed of non-conjugated dienes.

Bioassay of the two constituents indicated that B was biologically active and suggested that the active compound was a non-conjugated diene and structurally a diterpene.

Constituents A and B were analyzed by gas-liquid chromatography and resolved into four and three peaks, respectively. Preparative gas-liquid chromatography allowed for the separation of peaks to greater than 95% purity. Mass spectra were analyzed and compared to spectra of known resin acid standards (Zinkel, et al. 1971). All seven peaks were found to be diterpenes of the grindelic type. Constituent A was composed of the conjugated abietic variety of resin acid methyl esters: abietate, neoabietate, palustrate, and and the aromatic resin acid methyl ester, dehydroabietate. These did not cause reproductive failure in mice. Constituent B was composed of the non-conjugated pimeric variety of resin acid methyl esters: pimarate, isopimarate, and sandaracopimarate. When ingested these resin acids interrupted implantation and resulted in embryonic resorptions in mice during early gestation.

At present, it is not known whether a synergistic effect exists among these compounds or whether they have their affect individually. In addition, neither is the target tissue known nor the biochemical mechanism understood for this effect on mice during early gestation.

Characteristics of Reproductive Failure

The characteristics of the reproductive failure observed are as follows: a) interrupted implantation and b) embryonic resorptions. These include fewer embryos, uneven spacing of blastocysts, embryos smaller in size and weight and lacking definition of form, adhesiveness and physical attachment.

Implantation may be interrupted by the following factors: a) embryotoxins; b) altered hormone(gonadotropin) levels; c) maternal immune system; and d) lack of uterine implantation factor. Embryotoxins can vary in nature, origin and effect. Aminopterin, 6-amino-nicotinamide and actinomycin D (all antimitotic substances) affect rat embryos on day 5-9 of gestation, but do not seem to affect the embryos as greatly after day 10 and not at all before day 5 of gestation. This change in sensitivity is related to a change in nutritional requirements of a 10 day embryo

(Beck and Lloyd, 1966). The insensitivity of the pre-implantation embryo may be due to a better repair mechanism and a lesser dependence on an external food supply. Thus the toxic agents act by reducing the amount of nutrition available to the embryo and the effect is not felt until after implantation (Gebhardt, 1969). Diterpene resin scids in the hexane extract may act in a similar way with the effect occurring prior to implantation but the actual signs of this effect not being observed until after implantation.

Indomethacin, an anti-inflammatory, anti- pyretic and analgesic drug, interrupts implantation by inhibiting the increase of capillary permeability and affecting the spacing of blastocysts (Kennedy, 1977) both responses which are mediated by prostaglandins (Williams and Morely, 1973; Vane and Williams, 1973). Thus, indomethacin itself is not toxic to blastocysts but rather is an antagonist of prostaglandin synthesis which may be involved in implantation in rodents (Saksena, et al. 1976). In the same manner diterpene resin acids of ponderosa pine needles may themselves not be toxic to blastocysts, but may be antagonistic toward prostaglandin or steroid biosynthesis.

Components of rabbit uterine fluid have been shown to be embryotoxic. They are estrogen-induced and inhibit RNA

synthesis by the blastocyst (Guilbert-Blanchette and Lambert, 1978) Stone, et al. (1977) report the presence of such an embryo toxin located in oviduct fluid of rabbits. As of yet, no such uterine toxin in mice has been reported.

Changes necessary for, and indicative of, impending implantation are induced by the synergistic action of estrogen and progesterone on the blastocyst (Yasukawa and Meyer, 1966). It is well established that the conditioning of the uterus for blastocyst interaction begins with estrogen secretion (Nilsson, 1967), which stimulates proliferation of uterine stroma. After exposure to progesterone, the stimulation is altered; epithelia secrete materials which stimulate post-blastocyst development; proliferation of stromal cells becomes more intense with some cells acquiring the capability of transforming into decidual cells. Altered gonadotropin levels result in failure of stromal and epithelial response which lead to failure of ovum implantation due to inadequate secretion and lack of decidual cell formation (Shelesnyak and Marcus, 1971). The diterpene resin acids in the hexane extract of ponderosa pine needles may in some manner alter hormonal levels at or prior to the time of implantation.

Immunological aspects have been hypothesized to be

involved in implantation (Tyler, 1961; Shelesnyak, et al. 1967). For immunological events to be a factor two requirements must be satisfied: 1) the blastocyst must be antigenic; 2) the pregnant female must be immunologically competent (Kirby, 1970). There is no doubt that at the time of implantation the blastocyst is antigenic (Kirby, et al. 1966). If for some reason the uterus becomes specifically immunized against the blastocyst, the fertilized egg is destroyed at some time during implantation and implantation is disrupted (Kirby, 1970). During pregnancy, transplantation antigens escape from the conceptus and the mother recognizes and responds to them (Breyere and Barrett, 1960). Should these events not occur, immunological rejection by the maternal system would result and implantation would not be accomplished (Poppa, et al. 1964).

Observations of implantation in the mouse provide evidence for an estrogen-dependent factor of uterine origin which has been termed implantation initiating factor (IFF) (Mintz, 1970). It acts on receptors at the blastocyst cell surface and causes initial attachment (adhesion) to the uterine wall (Mintz, 1972). This proteinase is also able to lyse the zona pellucida. In addition, a second proteinase secreted by the trophoblast may act to cause a

deeper lytic invasion of the uterine tissue. If implantation initiating factor is absent, implantation is physiologically delayed or fails totally (Pinsker, et al. 1974). A similar type of protein, found in rabbits, termed uteroglobin (Beier, 1966), is produced and released in response to progesterone (Beier, 1970a). It has been shown that passage of uteroglobin into the blastocyst is essential for implantation in the rabbit and that any interference with the passage of this uterine component into the blastocyst may inhibit implantation and provide a method of contraception (Beier, 1976). Independently, this same uterine protein was discovered by Krishnan and Daniel (1967) and termed blastokinin. This protein fraction was found to induce and regulate blastocyst development. The production of blastokinin is accelerated at the time of implantation and it has been suggested that blastokinin is eminent for implantation to be accomplished (Daniel, 1976).

The hexane extract of ponderosa pine needles may interrupt implantation in any of the above mentioned ways; most likely by altering the normal gonadotropin levels during the pre-implantation stages of gestation. The normal physiological changes necessary for implantation may not occur and implantation will be interrupted.

Many of the physical characteristics observed by day 8 of gestation may be due to any of the above discussed factors. For example, the uneven spacing of blastocysts may be due to the presence of an inhibitor of prostaglandin synthesis (Kennedy, 1977) or the presence of an unfertilized ova (Psychyos, 1970) or a lack of synchrony between the uterine epithelium and the blastocyst (Psychyos, 1976; Shelesnyak and Marcus, 1970) which eventually could lead to embryo mortality (Schacht and Foote, 1978). Due to the presence of uterine dye sites, the possibility of unfertilized ova does not seem to be a reasonable explanation for the uneven spacing of blastocysts observed in the hexane extract treated mice. However, the presence of a prostaglandin synthesis inhibitor in the hexane extract of ponderosa pine needles is a possibility as is the lack of synchrony between uterus and blastocysts.

The lack of adhesiveness is not suprising since this stage of implantation is a progressive one (Schlafke and Enders, 1975). At the onset, any factor affecting the surface of the uterus or blastocyst or increasing motility of the uterus (Chan, 1977) would be deleterious to implantation (Enders, 1976).

Though these characteristics and others mentioned

above (embryos fewer in number; smaller in size and weight; lacking definition of form and physical attachment) may be due to the diterpene resin acids of ponderosa pine needles; they are most likely a direct result of the resorption process.

The next step of a study of pine needle induced reproductive failure in mice during early gestation could involve the elucidation of the biochemical mechanisms by which these effects occur.

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