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Embryonic Screening and Isolation of Pine Needle Abortive Factors

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EMBRYONIC SCREENING AND ISOLATION OF PINE NEEDLE ABORTIVE FACTORS

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Cattlemen and veterinarians in the western states and Canada have noted the frequent occurrence of reproductive failure in cattle grazing ranges with tree stands of ponderosa pine (Pinus ponderosa). Consumption of the needles or buds from this tree has caused pregnant stock cows to abort or deliver weak calves. Retained fetal membranes are also frequently associated with pine needle abortion.

Surveys conducted by the Black Hills Area Resource and Development Project in 1974 covering eight counties in western South Dakota and eastern Wyoming estimate that at least 600 calves were lost through pine needle abortion in a three year period in that area alone. Pine needle abortion is a frequent cause for economic loss to these livestock producers. Since no disease can be intelligently prevented or controlled until it is understood, more definitive research is needed to solve the problem. Research attempts to further define the disease and to isolate the causative agent are continuing in various laboratories. Some of the investigations are designed to produce the disease at will under laboratory conditions. If the causative factor or factors are discovered and prevention is then possible, the economic benefit to ranchers and to the meat industry in general is obvious.

Biological screening for the potential active factor causing the abortions is difficult. So many minute extracted fractions from pine needles require a biological testing method. A simple, inexpensive, and reproducible screening technique therefore is essential. Further fetal and maternal effects must be identified and relative intensity of each

measured. Early embryonic effects, except in small laboratory animals, have not received much attention. Chick embryo bioassays are a logical choice for a screening technique. The egg produces an environment for the embryo that is free from outside contamination that could interfere with treatment results. Difficulty exists with small laboratory mammals because they usually resorb the fetuses early after embryonic death. Cattle are the main concern with pine needle abortion, and since they are a monotocus species, a bioassay on a polytocus species such as mice and rats would be disadvantageous.

The primary objective of this study is to test the validity of the chick embryo bioassay screening method with pine needle isolates. An attempt is made to determine if active components appear in lipid or carbohydrate fractions.

LITERATURE REVIEW

Pine Needle Abortion

Occurrence. Folklore has it that Indian women of Washington and Oregon prepared a water extract of ponderosa pine needles to drink to cause abortions (Tucker, 1961). Pine needles have been suspected as a cause of reproductive failure in livestock as early as 1927 (Bruce, 1927). Reports of pine needle abortion in cattle were at first discounted because of a number of other causes of abortion in cattle, such as the infectious diseases brucellosis, vibriosis, leptospirosis, and trichomoniasis, and the nutritional disorders vitamin A deficiency and phosphorus deficiency (Tucker, 1961). The needle from the western yellow or ponderosa pine were experimentally shown in 1952 by McDonald to cause abortions when grazed by cattle.

It has not been established that abortions occur wherever Pinus ponderosa grows. Today, the frequency of occurrence of pine needle abortion is unknown. Field reports of practicing veterinarians, county agents, and ranchers claim serious calf losses in ponderosa pine ranges of South Dakota, Montana, Idaho, Colorado, Washington, Oregon, California, and Western Canada. Pinus ponderosa is extremely adaptable, for the tree endures many climates, adapts itself to varying conditions, and develops a type for each habitat and region (Rogers, 1917). The tree usually grows in rather open parklike stands, from the Black Hills of the Dakotas northwestward to British Columbia and southward in the Pacific and Rocky Mountain regions to northern Mexico and southern California (Grimm, 1967). The typical tree in affected states is

extremely drought resistant. Its usual height is from 70 to 80 feet, but can reach 200 feet and has many short, thick, forked branches in a spine-like head. The leaves are two or three in a bundle, stout, dark yellow-green, 5-11 inches long, and deciduous during their third season (Rogers, 1917). Their leaves give their name to the species for ponderosa pine is also called yellow pine or bull pine.

Chemical and physical structure of pine needles. If the assumed abortive factor lies within the needle and bud of Pinus ponderosa, an exhaustive compositional description is necessary. Some analytical data is available although incomplete. Cogswell (1974) determined percent protein, moisture, ash, ether extract, acid detergent fiber (ADF), and acid detergent lignin (ADL) on 12 collection periods of pine needles (July, 1971 to June, 1972). Analysis of the pine needles varied little with season. Average results over the 12 collection periods were:

moisture	50.30%
ash	2.16%
protein	6.68%
ether extract	9.16%
ADF	33.96%
ADL	14.21%

With ADF substituted for crude fiber, an approximate value for nitrogen free extract (NFE) of 48 percent was calculated from the analysis above. The NFE indicates a high amount of available carbohydrates. Pine needles accumulate considerable amounts of available carbohydrates throughout the winter for energy storage (Hepting, 1945).

The needles seem to be rich in components extracted with ether. In addition to fats, the ether extract fraction may remove other non-fat

materials such as chlorophyl, xanthophyl, cholesterol, resins, alkali substances and breakdown products of triglycerides (Kamstra, 1975). If the ether fraction of pine needles was largely triglycerides, this would also contribute to the energy value.

Pine needles have a low ratio of surface to volume; they have a thick cuticular layer, an epidermis with heavy cell walls, and sunken stomata whose front cavities contain whitish alveolar material, apparently wax (Esau, 1953). These factors make them conserve water for better drought resistance. Beneath the epidermis is a well developed hypodermis, several layers thick, containing elongated sclerenchyma cells. Thus, needles are strong and rigid. Annual shoots and needles are often covered with waxy bloom (Mirov, 1967).

Pines possess a well developed resin-producing system that makes them more resistant to infections and physical injuries. Pine needles have varied numbers of resin ducts. When severed, these ducts exude oleresin that appears to be much thinner, i.e., richer in volatile oil, than the oleresin obtained from the resin ducts of sapwood. The resin ducts of needles are not connected with the resin ducts of wood resulting in different compositions of volatile oil (Mirov, 1967).

With regard to the chemical structure of the oleresin, it can be said that the volatile oil and the resin acids belong to the terpenoids, derived from hydrocarbons known as terpenes. Most of these hydrocarbons are unsaturated and are built up of isoprene molecules (C_5H_8). Depending upon the number of isoprene moieties in

them, they are called monoterpenes ($C_{10}H_{10}$), sesquiterpenes ($C_{15}H_{24}$), and diterpenes ($C_{20}H_{32}$). The volatile oil belongs chiefly to the mono- or sesquiterpenes, the resin acids to the diterpenes (Wenzl, 1970).

Oleresin can be separated into two components: rosin and turpentine. Rosins are usually restricted to the solid resinous material obtained from oleresin (Browning, 1967). Turpentine is the light, volatile essential oil obtained from oleresin. Turpentines consist chiefly of cyclic terpenes and occasionally of diphatic hydrocarbons (Mirov, 1957).

Implication is made that turpentine consumed in sufficient quantities is capable of inducing abortion (Cogswell, 1974; Dreisback, 1971). Cogswell concluded that previous research provided little evidence that turpentine alone is the abortive factor.

Mirov (1967) states that turpentine composition in individual pine trees varies little throughout the growing season.

Zavarin et al. (1971) investigated the influence of season and needle age on yield and composition of the P. ponderosa needle oil. Oil yields throughout the year averaged 0.13% on the basis of tissue green weight. The average composition was:

α-pinene	11.9%	
β-pinene	70.2%	
3-carene	8.0%	
myrcene	5.0%	
limonene	1.8%	
β-phellandrene	2.2%	
methyl chavicol	6.4%	(total monoterpenes = 100%)

The amount of methyl chavicol and total monoterpenoids was highest in summer, lower in juvenile than in mature first-year needles, and decreasing thereafter with needle age. A possible intermediate in the formation of methyl chavicol could be the active component causing pine needle abortion (Cogswell, 1974).

Characterization of pine needle abortion. It is believed that the abortions induced in cattle grazing pine needles of Pinus ponderosa occur during the last trimester of pregnancy. Pine needle abortions usually appear one to three days after pregnant cattle have eaten the needles or buds. Abortion within a herd will continue for up to two weeks even though cattle are withdrawn from feed. Abortion or premature calving usually occurs suddenly. If calves live, they appear very weak (Olson, 1976). There is also a high incidence of retained placentas in affected cows.

Pine needle abortion is further characterized by weak parturition contractions, excessive uterine hemorrhage, and incomplete dilation of the cervix. Septic metritis is constant after the abortion and may be followed by peritonitis (Stevenson et al., 1972). Marked, rapid swelling of external genital organs and unusual udder development may accompany the eating of pine needles by a pregnant cow. Cortical necrosis of the kidney, pulmonary congestion, and excessive hemoglobin breakdown in tissues have been noted in some aborted fetuses (James et al., 1977). In certain cases the toxicity seem to affect only the placenta, and the cow suffers only from complications of the abortion. The more acute cases seem to indicate that there may be a toxic reaction

to the mother (Stevenson et al., 1972). The general body condition appears to be unrelated as abortions occur in thrifty as well as poor cows. Apparently, the agent neither acts as a contraceptive nor affects fertilization and implantation in cattle (Chow et al., 1972). Pine needle abortion still is not adequately described to differentiate it from other types of abortion.

Abortions that could be associated with pine needles have not been observed during the early gestational stages in cattle. Range cattle that graze on ponderosa pine ranges during early pregnancy usually do not abort (Stevenson et al., 1972). It is not known whether the cow is less susceptible during early pregnancy or if cattle do not graze these pines during late spring, summer, and early fall.

Although ranchers assume that the abortive factor to be present in pine needles only during late winter or early spring, Chow (personal communication) found no seasonal variation in abortive potential. Cogswell's (1974) research with mice and rats also indicated the abortive factor to be present throughout the year. However, aqueous and acetone test rations prepared from January collections of pine needles did cause a greater reduction in average litter size in rats than in July and October collections. This would seem to indicate that larger concentrations of the abortive factor could occur during the winter.

The observations of most ranchers and some veterinarians practicing in areas where pine needle abortions occur suggest that stress conditions seem to be related to consumption of pine needles and

the subsequent abortion. Many ranchers seem to feel that cows graze needles most readily after snow, cold, and wind (Stevenson et al., 1972). However, cattle have been observed eating pine needles and buds by preference, even though they had access to good quality feed (Olson, 1976). Experiments with cows indicate that the pine-induced abortion occurs much more readily if cows are under stress (James et al., 1977).

Occurrence of pine needle abortion with cattle is unpredictable. Cattle that have had regular access to pine needles or buds seem to have fewer problems with pine needle abortion than those who have not had previous exposure. Not all pregnant cattle will abort after eating pine needles, but the disease had been known to affect as many as 50 percent of a cow herd (Olson, 1976).

Cattle are the principal species known to be affected, but the disease has also been suspected in grazing sheep and deer. Mule deer consume ponderosa pine needles throughout the grazing season. They relish green needles from lower branches of live trees, but also eat quantities of dry needles from downed trees or slash (Currie et al., 1977). Abortion in deer is suspected but no evidence has been found.

James et al. (1977) state that sheep abort after they eat pine needles. However, Call and James (1976) conducted an experiment in which they concluded that consumption of pine needles by sheep did not interfere with pregnancy. Pregnancy and lambing rates were not affected in 30 mature ewes by the feeding of pine needles. Estrous cycles were also not altered in these ewes fed pine needles 11 to 13 days prior to breeding. However, pine needles that were collected in the same area

subsequently induced abortion in cattle. There was no indication of any toxic effects on ewes consuming a 37 percent pine needle diet.

Approaches and Theories

Hormonal imbalance. Due to the numerous reports of beef cows aborting when consuming yellow pine needles, it has been suspected that these needles may contain estrogenic and/or anti-estrogenic substances. An anti-estrogen, as defined by Ostrovsky (1960), is any substance that can cause a decrease in uterine weight or prevent vaginal cornification. Allen and Kitts (1961) studied the possibility of anti-estrogenic effects on mice. An aqueous pine needle extract depressed uterine weight and an ether extract increased the incidence of mortality. It has, however, been shown that pine needles do not have an anti-estrogenic effect in ruminants (James, unpublished data; Call and James, 1976).

Toner (1971) found an increased ($P<.05$) plasma estrogen level after pine needles were drenched via stomach tube for three days. If ponderosa pine needles had the ability to increase estrogen synthesis, they could possibly upset the normal uterine progesterone controlled environment necessary to maintain pregnancy (Cogswell, 1974). The presence and amount of estrogen-like substances in pine needles is still uncertain.

Mycotoxicosis. It has been suggested that abortion or fetal resorption may not be caused by the components of pine needles but by the toxic metabolites produced by fungi growing on pine needles. This theory would be one of mycotoxicosis. Chow et al. (1974) did a study

to determine whether metabolites produced by fungi which had been observed on pine needles could be the cause of reproductive failure. An aqueous-fungi fraction was prepared following incubation and compared to aqueous fractions. The aqueous-fungi preparation showed a significantly greater disruptive effect on pregnancy than did the aqueous fraction. Complete fetal resorption in mice was observed after the mice were fed seven days on the feed containing pine needle aqueous fractions contaminated with fungi. Two fungi were thought to be isolated, Aspergillus niger and Torulopsis candida. These two fungi were thought to have synergistic effects because when isolated and incubated, the fungi did not have much of an effect on mice. Meanwhile, Chow has not been able to reproduce the fungal toxins once isolated (Chow, personal communication).

Anderson and Lozano (1977) observed that two, and possibly three, toxic compounds are associated with pine needles. In mice, the toxin of most importance was heat stable, insoluble in water, but soluble in most organic solvents. A heat labile toxin, suggestive of a mycotoxin was demonstrated in water extracts, was found only in fresh green needles, and exerts its effect early in gestation. A third toxin lethal to the female mouse in late gestation and not water soluble may be present. The latter toxin had no observable effect on fetal mice.

Previous work has shown the abortifacient in pine needles to possess heat labile characteristics (Tucker, 1961; Stevenson et al., 1972); and also is known to exist in a water soluble fraction (Chow et al., 1972; Cogswell, 1974). This could suggest that the secondary

toxin (heat labile) could produce the abortions. However, if the secondary toxin is only present in green needles, Tucker (1961) states that drying does not destroy the toxic agent, but heating does.

It has been theorized that mycotoxicosis may be responsible for some of the 75 to 80 percent undiagnosed bovine abortions (Chow, CRIS release, 1978). The data suggests that mycotoxins could play a role in pine needle toxicity but whether this is the same toxicity that causes the majority of the fetal abortions is not known. The familiar problems of difficulties in toxin identification and obtaining repeatable results occur in this area.

Biological response in laboratory animals. Because of the time and expense involved, laboratory animals have been used rather than large animals as a measure of biological response. This theory assumes that all animals respond similarly. The majority of the research reported on pine needle toxicity has dealt with mice and rats rather than domestic animals (Allen and Kitts, 1961; Allison and Kitts, 1964; Cook and Kitts, 1964; Chow et al., 1972; Chow et al., 1974; Cogswell, 1974; Anderson and Lozano, 1977). The use of mice as a biological assay was quite successful because pine needles caused disruptive effects on pregnancy through death and resorption of the fetuses.

With mice, disruption of pregnancy seems to occur during the stage when the placenta starts to grow. At that time, cell division and growth are rapid. The effect of the abortive agent is much less severe when feeding is started late in pregnancy. Since the result is more severe the longer the mice are on feed, the effect seems to be

cumulative (Chow et al., 1972). Cogswell (1974) noted a detrimental effect of pine needle fractions upon implantation in the mouse.

Reduced litter size in rats fed pine needle extract was shown by Cogswell (1974). The feeding of the aqueous and acetone fractions of ponderosa pine needles to rats was, however, toxic to both the dam and the fetus.

Chick embryos. No information was found on the use of chick embryo bioassays for pine needle abortion. However, its successful use in toxicology and disease research merits its literature and experimental investigation. The developing chick embryo has provided a valuable biological model for the study of toxicology and teratogenesis. The manifestations of toxicity in the embryo are growth retardation, functional deficit, malformation, and death (Schardein, 1976). The chick embryo is usually chosen because it is readily available and adaptable to studies of either chemicals or drugs for determining their possible injurous or teratogenic effects on rapidly proliferating and differentiating embryonic tissue. Hundreds of chicken embryos may be observed in a minimum of space, and over a comparatively short period of time. The feasibility of using such large numbers is valuable also in the statistical evaluation of toxicology data (McLaughlin et al., 1963).

The earliest work in chick embryo bioassays found in literature was done in 1893 (McLaughlin et al., 1963). The interest then was mainly in the teratogenic effect of chemicals. Since 1900, the fertile embryo has been used extensively in toxicology work. A method was

described by Marliac (1962) with simple injection of different chemicals into chicken eggs as a toxicology test. Toxicology measurement of some chemicals was also demonstrated by injection into chicken eggs (McLaughlin and Mutchler, 1962).

The chick embryo became very valuable as a test organism for toxic substances in food since there are many substances in foodstuffs, both naturally occurring and additive, which have, or are suspected of having adverse effects on the consumer (Platt et al., 1962).

The toxicology of drugs such as insulin and tetracycline have been evaluated using the chick embryo technique (Verrett and McLaughlin, 1963). This technique has been used extensively to evaluate the toxicology of fertilizers, pesticides, and herbicides (Marliac and Mutchler, 1963; Strange et al., 1976).

The chick embryo bioassay has been used as an interferon bioassay. Sedmak and Grossberg (1973) state that this assay of the production of the influenza virus enzyme, neuraminidase, in cultured chicken embryo cells provides a highly precise, rapid, simple, and economical means to measure interferon action.

Buddingh and Womack (1941); DeRopp (1944); Goodpasture and Anderson (1937) all studied the effect of Brucella abortus in the developing chick embryo. Brucella abortus is an infectious disease of cattle that is characterized by abortion, birth of weak or dead full-term calves, retained fetal placentas, and temporary or permanent infertility (Manthei, 1968). The results of this disease are very similar to pine needle abortion.

The multiplication of Br. abortus within the chorionic epithelium of the cow's placenta is usually the first indication that this microorganism could adjust itself to an intracellular environment (Smith, 1919). Goodpasture and Anderson (1937) observed this same type of behavior with infection of the chorio-allantois of the developing chick embryo. Buddingh and Womack (1941) observed that embryos infected by this organism do not survive longer than 96 to 120 hours. These experimental observations add to the constantly increasing list of bacteria and viruses which can be successfully cultured and which induce characteristic infections in the chick embryo.

In the Division of Toxicology, Food and Drug Administration (FDA), the fertile chicken egg test demonstrates to be extremely valuable for confirming the presence of such highly toxic materials as mycotoxins and dioxins. This test has been used routinely since 1963 and, on the basis of this experience, the bioassay for aflatoxin B_1 was adapted by the Association of Official Analytical Chemists as a procedure (Verrett et al., 1973). Data obtained in coordinate studies from five laboratories under contract with the FDA indicate that the technique gives reproducable and reliable results in the hands of different investigators (Friedman, 1974).

The development of the chicken embryo technique as a definite bioassay for aflatoxins was described by Verrett et al. (1964) after he injected fertile eggs by both the yolk and the air cell route. The air cell injection route proved to be more sensitive than the yolk injection route. They also found that sensitivity to aflatoxin decreased rapidly

with increasing age. They reported that well over 400 samples were examined for aflatoxin contaminates by this method, and its correlation with chemical assays was excellent. One of the few dose-response curves for aflatoxin toxicity in biological studies has been reported using this method.

The adaption of a particular bioassay usually rides on its value. These experimental observations by many investigators find the chick embryo bioassay quite favorable to many situations.

METHODS OF PROCEDURE

Fertile Egg Injection

Hatching eggs of White Leghorn strain were obtained from the Lakeview Hatchery at Clear Lake, South Dakota. These eggs were incubated at 37.2° C in Jamesway incubators located at the Poultry Research Unit. After four days of incubation, the eggs were hand candled to select viable embryos for treatment. To help eliminate contamination, the blunt end which contains the air cell was cleaned and washed with alcohol prior to treatment injection. A blunt needle was used to provide an orifice to the air cell. Pre-determined levels of treatment material were injected by a syringe and the egg was securely re-sealed with tape.

Initial experiments were designed to determine dosage, concentration, and desirable treatment carrier. Carrier toxicity and total volume limits dosage levels. It is generally assumed that a volume limit of 0.2 ml of any solution injected into the air cell of an egg will not cause suffocation of the embryo. McLaughlin et al. (1963) conducted a series of experiments to evaluate the toxicology of various chemicals injected into fertile eggs. He reported that water, propylene glycol, corn oil, isotonic saline solution, and isotonic glucose solution showed no toxicity or a very low order of toxicity. Acetone, methanol, ethanol, n-butanol, ethylene glycol, isopropanol, hydrochloric acid, ethyl acetate, malathion, heptachlor, and styrene showed an intermediate order of toxicity. Strange et al. (1976) showed that acetone, which has an intermediate order of toxicity, could be used as a treatment

carried in very small doses. It could be assumed that other chemicals of intermediate order of toxicity could also be used as a carrier in the various treatments. Although water or other substances with very little toxicity would be the best choice for making treatment solutions, solubility of treatment materials must be considered. If solubility becomes important, a chemical with an intermediate order of toxicity could be used to dissolve the material.

Two-tenths (0.2) ml of treatment solution was injected using water as a carrier. If organic solvents were used as the treatment carrier, only 10-20 ul of solution was injected depending on the solvent used. Only the treatment carriers were injected into eggs which served as controls.

The eggs were candled every three or four days and embryo development observed. Embryonic death was evidenced early by the presence of blood rings formed by degeneration of the blood into coagulated rings. Later embryonically, death was also characterized by the degeneration of blood and their vessels.

Results were reported and statistically analyzed by chi square (x^2) according to methods outlined by Lapin (1975).

Chick Embryo Bioassay Methods - Efficacy of Crude Extracts

Experiment 1. Freeze-dried pine needle preparations from previous work which caused reproductive failure in laboratory animals were used in this study. Pine needles collected in July of 1971 from a pine enclosed Bureau of Land Management pasture located near Sturgis, South Dakota, were extracted with water and the preparation freeze-dried

as outlined by Cogswell (1974). This freeze-dried material was dissolved in distilled water in concentrations of 0 mg/ml, 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml. Two-tenths (0.2) ml of each concentration was injected into 30 eggs.

Experiment 2. Ponderosa pine needles were collected from the Arneson ranch near Hot Springs, South Dakota, in October of 1977. Black Hills spruce (Picea glauca) needles were collected in June of 1978. Two 100 g samples of pine needles and a 100 g sample of spruce needles were cut into 2.5 cm segments and each sample was macerated separately in a Waring blender with 400 ml of distilled water. The liquid portion was decanted into a buchner funnel and the residue extracted again with an additional 400 ml of distilled water. The filtrate and washings of one of the pine needle samples was autoclaved for 30 minutes at 121° C and 50 lbs of pressure. This served as a negative control since the abortive factor is believed to be subjected to destruction through autoclaving. The filtrate and washings of all three samples were then freeze-dried in a Vir Tis Freeze-Drier for 20 hours. The crystalline dried residue from this extracted material was placed into plastic bags and stored in a desiccator.

Experiment 3. The sensitivity of the chick embryo method was tested with salts of cobalt and selenium. To 20 mg freeze-dried aqueous extracts, 1 ml of 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml, and 10 mg/ml, respectively, of cobalt chloride solutions in water were added. A similar mixture series was prepared with sodium selenite using only the two smallest concentrations shown for cobalt chloride. An additional

mixture was prepared in which 1 ml of 1 mg/ml of both cobalt chloride and sodium selenite were added to the 20 mg/ml pine needle extract solution. Each solution containing cobalt chloride was injected into 30 eggs in 0.2 ml doses. The same amount of solution containing sodium selenite was injected into 20 eggs. Untreated 20 mg/ml solutions of freeze-dried pine needle extract served as controls.

Chemical Fractionation of Pine Needles

Pine needles were chemically fractionated into a lipid fraction and carbohydrate fraction. A protein fraction was not obtained due to the difficulty of purification.

Lipid fraction preparation. Pine needles were ground in a Wiley mill containing a 2 mm sieve. Twenty-four grams of ground pine needles were placed in 12 fat extraction thimbles and extracted for 10-12 hours with chloriform/methanol (2:1, v/v) on a Labconco-Goldfisch fat extraction apparatus. Total lipids will be extracted using this chloriform/methanol mixture (Folch et al., 1957). After all the solvent was allowed to evaporate from the extract, it was washed with water, placed in plastic bags, and frozen.

Carbohydrate fraction preparation. The pine needles now minus the lipids were removed from the fat extraction thimbles and spread out on cloth to evaporate any solvent left on the needles. They were then washed with cold water and again allowed to dry. The pine needles were evenly distributed into six berzelius beakers with 200 ml of water in each beaker. These were refluxed for 1.5 hours on a Labconco crude fiber digestion apparatus to make a boiling water extract for an aqueous

solution of pine needles. This solution was filtered twice through Whatman no. 3 filter paper in a buckner funnel.

Additional purification of the carbohydrate was obtained using ion exchange. A cation exchange column was prepared using Dowex 50W-X12 (200-400 mesh) packed to 200 ml in a 500 ml buret. An anion exchange column was prepared the same way using Dowex 1-X2 (200-400 mesh). The columns were preluded with 2N HCl. The aqueous pine needle filtrate was added to the cation column and slight suction was applied. The filtrate collected was then added to the anion column and again slight suction was applied. The effluent from this column was neutralized with 2N NaOH. Water was evaporated from the ion exchange effluent with a rotary vacuum evaporator. The residue was weighed and labeled as the carbohydrate fraction.

Bioassay Testing of Fractions

Experiment 4. Total lipid extract of pine needles was tested in this study. The chloriform/methanol (2:1, v/v) extract of pine needles previously described was dissolved in either acetone or 80% ethanol for injection into chicken eggs. The lipid extract was dissolved in 80% ethanol in concentrations of 5 mg/ml, 10 mg/ml, 15 mg/ml, and 20 mg/ml. Each of the concentrations plus an untreated 80% ethanol control was injected into 30 eggs in 20 ul doses. The lipid extract was also dissolved in acetone in a concentration of 20 mg/ml. This concentration plus an untreated acetone control was injected into 30 eggs in a 10 ul quantity. Very small amounts of solution were injected due to the toxicity of the solvent to the embryo.

Experiment 5. Purified carbohydrate extract of pine needles was used in this study. The carbohydrate extract described earlier was dissolved in distilled water in concentrations of 5 mg/ml, 10 mg/ml, 15 mg/ml, and 20 mg/ml. Distilled water controls were treated with 2N HCl to achieve the same pH as the carbohydrate extract emerging from the resin and then subsequently neutralized with 2N NaOH forming a saline solution. Each of these concentrations plus the pH adjusted distilled water control was injected into 30 eggs in 0.2 ml doses.

Paper Chromatography Separation of Sugars From Pine Needles

Paper chromatography as outlined by Myhre and Smith (1960) was used to identify the neutral sugar composition of the carbohydrate extract. The purified carbohydrate extract from pine needles was hydrolyzed in 10 ml of 1N H_2SO_4 at a slow boil for 9 hours. This was centrifuged and the supernatant decanted. $BaCO_3$ was used to neutralize the hydrolyzed material. The concentration was increased by removing water with the rotary vacuum evaporator.

One 25 λ (lambda) of this hydrolyzate was spotted on sheets of Whatman no. 3 filter paper cut to 25.5 x 57 cm. Common monosaccharide standards at a prepared concentration of 5 mg/ml were also spotted with a 25 λ pipette on the same paper. It was necessary to limit the size of the 25 λ application by repeating spotting and drying periods.

The papers were hung in a descending chromotography chamber containing a saturated atmosphere of butanol-pyridine-water (6:4:3, v/v). This same solvent was added to the trough containing the edge of the filter paper and irrigated for 34 hours. After completion, the paper

was removed and allowed to dry at room temperature. The dried chromatograms were sprayed with aniline hydrogen phthalate developed by Partridge (1949) made by dissolving 1 ml of aniline and 1.60 g of phthalic acid into 100 ml of water saturated n-butanol. Colored spots developed after 15 minutes of drying in a 100°C oven. A R_f value was determined for each spot. R_f is defined by Consden et al. (1944) as the ratio of the distances traveled by the substance and the liquid front from the point of application.

RESULTS AND DISCUSSION

Chick Embryo Bioassay of Crude Pine Needle Extracts

Due to the past success by many researchers using the chick embryo bioassay for testing the presence of toxins and some abortive diseases, this technique was selected to test for toxic components of pine needles. Once activity was noted in crude extracts, experiments proceeded to more purified sub-fractions of pine needles.

Experiment 1. Freeze-dried crude pine needle aqueous extract was dissolved in distilled water in concentrations as shown in tables 1 and 2 and injected into fertile eggs. After the embryos had reached the half-way point in incubation (11 days), the 20 mg/ml concentration of extract in water caused an increased ($P<.05$) death response over the distilled water group (table 1). The three lower concentrations showed no increase in death response at this time in the incubation period. At the end of incubation (21 days), death loss numbers increased slightly with concentration; but, compared to controls, these differences were not significant (table 2).

Cogswell (1974) demonstrated that this pine needle extract caused reproductive failure in rats. This same extract, however, produced only a small number of embryonic deaths in fertile chicken eggs.

TABLE 1. EXPERIMENT 1 - CHI SQUARE ANALYSIS OF TREATMENT EFFECT COMPARED TO CONTROLS AT 11 DAYS OR ONE-HALF THE INCUBATION PERIOD.

Treatment (0.2 ml)	No. dead embryos	No. viable embryos	X^2
Dist H_2O	1	29	
5 mg/ml ext.	1	29	0
10 mg/ml ext.	2	28	0.35094
15 mg/ml ext.	3	27	1.07143
20 mg/ml ext.	6	24	4.04310*

*P<.05, 1 d.f.

TABLE 2. EXPERIMENT 1 - CHI SQUARE ANALYSIS OF TREATMENT EFFECT COMPARED TO CONTROLS AT THE END OF THE INCUBATION PERIOD.

Treatment (0.2 ml)	No. dead embryos	No. viable embryos	X^2
Dist. H_2O	3	27	
5 mg/ml ext.	4	26	0.16173
10 mg/ml ext.	6	24	1.17647
15 mg/ml ext.	8	22	2.78293
20 mg/ml ext.	8	22	2.78293

1 d.f.

Experiment 2. Since ponderosa pine is the only coniferous tree known to cause abortions in cattle at this time, Black Hills spruce (Picea glauca) was chosen as a representative coniferous tree to be compared with collections of ponderosa pine needles. Experimenting with preparations thought not to contain abortive potential is an excellent means of testing the validity of a testing technique. Since steam distillation, pelleting, and extreme heat have been known to destroy the toxic agent in pine needles (Tucker, 1961; Stevenson et al., 1972), it is suggested that autoclaving will also destroy the pine needle's abortive potential. Preparations of ponderosa pine, Black Hills spruce, and autoclaved ponderosa pine were compared to distilled water controls in fertile eggs as shown in tables 3 and 4.

The 20 mg/ml concentration of freeze-dried aqueous pine needle extract showed a significantly greater ($P<.01$) death response than the distilled water control (table 4). This same extract also had a larger ($P<.01$) death response than the same concentration (20 mg/ml) of the autoclaved pine needle extract and the spruce needle extract. The 10 mg/ml concentration of the freeze-dried aqueous pine needle extract did not show a significant difference compared to the distilled water controls plus the same concentration of autoclaved pine needle extract and the spruce needle extract (table 4). Table 3 shows the treatment response throughout the incubation period.

Both concentrations of the autoclaved pine needle extracts and the spruce needle extracts showed no significant differences compared to the distilled water controls (table 4). These experiments would

TABLE 3. EXPERIMENT 2 - NUMBER OF VIABLE EMBRYOS AT VARIOUS DAYS TREATED WITH FREEZE-DRIED AQUEOUS EXTRACTS

Days from incubation		7	10	14	18	21
Days from treatment		3	6	10	14	17
Treatment (0.2 ml)	No. eggs					
Dist. H_2O	30	28	25	24	24	24
10 mg/ml Pine needles	30	20	20	20	20	20
20 mg/ml Pine needles	30	21	19	15	14	12
10 mg/ml Autoclaved P.N.	30	24	21	21	20	19
20 mg/ml Autoclaved P.N.	30	27	24	24	24	23
10 mg/ml Spruce	30	27	23	23	23	23
20 mg/ml Spruce	30	24	24	24	24	23

TABLE 4. EXPERIMENT 2 - CHI SQUARE ANALYSIS OF TREATMENT EFFECT COMPARED TO CONTROLS

Treatment (0.2 ml)	No. dead embryos	No. viable embryos	X^2
Dist. H_2O	6	24	
10 mg/ml Pine needles	10	20	1.18182
20 mg/ml Pine needles	13	12	10.00000*
10 mg/ml Autoclaved P.N.	11	19	2.05198
20 mg/ml Autoclaved P.N.	7	23	0.26072
10 mg/ml Spruce	7	23	0.26072
20 mg/ml Spruce	7	23	0.26072

*$P<.01$, 1 d.f.

suggest that autoclaving does destroy the abortive factor and that spruce needles do not contain abortive potential.

The 20 mg/ml pine needle solution in this experiment was much more lethal to the chick embryos than the same concentration in experiment 1. The extracts in the first experiment were obtained from pine needles collected in 1971. The extracts in this experiment were obtained from pine needles collected in 1977; so, there was a difference in storage time of extracts. Also, the pine needles in the two experiments were collected at different locations. The collections for the first experiment were near Sturgis, South Dakota, and the collections for the second experiment were near Hot Springs, South Dakota. It has been suggested that pine needles from some areas have a greater abortive effect on livestock than other areas.

Experiment 3. Cobalt and selenium were used in this study to test the method sensitivity of toxic and nontoxic mineral additions. The alleviation of toxicity to chick embryos when injected with pine needle extract was also of interest.

Alleviation of toxicity or an antidote for pine needle abortion would be even more beneficial to cattle raisers than knowning the factor causing the abortion. Ranchers have used mineral supplements which include selenium and cobalt although no relationship between pine needle abortion and mineral supplements is known at this time. There is speculation that supplemental cobalt might overcome the pine needle's detrimental effect on pregnancy. Selenium also has been known to improve poor reproductive performance (Hartley et al., 1960). However,

excess levels of selenium reduce hatchability of chickens (Scott et al., 1976).

The pine needle preparation at the same concentration (20 mg/ml) used in experiment 2, which showed an increase in embryonic death, was treated with different levels of cobalt and selenium and injected into fertile eggs. All levels higher than 0.1 mg/ml $CoCl_2$ and 0.01 mg/ml Na_2SeO_3 were not tolerated by the embryo (table 5). The 1:1 mixture of $CoCl_2$ and Na_2SeO_3 at a combined concentration of 1 mg/ml seemed to be less toxic than the higher individual levels of cobalt and selenium. Almost no improvement in embryonic death response was accomplished with cobalt chloride ($CoCl_2$) and sodium selenite (Na_2SeO_3) treated pine needle extract (table 5). As in experiment 2, the freeze-dried aqueous pine needle extract at a concentration in water of 20 mg/ml, which were used as controls in this experiment, again showed high lethality.

Chick Embryo Testing of Pine Needle Fractions

Experiment 4 - Lipid fraction. Turpentine, estrogen, or one of the lipid or non-polar components in pine needles, in many cases, has received partial blame for pine needle abortion. Chloriform and methanol which was used to extract total lipids would also extract turpentine, sterols, and other non-polar components in pine needles.

Total lipid extract dissolved in either acetone or 80% ethyl alcohol was injected into fertile eggs and compared with controls. There were no significant differences between any of the four concentrations of lipid extract dissolved in 80% ethyl alcohol and the

TABLE 5. EXPERIMENT 3 - NUMBER OF VIABLE EMBRYOS AT VARIOUS DAYS TREATED WITH COBALT AND SELENIUM SUPPLEMENTED PINE NEEDLE EXTRACT

Days from incubation		7	10	14	17	20	21
Days from treatment		3	6	10	13	16	17
Treatment (0.2 ml)	No. eggs						
20 mg/ml P.N. extract	30	12	10	10	10	8	7
0.01 mg/ml $CoCl_2$	30	14	11	11	11	9	9
0.1 mg/ml $CoCl_2$	30	18	12	8	8	7	7
1 mg/ml $CoCl_2$	30	4	0	0	0	0	0
10 mg/ml $CoCl_2$	30	0	0	0	0	0	0
1 mg/ml $CoCl_2/Na_2SeO_3$	30	17	12	10	9	6	5
20 mg/ml P.N. extract	20	8	8	6	6	5	4
0.01 mg/ml Na_2SeO_3	20	9	7	7	5	5	4
0.1 mg/ml Na_2SeO_3	20	3	2	2	2	2	1

controls of untreated 80% ethyl alcohol (table 7). Data in table 7 show the 20 mg/ml concentration of lipid extract dissolved in acetone also produced no increases in death response compared to controls of untreated acetone but actually decreased it ($P<.01$). These results along with those in experiment 2 show no support of a link to pine needle abortion with lipids or other non-polar materials that are extracted along with lipids. Due to the fact that non-polar materials are not soluble in water, lipids or other non-polar material should not be an ingredient in the aqueous pine needle extract used in experiment 2.

Results relative to the last day of incubation shown in table 6 indicate that 35 percent of the chicken embryos died when the eggs were injected via the air cell with 10 ul of acetone. This negates work done by Strange et al. (1976) who reported a 98 percent hatching rate with chicks that were injected via the air cell with 15 ul of acetone. Figures in table 6 also indicate that 40 percent of the chicks died when the eggs were injected with 20 ul of 80% ethanol. McLaughlin et al. (1963) reported a 95 percent hatching rate with chicks that were injected with 50 ul of undiluted ethyl alcohol into the yolk sac of fertile eggs. Although the injection sites were different, there was still a very large difference in results.

TABLE 6. EXPERIMENT 4 - NUMBER OF VIABLE EMBRYOS AT VARIOUS DAYS TREATED WITH TOTAL LIPID EXTRACT FROM PINE NEEDLES

Days from incubation		7	10	14	17	20	21
Days from treatment		3	6	10	13	16	17
Treatment	No. eggs						
EtOH (20 ul)	30	21	18	18	16	16	12
5 mg/ml EtOH sol. (20 ul)	30	24	21	21	21	17	14
10 mg/ml EtOH sol. (20 ul)	30	26	22	20	20	17	13
15 mg/ml EtOH sol. (20 ul)	30	23	21	18	18	15	11
20 mg/ml EtOH sol. (20 ul)	30	21	20	19	16	13	10
Acetone (10 ui)	30	22	17	14	13	8	7
20 mg/ml Ac. sol. (10 ul)	30	27	26	25	25	24	20

TABLE 7. EXPERIMENT 4 - CHI SQUARE ANALYSIS OF TREATMENT EFFECT COMPARED TO CONTROLS

Treatment	No. dead embryos	No. viable embryos	X^2
EtOH (20 ul)	18	12	
5 mg/ml EtOH sol. (20 ul)	16	14	0.27149
10 mg/ml EtOH sol. (20 ul)	17	13	0.06857
15 mg/ml EtOH sol. (20 ul)	19	11	0.07051
20 mg/ml EtOH sol. (20 ul)	20	10	0.28708
Acetone (10 ul)	23	7	
20 mg/ml Acetone sol. (10 ul)	10	20	11.38047*

*P<.01, 1 d.f.

Experiment 5 - Carbohydrate fraction. Purified carbohydrate extract, in aqueous concentrations shown in tables 9 and 10, was injected into fertile eggs and compared to controls. All the carbohydrate extract concentrations except the highest (20 mg/ml) had an increased ($P<.05$) death response compared to the controls (table 9). When the 20 mg/ml concentration was repeated using a larger population (60 eggs), it also had an increased ($P<.05$) death response over the controls (table 9). Figures in table 8 show that most deaths appeared to occur within 7 days after treatment.

There seems to be no increasing death response with increasing concentration. This does not follow the trend from injecting water extracts of the crude pine needles. If the abortive factor is contained entirely within the purified carbohydrate fraction, it would seem to be more concentrated and when injected would cause at least as many deaths as injection of crude pine needle water extracts. However, the carbohydrate fraction did demonstrate lethality to chick embryos at all levels. It is possible that the abortive factor could be some material such as a glycoprotein or other plant components that is bound to a carbohydrate unit.

Pine needles contain approximately 48 percent NFE (nitrogen free extract) indicating a comparatively high available carbohydrate level. No simple sugars were found in the purified carbohydrate extract prior to hydrolysis. Soluble carbohydrates must occur either as oligosaccharides or as polysaccharides other than cellulose or hemicellulose. According to Robinson (1975), polysaccharides in plants

TABLE 8. EXPERIMENT 5 - NUMBER OF VIABLE EMBRYOS AT VARIOUS DAYS TREATED WITH PURIFIED CARBOHYDRATE EXTRACT FROM PINE NEEDLES

Days from incubation		7	11	14	17	20	21
Days from treatment		3	7	10	13	16	17
Treatment (0.2 ml)	No. eggs						
pH adjusted dist. H_2O	30	26	26	26	25	24	24
5 mg/ml ext.	30	22	21	20	19	17	15
10 mg/ml ext.	30	20	16	15	15	15	14
15 mg/ml ext.	30	24	20	18	18	18	15
20 mg/ml ext.	30	27	24	23	22	22	20
pH adjusted dist. H_2O	60	58	52	48	48	48	45
Repeated 20 mg/ml ext.	60	44	43	40	38	38	34

TABLE 9. EXPERIMENT 5 - CHI SQUARE ANALYSIS OF TREATMENT EFFECT COMPARED TO CONTROLS

Treatment (0.2 ml)	No. dead embryos	No. viable embryos	X^2
pH adjusted dist. H_2O	6	24	
5 mg/ml ext.	15	15	5.93406*
10 mg/ml ext.	16	14	7.17703**
15 mg/ml ext.	15	15	5.93406*
20 mg/ml ext.	10	20	1.36364
pH adjusted dist. H_2O	15	45	
Repeated 20 mg/ml ext.	26	34	4.48285*

*P<.05, 1 d.f.
**P<.01

such as starch, pectic substances, gums, mucilages, and fructans are soluble in boiling water and should not be removed by ion exchange. An iodine test for starch was used on the ion exchange effluent of the pine needle extract and showed negative results. Possibly starch and other polysaccharides are too complex to pass through the small mesh size (200-400 mesh) of the ion exchange resins. Fragments of these polysaccharides should be present in the effluent and thus would also be in the purified carbohydrate extract that was injected into the fertile eggs.

Neutral Sugar Components of the Carbohydrate Fraction

According to the R_f value and the characteristic color of the chromatographic spot, xylose, glucose, and galactose were positively identified as unknown number 1, 3, and 4 respectively (table 10). As shown in table 10, one spot designated as unknown #2 has a R_f value similar to arabinose, fructose, and mannose standards but the color of the spot was different. The color reagent characterizes aldo-hexoses as green spots and aldo-pentoses as red spots but this particular spot portrayed a brown spot. When the pine needle carbohydrate hydrolyzate was spotted on a chromatogram along side a mixture of five standard sugars (xylose, arabinose, fructose, glucose, and galactose), all unknown spots coincided with adjacent standards. The unknown spot (#2) would appear to be either arabinose or fructose except the color was brown rather than bright orange which was portrayed with the fructose and arabinose mixture. When the standard mixture included mannose as

TABLE 10. SEPARATION OF SUGARS IN THE CARBOHYDRATE EXTRACT OF PINE NEEDLES*

Sugar	R_f	Color of spot
In Hydrolyzate		
Unknown #1	.890	Red
Unknown #2	.786	Brown
Unknown #3	.669	Green
Unknown #4	.575	Green
Standards		
Galactose	.573	Green
Glucose	.667	Green
Xylose	.891	Red
Arabinose	.790	Red
Mannose	.800	Green
Fructose	.781	Green
Sorbose	.639	Green
Ribose	.950	Red
Mannose-Arabinose 1:1	.790	Brown
Fructose-Arabinose 1:1	.788	Orange
Fructose-Mannose-Arabinose 1:1:1	.788	Brown

*Separation occurred using a solvent of n-Butanol:Pyridine:H_2O. Color reagent used was Aniline Hydrogen Phthalate.

well as arabinose and fructose, a brown color corresponding to unknown #2 was obtained with a similar R_f number. Therefore, it would appear that unknown #2 is a mixture including arabinose, mannose, and a possibility of fructose which can not be clearly separated using this solvent system. Other solvent systems and sprays attempted were also unsuccessful in separation of the components comprised of unknown spot #2.

The neutral sugars in this carbohydrate extract are very similar to the constituents of hemicellulose. Jermyn (1955) classifies most hemicelluloses as polymers of xylose, arabinose, mannose, galactose and possibly some other monose units. Hirst (1962) states that the molecular structure of hemicellulose in many trees also involves glucose residues. No comprehensive work covering the hemicelluloses and the simple sugar composition of the genus Pinus has yet been expressed in literature (Mirov, 1967). Smith and Zavarin (1960) show that different parts of a tree differ markedly in amounts of simple sugars. Arabinose, glucose, galactose, and xylose seem to be present in the heartwood and outer bark of Pinus ponderosa. Glucose, fructose, and sucrose seem to be characteristic for the sapwood and inner bark. There is very little previous reference to the simple sugar components of the needles and buds.

SUMMARY AND CONCLUSIONS

A series of experiments was conducted to determine the value of chick embryo bioassays for measuring the detrimental effect of pine needle extracts and their sub-fractions. Ponderosa pine extracts, non-abortive standards, and accessory factors for alleviating toxicity were tested for sensitivity to the chick embryo. Effort was made to separate the principal components of pine needles with special emphasis on the separation and further identification of the carbohydrate constituents. The following general conclusions were made on the basis of the results obtained.

1. In experiment 1, a freeze-dried aqueous pine needle extract in a concentration of 20 mg/ml in water showed an increased ($P<.05$) death response to chick embryos at the half-way point in incubation (11 days). At the end of incubation more deaths occurred as the concentrations were increased from 0.5 mg/ml to 20 mg/ml; however, the differences were not significant. This material previously caused reproductive failure in rats shortly after being prepared (July, 1971). The lack of response in the present study may be related to extract storage time or difference in bioassay method sensitivity.

2. In experiment 2, it was demonstrated that pine needle extracts do have a detrimental effect on chick embryos. A 20 mg/ml concentration of freeze-dried pine needle aqueous extract from more recent collections of pine needles (October, 1977) displayed an increase ($P<.01$) in death response with chick embryos. Similar preparations

thought not to contain abortive potential such as autoclaved pine needles and Black Hills spruce did not significantly increase the death response and therefore did not have detrimental effects on chick embryos. The merit of the chick embryo bioassay for pine needle abortion was demonstrated in this experiment.

3. In experiment 3, the chick embryo showed signs of sensitivity to cobalt and selenium additions to injected pine needle extract. All levels higher than 0.1 mg/ml of $CoCl_2$ and 0.01 mg/ml of Na_2SeO_3 were not tolerated by the embryo. Cobalt and selenium salts did not reduce the large death rate caused by the 20 mg/ml concentration of aqueous pine needle extract. The toxicity of pine needle extracts was not alleviated with cobalt and selenium; in fact, they were toxic in themselves.

4. In experiment 4, the lipid fraction of pine needles demonstrated to be no more lethal to chick embryos than the controls. All levels of lipid extract dissolved in 80% ethanol produced no significant increases in death response compared to controls of untreated 80% ethanol. The concentration of lipid extract dissolved in acetone also produced no increases in death response compared to controls of untreated acetone but actually decreased it ($P<.01$). Data did not support an abortive factor link to lipids or other non-polar materials in pine needles.

5. In experiment 5, a purified carbohydrate extract of pine needles showed lethality to chick embryos. The carbohydrate fraction contained oligosaccharides and possible fragments of polysaccharides

excluding cellulose and most hemicelluloses. All concentrations in water of this carbohydrate extract had an increased ($P<.05$) death response to chick embryos. However, there was no increasing death rate with increasing concentrations. Some components of the carbohydrate fraction of pine needles could possibly play a role in pine needle abortion.

6. Chromatographic separation of neutral sugars in the purified carbohydrate extract revealed xylose, galactose, glucose, arabinose, mannose, and possible traces of fructose.

In conclusion, the search for the abortive factor in pine needles is still clouded by contradictions. For example, recent reports by Anderson and Lozano (1979) imply that autoclaving pine needles enhances the toxic effects on mice which is suggestive of a heat stable toxin in pine needles. The chick embryo bioassay tests support previous research (Allen and Kitts, 1961; Allison and Kitts, 1964; Anderson and Lozano, 1977; Chow et al., 1972) which describes the abortive factor to be water soluble and heat labile. It is also postulated that the active factor is not included in the lipid fraction but may be partially included in the carbohydrate fractions.

Two avenues of research appear to be needed. There must be a definition of pine needle abortion as it occurs in the animal; and, actual isolation of the abortive factor must be accomplished. The chick embryo bioassay has proven to be a good screening technique but it should only be regarded as a means for further investigation.

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