



A phytoestrogen from *Pinus ponderosa* assayed by competitive binding with 17β -estradiol to mouse uterine control
by William Douglas Wagner

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry
Montana State University
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Abstract:

This work describes the isolation of a phytoestrogenic fraction from *Pinus ponderosa*, western yellow, or Ponderosa pine. Ranchers and veterinarians report a high incidence of abortion and weak calf losses with cattle ingesting Ponderosa pine needles. Phytoestrogens from other plant species are known to induce similar responses to those observed with pine needle abortion.

The object of this study was to isolate and characterize the principal phytoestrogenic activity in the needles of *Pinus ponderosa*. Compounds elicit estrogenic responses in target tissue by initially binding to a cytoplasmic estrogen receptor protein. Components of Ponderosa pine were assayed for competitive binding with 17β -estradiol to mouse uterine cytosol. The biological activity of a phytoestrogen fraction was determined using a 24 hour uterine growth assay in immature mice.

Ponderosa pine needles contain a phytoestrogenic fraction (0.7 mg/g ground needles), that competes with 17β -estradiol for specific binding to mouse uterine cytosol. The phyto-estrogen is obtained by fractionating the aqueous extract with 90-95% aqueous acetone and elution from a polyvinylpyrrolidone column with 90% methanol. The uterotrophic activity of the phytoestrogen and 17β -estradiol were additive. The impeded estrogenic activity isolated from *Pinus ponderosa* was comparable to that found in other plants known to cause reproductive failure in cattle.

The phytoestrogenic fraction obtained in this study had characteristics of a nonphenolic, high molecular weight complex carbohydrate retained in dialysis tubing with a 14,000 M.W. cutoff. This is atypical of the many smaller phenolic compounds known to possess estrogenic activity. Toxicity of this phytoestrogen to immature mice was higher than any fraction of Ponderosa pine reported to date.

Investigation of the effect of this novel phytoestrogen on bovine reproduction might clarify the etiology of the pine needle abortion syndrome. Further study of the pharmacology of this fraction may contribute to the basic model of hormone-receptor interaction.

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BINDING WITH 17 β -ESTRADIOL TO MOUSE UTERINE CONTROL**

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William Douglas Wagner

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

of

Doctor of Philosophy

in

Biochemistry

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APPROVAL

of a thesis submitted by

William Douglas Wagner

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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DEDICATION

This work is dedicated to Dr. Edward L. Moody

To ponder here, o'er spells and signs,
Symbolic letters, circles, lines;
And from their actual use refrain,
Were time and labour lost in vain . . .

—Goethe

VITA

William Douglas Wagner was born November 29, 1953 in Minneapolis, Minnesota, the youngest of four children born to Paul and Thelma Wagner.

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William married Karen Kay Witzel August 17, 1974 and they were blessed with a daughter, Karla Marie, born September 7, 1981.

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ABSTRACT

This work describes the isolation of a phytoestrogenic fraction from *Pinus ponderosa*, western yellow, or Ponderosa pine. Ranchers and veterinarians report a high incidence of abortion and weak calf losses with cattle ingesting Ponderosa pine needles. Phytoestrogens from other plant species are known to induce similar responses to those observed with pine needle abortion.

The object of this study was to isolate and characterize the principal phytoestrogenic activity in the needles of *Pinus ponderosa*. Compounds elicit estrogenic responses in target tissue by initially binding to a cytoplasmic estrogen receptor protein. Components of Ponderosa pine were assayed for competitive binding with 17β -estradiol to mouse uterine cytosol. The biological activity of a phytoestrogen fraction was determined using a 24 hour uterine growth assay in immature mice.

Ponderosa pine needles contain a phytoestrogenic fraction (0.7 mg/g ground needles), that competes with 17β -estradiol for specific binding to mouse uterine cytosol. The phytoestrogen is obtained by fractionating the aqueous extract with 90-95% aqueous acetone and elution from a polyvinylpyrrolidone column with 90% methanol. The uterotrophic activity of the phytoestrogen and 17β -estradiol were additive. The impeded estrogenic activity isolated from *Pinus ponderosa* was comparable to that found in other plants known to cause reproductive failure in cattle.

The phytoestrogenic fraction obtained in this study had characteristics of a nonphenolic, high molecular weight complex carbohydrate retained in dialysis tubing with a 14,000 M.W. cutoff. This is atypical of the many smaller phenolic compounds known to possess estrogenic activity. Toxicity of this phytoestrogen to immature mice was higher than any fraction of Ponderosa pine reported to date.

Investigation of the effect of this novel phytoestrogen on bovine reproduction might clarify the etiology of the pine needle abortion syndrome. Further study of the pharmacology of this fraction may contribute to the basic model of hormone-receptor interaction.

INTRODUCTION

Many plants contain toxic or pharmacologically active components. This work describes the isolation of a phytoestrogenic fraction from *Pinus ponderosa*, western yellow or Ponderosa pine.

Ranchers and veterinarians report a high incidence of abortion and weak calf losses with cows ingesting Ponderosa pine needles (MacDonald, 1952; Faulkner, 1969). Pine needle-induced abortion has been observed in cattle under controlled conditions (Stevenson, James, and Call, 1972; James, Call, and Stevenson, 1977). Nutritional and environmental stress of winter range conditions is a reported factor in both the consumption of the pine needles and the subsequent abortion. Call and James (1976) report that sheep also abort after eating Ponderosa pine needles.

The symptoms associated with pine needle-induced abortion include a high incidence of retained placenta, bloody discharge from the reproductive tract resulting from uterine hemorrhaging, vaginal swelling, unusual udder development, nymphomania, and apparent intoxication (James et al., 1977). The swelling of the genitals quickly recedes and no abortion occurs if the cattle are separated from the needles. Some cows show estrus shortly after pine needle-induced abortion (Faulkner, 1969). These symptoms are consistent with potential phytoestrogenic activity in the needles of Ponderosa pine (Samuel, 1967; Cox and Braden, 1974; Labov, 1977).

Feeding studies also indicate that components in Ponderosa pine interfere with steroid hormone action in target tissues of laboratory ruminants. These studies describe the effect of whole needles and various extracts mixed with basal lab chow on the uterine

weight of immature female mice and on the disruption of pregnancy in mice fed over a period of several days.

Allen and Kitts (1961) reported uterine weights significantly decreased in immature mice fed the acetone extract of Ponderosa pine needles at 8 grams of needle equivalents over a 3 day period. The diethyl ether soluble fraction of this extract was toxic to the mice at this dose but did not affect uterine weight. Disruption of pregnancy was observed in mice fed 18 grams of needle equivalents of this acetone extract over 7 days. These results were confirmed by Cook and Kitts (1964), who also found the activity to be heat labile. Allison and Kitts (1964) reported the pine needle extract to inhibit uterine growth induced by diethylstilbestrol in immature female mice. They cited this activity as antiestrogenic.

Chow et al. (1972) also found the aqueous extract of pine needles to decrease uterine weight in immature female mice at a consumption rate of 9 grams of needle equivalents over 4 days. This aqueous extract also disrupted pregnancy in mice fed one gram of needle equivalents mixed in each gram of lab chow. The disruption of pregnancy did not occur if the aqueous extract was autoclaved. The acetone extract of needles previously extracted with water showed no effect on pregnancy. Chow, Hamar, and Udall (1974) suggested the embryotoxic activity may be a mycotoxin from fungal growth present on the needles. They observed the embryocidal activity was regenerated if the autoclaved aqueous extract supported fungal growth for two weeks at room temperature. This observation was reproduced by Anderson and Lozano (1976). This regenerated activity disrupted pregnancy in mice fed 49 grams of needle equivalents over an 11 day period.

The feeding of the whole needles was reported more toxic than the aqueous extract (Anderson and Lozano, 1979). The embryocidal activity they reported was soluble in methanol, ethanol, chloroform, hexane and n-butanol. Autoclaving the needles or the extracts enhanced the toxic activity by 30 percent (%). The dose resulting in 50% embryonic mortality was 9 grams of needle equivalents/mouse fed over a 6 day period.

Embryocidal activity in the hexane extract of Ponderosa pine needles was confirmed by Kubik and Jackson (1981). Oral administration of 125 mg of the hexane extract/day during the first five days of gestation resulted in a survival rate of embryos to day 8 of gestation 35% that of control mice. This represented 8 grams of pine needle equivalents per mouse fed over 4 days. The active components in the hexane extract were a mixture of resin acids. The dose causing 50% embryonic mortality was 22.4 mg of the resin acid mixture/mouse/day (2 grams of needle equivalents/mouse/day) over four days.

Weideman (1973) and Cogswell (1974) reported that the most pronounced embryocidal activity of Ponderosa pine occurs during feeding on days 4 and 5 of gestation in the mouse. This was confirmed by Anderson and Lozano (1979) and Kubik and Jackson (1981). This is the time of implantation of the blastocyst to the endometrium, i.e., the embryo is attaching to the uterine wall. This process of implantation is known to be preceded by a sharp rise in peripheral 17β -estradiol levels (McCormack and Greenwald, 1974). Administration of 17β -estradiol disrupted implantation in mice (Sartor et al., 1978). They showed disruption of implantation resulted from 10 μ g of estradiol injected on day 4 of gestation. The disruption of implantation in mice following Ponderosa pine needle ingestion may be caused by estrogenic activity in the needles.

All symptoms reported with Ponderosa pine needle ingestion, except general toxicity, are consistent with the phytoestrogenic activity previously found in a large number of plant species (Farnsworth et al., 1975). Bradbury and White (1954) listed over 50 species of plants shown to possess phytoestrogenic activity and many others have been reported since that time (Moule, Braden, and Lamond, 1963; Cox and Braden, 1974; Labov, 1977).

The three main classes of known phytoestrogens are the isoflavones, coumestans and resorcylic acid lactones (Labov, 1977). These non-steroidal estrogens typically induce estrogenic responses with low potency yet inhibit 17β -estradiol or diethylstilbestrol from eliciting full response in target tissue. Reports of phytoestrogenic activity are most com-

monly based on the induction of uterine growth or the cornification of vaginal epithelium in laboratory ruminants (Folman and Pope, 1966; Terenius, 1971).

Non-steroidal phytoestrogens of this type initially compete with 17β -estradiol for binding to the cytoplasmic estrogen receptor protein in the target tissue (Shutt and Cox, 1972; Shemesh, Lindner, and Ayalon, 1972; Baulieu, 1978; Jordan et al., 1978; Rochefort, Garcia, and Borgna, 1979). The receptor-ligand complex transforms into a form which migrates into the nucleus of the target cell (DeSombre, Mohla, and Jensen, 1975). The intricate mechanism by which the estrogen-receptor complex regulates transcription and the induction of protein synthesis remains unknown (Cidlowski and Muldoon, 1978; Korach, 1979).

This study is based on the hypothesis that Ponderosa pine needles contain at least one phytoestrogenic component. The symptoms associated with pine needle ingestion include abortion, high incidence of retained placenta, vaginal swelling, bloody discharge from the reproductive tract resulting from uterine hemorrhaging, unusual udder development, and nymphomania observed in cattle. All of these symptoms along with disruption of pregnancy and changes in uterine growth in laboratory ruminants are consistent with estrogenic activity in the needles of Ponderosa pine (Labov, 1977).

The non-steroidal phytoestrogenic compounds previously identified are known to compete with 17β -estradiol for binding to uterine cytoplasmic estrogen receptor protein. Although the affinity of the estrogen receptor protein is considerably less for the known phytoestrogens than for 17β -estradiol (Shutt and Cox, 1972), fractions containing phytoestrogenic components should display enhanced binding affinity relative to other inactive fractions. Thus, competitive binding assays with 17β -estradiol were used to screen potential phytoestrogens in various fractions of Ponderosa pine needle extracts.

The objective of this study was to isolate and characterize the principal component(s) in *Pinus ponderosa* eliciting phytoestrogenic activity. An isolation procedure was developed

based on enhanced tritiated 17β -estradiol displacement from mouse uterine cytosol resulting with each fractionation step. Fractions void of estradiol displacing activity to estrogen specific sites in the uterine cytosol were considered unlikely to contain phytoestrogenic components. The value of each fractionation step was assessed by the enhancement of the relative estradiol displacement of the new fraction.

Ponderosa pine needles were extracted using various solvents ranging in polarity from hexane to water. The extracts were assayed for tritiated 17β -estradiol displacement activity to mouse uterine cytosol. Isolation of the main estradiol displacing activity was attempted using a variety of fractionation procedures.

The biological activity of the potential phytoestrogenic fraction(s) was determined. The effect of interperitoneal injection and oral administration of the phytoestrogen(s) on uterine growth in immature mice was assayed.

MATERIALS AND METHODS

Extraction of Ponderosa Pine Needles

The lower branches of *Pinus ponderosa* were collected near Grey Cliff, Montana, and stored at 4 C until used within two months. The needles and buds were removed and cut into 1-2 cm lengths and finely ground with an electric coffee grinder.

Extractions of groups of needles were made with 10 ml of extraction solvent for each gram of needles to allow full exposure of the needles to solvent. Preliminary extractions were made with hexane, carbon tetrachloride, benzene, n-butanol, acetone, ethanol, acetonitrile, p-dioxane, and water. All solvents were redistilled before use. The extractions were performed by constant shaking at room temperature for 24 hours. The extracts were filtered through Whatman No. 1 filter paper and the solvent was removed by rotary evaporation under reduced pressure at 37 C. The extracts were weighed and redissolved in the appropriate solvent and stored at 4 C until used in an estradiol displacement assay.

Soxlet extraction of the needles with hexane, acetone, or water was performed by refluxing the solvent through the ground needles for four hours. Filtration and weighing of these extracts were performed according to the procedure described above.

Aqueous extracts were found to contain the most phytoestrogen so procedures from here on are based on aqueous extracts.

Acetone Fractionation of the Aqueous Extract

Aqueous extraction was repeated three times and the extracts were combined and condensed by rotary evaporation. The extract was made 90% by volume in acetone. The gummy precipitate was extracted four times with 90% acetone by vigorous rotation in a

37 C water bath for 30 minutes. The 90% acetone soluble decantate was taken to dryness by rotary evaporation, weighed, and extracted four times with 100% acetone at 37 C for 30 minutes. The acetone insoluble material was extracted four times with 95% acetone at 37 C. The solvent was removed by rotary evaporation, the fractions were weighed, redissolved in water and used in an estradiol displacement assay.

Poly-N-vinylpyrrolidone Column Chromatography

This procedure is based on the method reported by Mousedale and Knee (1979). Cross-linked poly-N-vinylpyrrolidone (obtained from Aldrich, Milwaukee, WI) of 40-80 mesh particle size was thoroughly washed in 10% aqueous HCl and exhaustingly rinsed with methanol. A slurry of poly-N-vinylpyrrolidone, PVP, in 90% methanol in water was packed in a glass column 20 cm × 1.4 cm inner diameter (31 ml bed volume) and equilibrated by elution of 250 ml of 90% methanol. The 90-95% acetone soluble fraction of the pine needle aqueous extract (300 mg) was applied to the column in 0.8 ml of 90% methanol and eluted with 90% methanol from a reservoir 25 cm above the column bed resulting in a flow rate of 0.4 ml/min. Fractions (1.1 ml) were collected using a Gilman microfractionator. Fractions were evaporated to dryness under a stream of nitrogen at 35 C, weighed, and redissolved in water conc. An aliquot of the fractions were used in an estradiol displacement assay.

Dialysis

The PVP column 16.5-19.7 ml elution fraction (3.3 mg) was dissolved in 0.6 ml of distilled water and sealed in 45 mm Spectropor semi-permeable membrane tubing with a 12,000 to 14,000 molecular weight cutoff (Spectrum Medical Industries, Inc., Los Angeles, CA) which was pre-soaked in distilled water for two days at 4 C. This fraction was dialyzed against four changes of 80 ml distilled water at twelve hour intervals. The dialy-

sate and sample were dried by rotary evaporation, weighed, and used in an estradiol displacement assay.

Preparation of Mouse Uterine Cytosol

Randomly bred immature female Dub:ICR mice weighing 10-15 g were sacrificed by cervical dislocation. The uteri were stripped of fat and immediately placed on ice. All subsequent operations were performed at 0-4 C. The uteri were cut into 0.5 cm lengths and homogenized with four volumes (ml/g) of TEK buffer (.01 M Tris, pH 7.4; 1.5 mM EDTA; .4 M KCl; 1 mM dithioerythritol) using a Virtis tissue homogenizer with a setting of 50 for three 10-second bursts. The suspension was processed in a Thomas teflon-glass hand tissue homogenizer.

The nuclear-myofibullar fraction was pelleted by centrifugation at 1055 X g for 10 minutes using a Sorvall RC2-B centrifuge with an SS-34 rotor. The supernate was made 7.5×10^{-9} M in [2,4,6,7-³H] 17 β -estradiol (94 Ci/mmol, New England Nuclear, Boston, MA) at a final volume of 10 ml/gram tissue (Puca, Nola, Sica, and Bresciani, 1975).

The high speed supernate was obtained by centrifugation at 87,000 X g for 60 minutes using a Beckman L2 ultracentrifuge with an SW-27 rotor. This cytosol was stored in the cold room at 4 C until used in the estradiol displacement assays.

Estradiol Displacement Assay

The tritiated estradiol displacement of the pine needle fractions on mouse uterine cytosol was assayed based on the method of Shemesh, Lindner, and Ayalon (1972). Aliquots (50 μ l) of the mouse uterine cytosol were pipetted into $\frac{1}{2}$ " X 2" polyallomer tubes (Beckman, Irvine, CA) along with 150 μ l of TEK buffer. Fifty μ l of test sample or TEK buffer was added and mixed by vortexing. The assay tubes were incubated at 4 C for 24 hours.

Unbound tritiated 17β -estradiol was removed by addition of 100 μ l of dextran-coated charcoal suspended in the TEK buffer (5% Norit A, .5% dextran). After 20 minutes, the tubes were centrifuged at 1000 \times g for 10 minutes (Kushinski and Anderson, 1975).

The supernate was carefully poured into scintillation vials along with 15 ml of toluene-based scintillation fluor (8 g 2,5-diphenyloxazole; 200 g naphthalene; 700 ml toluene; 300 ml 95% ethanol). The vials were counted in a Beckman LS-100 liquid scintillation counter using a preset tritium window to 1% error. The counting efficiency was 38.9%.

The determination of nonspecific binding of 17β -estradiol to the mouse uterine cytosol was made by heating the cytosol to 65 C for 5 minutes or by making the assay mixture 10^{-7} M in trans-diethylstilbestrol.

The percent of bound 17β -estradiol was computed by the following formula:

$$\frac{(\text{Sample cpm} - \text{Nonspecific cpm})}{(\text{Specific cpm} - \text{Nonspecific cpm})} \times 100\% = \% \text{ Bound}$$

The relative estradiol displacement activity for the phytoestrogen sample was estimated as the concentration of the sample in the assay mixture that results in 50% displaced 17β -estradiol.

Long-Term Uterotrophic Response Assay

The uterine growth in immature mice activity of the various phytoestrogen samples were tested by the method of Jordan, Dix, and Naylor (1978), and Terenius (1971). Immature female Dub:ICR mice, weighing 12-16 grams, were randomly placed in groups of five or more mice each. Each mouse was weighed immediately before administration of the test sample and identified with a colored mark at the base of the tail.

Intraperitoneal injection of 0.1 ml sample was performed with a 1 ml tuberculin syringe with a 27G \times $\frac{1}{2}$ inch needle. Oral administration by stomach tube was facilitated

by 4 cm × 1 mm polyethylene tubing connected to a 100 μ l syringe by a 27G × ½ inch needle.

The mice were sacrificed by cervical dislocation 24 hours after treatment. The uteri were removed and trimmed of fat, carefully blotted dry, and weighed. The uteri were placed in an oven at 100 C for 18 hours and reweighed.

The treatment groups were:

A. *Via Interperitoneal Injection*

1. NaCl 0.9% wt/vol (all samples administered intraperitoneally were dispensed in 0.9% NaCl solution).
2. Poly-N-vinylpyrrolidone (PVP) column elution 16.5-18.7 ml fraction of the pine needle extract at 700, 500, 350, 250, 175, 125, 87.5, and 62.5 μ g/15 g body weight.
3. 17 β -Estradiol at 5, 10, 25, 50, and 100 ng/15 g body weight.
4. 17 β -Estradiol at 10 ng/15 g body weight plus 175 μ g or 700 μ g of the PVP column elution fraction used in treatment 3.

B. *Via Stomach Tube*

5. Distilled water (all samples administered orally were dissolved in distilled water).
6. Poly-N-vinylpyrrolidone (PVP) column 16.5-18.7 ml elution fraction at 1400, 700, 350, and 175 μ g/15 g body weight.
7. The PVP column elution fraction, 700 μ g, used in treatment 2 plus interperitoneal injection of 10 or 50 ng/15 g body weight of 17 β -estradiol.
8. The 90% acetone insoluble fraction of the pine needle aqueous extract at 180 mg/15 g body weight.
9. The 95-100% acetone soluble fraction of the pine needle aqueous extract at [20 mg/15 g body weight].

Ultraviolet Absorption Spectra

Samples of known concentration were diluted at 1.2 ml of distilled water in quartz cuvettes of 1.0 cm pathlength. The absorption spectrum was scanned between 300 and 200 nm at 50 nm/min using a Varian Techtron UV-VIS model 635 dual beam spectropho-

tometer with a slit width of 1.0 nm. Fifty μl of 5% NaOH was added to sample and blank and the scan repeated after thorough mixing with the microsyringe needle. The spectra were documented by a recorder set at 1.0 optical density full scale.

Carbohydrate Analysis of Phytoestrogen Fraction

Addition of 1 ml of 5% phenol was followed by 3 ml of conc. sulfuric acid to 1 ml of duplicate samples of either distilled water, 100 to 2.5 $\mu\text{g/ml}$ D-glucose standard, or 750 to 94 $\mu\text{g/ml}$ of the poly-N-vinylpyrrolidone column 16.5-18.7 ml elution fraction in aqueous solution. The tubes were thoroughly mixed and sat 10 minutes at room temperature. They were then incubated at 37 C for 25 minutes. The absorbance of the PVP fraction and glucose standard samples were scanned between 650 and 400 nm.

Colorimetric determination of reducing sugars in the active PVP elution fraction was based on the method of Mattson and Tensen (1950). Color was developed by addition of 0.5 ml of 2% triphenyltetrazolium chloride in 5 N aqueous NaOH to 1 ml of distilled water, 75 to 10 $\mu\text{g/ml}$ D-glucose, or 75 to 10 $\mu\text{g/ml}$ of the PVP fraction. The tubes were heated at 94 C for 3 minutes after the addition of 0.5 ml of 2 N NaOH. The absorbance was read at 490 nm.

Determination of Hexoses by Condensation with Anthronol

Estimation of the hexose content of the phytoestrogen fraction was performed according to the method of Shields and Burnett (1960). Anthrone, 9,10-dihydro-9-oxo-anthracene, was dissolved in conc. sulfuric acid (.2% wt/vol) and added dropwise to 0.5 ml samples of 10-40 $\mu\text{g/ml}$ glucose or 20-50 $\mu\text{g/ml}$ phytoestrogen fraction at 0 C. The assay mixtures were placed in a boiling water bath for 8 minutes and reimmersed in an ice bath to stop the reaction. The absorbance spectra between 700-400 nm was recorded.

Hydrolysis and Gas-Liquid Chromatography of Alditol Acetates

Acid hydrolysis was performed by the addition of 0.5 ml of 2 N H_2SO_4 to 10 mg of the active PVP elution fraction dissolved in 0.5 ml distilled water and placed in a boiling water bath for 4 hours. The hydrolysate was centrifuged at $1000 \times g$ for 10 minutes and neutralized by addition of activated (HCO_3^- form) Amberlite IRA-410 anion exchange resin. The mixture was filtered through Whatman No. 1 filter paper and dried by rotary evaporation.

The alditol acetate derivatives of the hydrolysate were prepared according to the method of Gunner, Jones, and Perry (1961). The hydrolysate was redissolved in 2 ml of distilled water and 5 mg of $NaBH_4$ was added. The mixture was placed in the dark for 12 hours at 4 C. Activated Dowex 50 cation exchange resin was added until a pH of 4-5 was attained. The Dowex 50 was removed by filtration through Whatman No. 1 filter paper and the filtrate was dried by rotary evaporation. The $NaBH_4$ was removed by repeated addition of methanol and drying by rotary evaporation.

Two ml of 50% acetic anhydride in pyridine were added to the dried hydrolysate in a 50 ml round bottom flask. The flask was sealed with a glass stopper and placed in a 94 C water bath for 15 minutes. Two ml of distilled water was repeatedly added and dried by rotary evaporation. The reaction flask was rinsed thoroughly three times with 0.3 ml of chloroform. The alditol acetate derivatives of the hydrolysate and standard carbohydrates were stored at 4 C until analyzed by gas chromatography.

Alditol acetate samples were analyzed on a Varian 3700 gas chromatograph, using a 2 m \times 2 mm glass column packed with 3% ECNSS-M on Gas Chrom Q (110-120 mesh, Applied Science). Samples were chromatographed isothermally at 200 C with a flow rate of 30 ml/min or with a temperature program from 160 C to 200 C at 3 C/min with a flow

rate of 35 ml per minute. The injector temperature was 200 C and the flame ionization detector was set at 220 C with an attenuation of 8×10^{-10} A/mV.

The carbohydrate composition of the hydrolysate was determined by addition of standard alditol acetates co-chromatographed with the sample (Griggs et al., 1971). Peak areas were estimated by triangulation and used to determine the relative concentration of the sugars found in the hydrolysate.

Ninhydrin Determination of Free Amino Groups

The analysis of free amino groups within the phytoestrogen fraction was based on the method of Moore and Stein (1948).

Ninhydrin reagent was prepared by mixing 50 ml of 0.2 M citrate buffer, pH 5.0, containing 0.1 g $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ with 50 ml of 4% ninhydrin in methyl cellosolve. Samples (.1 ml) of the phytoestrogen (20 mg) or 0.5-2.0 mM leucine were mixed with 1.0 ml of the ninhydrin reagent. The reaction mixtures were heated 20 minutes in a boiling water bath and diluted with 5.0 ml of 50% vol/vol aqueous n-propanol. The absorbance was measured at 570 nM.

Additional Column Chromatography of the Phytoestrogen Fraction

Further attempts to resolve the phytoestrogen within the poly-N-vinylpyrrolidone column elution fraction were performed using Sephadex (Pharmicea, Piscataway, NJ 08854) and Biogel (BioRad Labs, Richmond, CA 94804). The phytoestrogen sample (10 mg in .2 ml) was applied to columns equilibrated by exhaustive elution with distilled water. The distilled water was degassed under reduced pressure.

Fractions eluting from these columns were used in the estradiol displacement assay.

Thermal Lability of the Phytoestrogen Fraction

Samples of the aqueous extract of *Pinus ponderosa*, the 90-95% soluble fraction, or the active poly-N-vinylpyrrolidone column elution fraction were heated in a boiling water bath for up to 4 hours. The estradiol displacement activity of the heat treated samples was determined.

RESULTS

The mouse uterine cytosol preparation used in the estradiol displacement assays contained 3.7 picomoles of 17β -estradiol specific high affinity binding sites/gram of uterus. This resulted in 2000 cpm for 100% bound 17β -estradiol compared to 500 cpm for 0% or nonspecific binding.

Relative Estradiol Displacement of the Ponderosa Pine Needle Extracts

The only extract displaying significant estradiol displacement activity with 17β -estradiol for mouse uterine cytosol was the aqueous extract. The relative estradiol displacement of a sample is expressed as the sample concentration in the assay mixture resulting in 50% displaced 17β -estradiol. Samples with apparent estradiol displacement activity greater than 2 mg/ml were considered inactive (Bradbury and White, 1954).

Extracts Without Significant Binding Activity

hexane	n-butanol	acetonitrile	acetone soxlet
carbon tetrachloride	acetone	p-dioxane	water soxlet
benzene	ethanol	hexane soxlet	

Estradiol displacement assays of the aqueous extract and subsequent fractions with enhanced estradiol displacement activity are shown in Figure 1.

Acetone Fractionation of the Aqueous Extract

The isolation scheme of the principal estradiol displacement activity from the aqueous extract is summarized in Figure 2. The yields per gram of dried needles and the apparent estradiol displacement activity are given at each separation step. Precipitation of 44% of

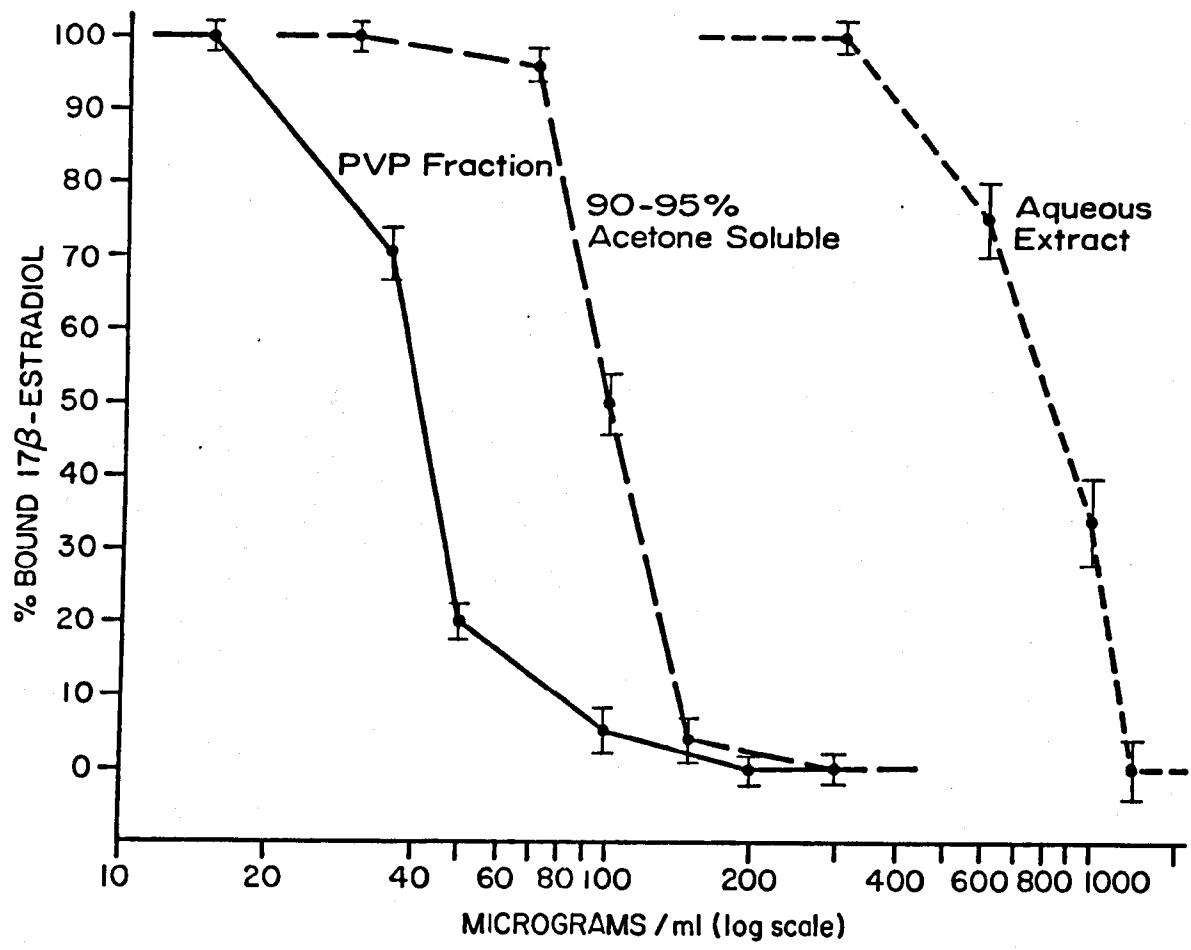


Figure 1. Estradiol displacement assays of the fractions of the aqueous extract of Ponderosa pine needles.

the aqueous extract with 90% acetone resulted in a slightly decreased estradiol displacement activity of 750 $\mu\text{g/ml}$. The 90% acetone insoluble fraction displayed negligible estradiol displacement activity.

A decrease in estradiol displacement to 100 $\mu\text{g/ml}$ for the 90-95% acetone insoluble fraction was observed. The estradiol displacement activity of the 100% acetone soluble fraction was considerably less (700 $\mu\text{g/ml}$).

Poly-N-vinylpyrrolidone Column Chromatography

Only 40% of the 90-95% acetone soluble fraction applied to the PVP column is recovered. The fraction with the most estradiol displacement activity elutes from the column at 16.5 to 18.7 ml (.53 to .60 bed volume of the column). This PVP column elution fraction has an apparent estradiol dissociation activity of 40 $\mu\text{g/ml}$. The estradiol displacement of this fraction is not enhanced by a second elution through the PVP column. The average yield of the PVP 16.5 to 18.7 ml elution volume fraction is .7 mg/g of needles. All other fractions eluting from the PVP column displayed estradiol displacement activities greater than 150 $\mu\text{g/ml}$ in the competitive binding assay. Figure 3 illustrates the poor resolution of the PVP column and the advantage of assaying the elution fractions for relative binding affinity to mouse uterine cytosol to determine which fractions are more active.

Dialysis of the Active PVP Column Fraction

The active fraction was retained in 12,000-14,000 molecular weight cutoff dialysis tubing and the estradiol displacement for binding to mouse uterine cytosol was unaffected by the dialysis.

Uterotrophic Activity of the Active Fraction

The response of uterine growth to intraperitoneal injection of 17β -estradiol is shown in Figure 4. The response appears saturated at a dosage of 50 ng of 17β -estradiol/15 g body

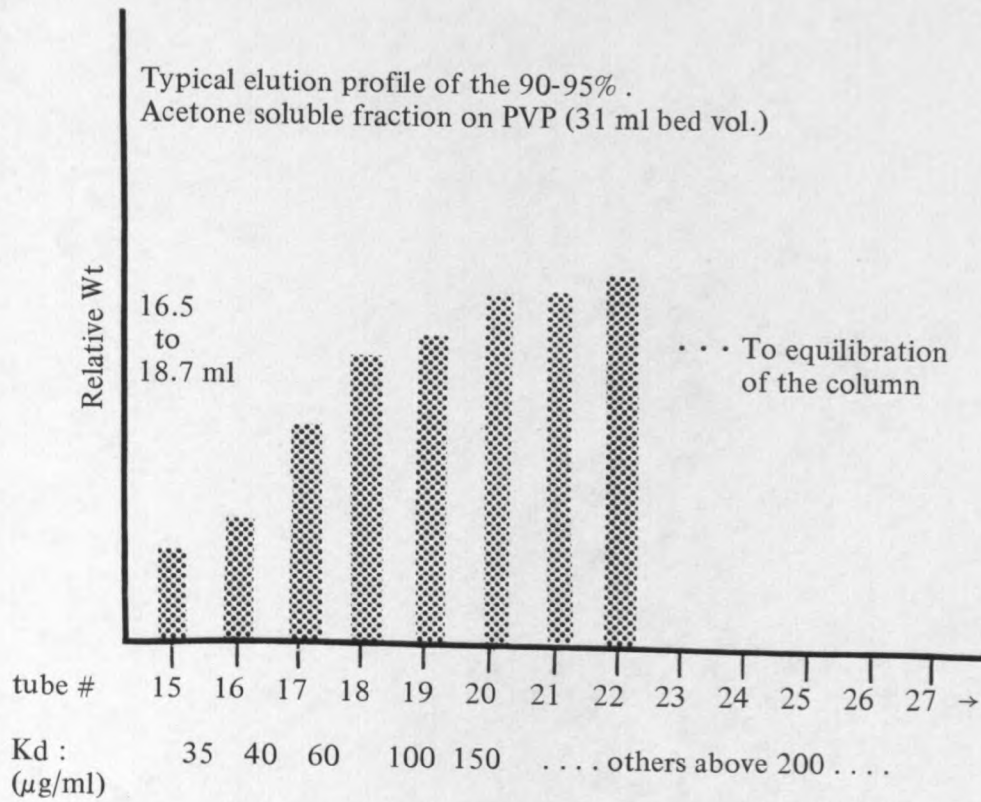


Figure 3. Illustration of the resolution of estradiol displacement activity resulting from poly-N-vinylpyrrolidone (PVP) column chromatography eluting with 90% methanol.

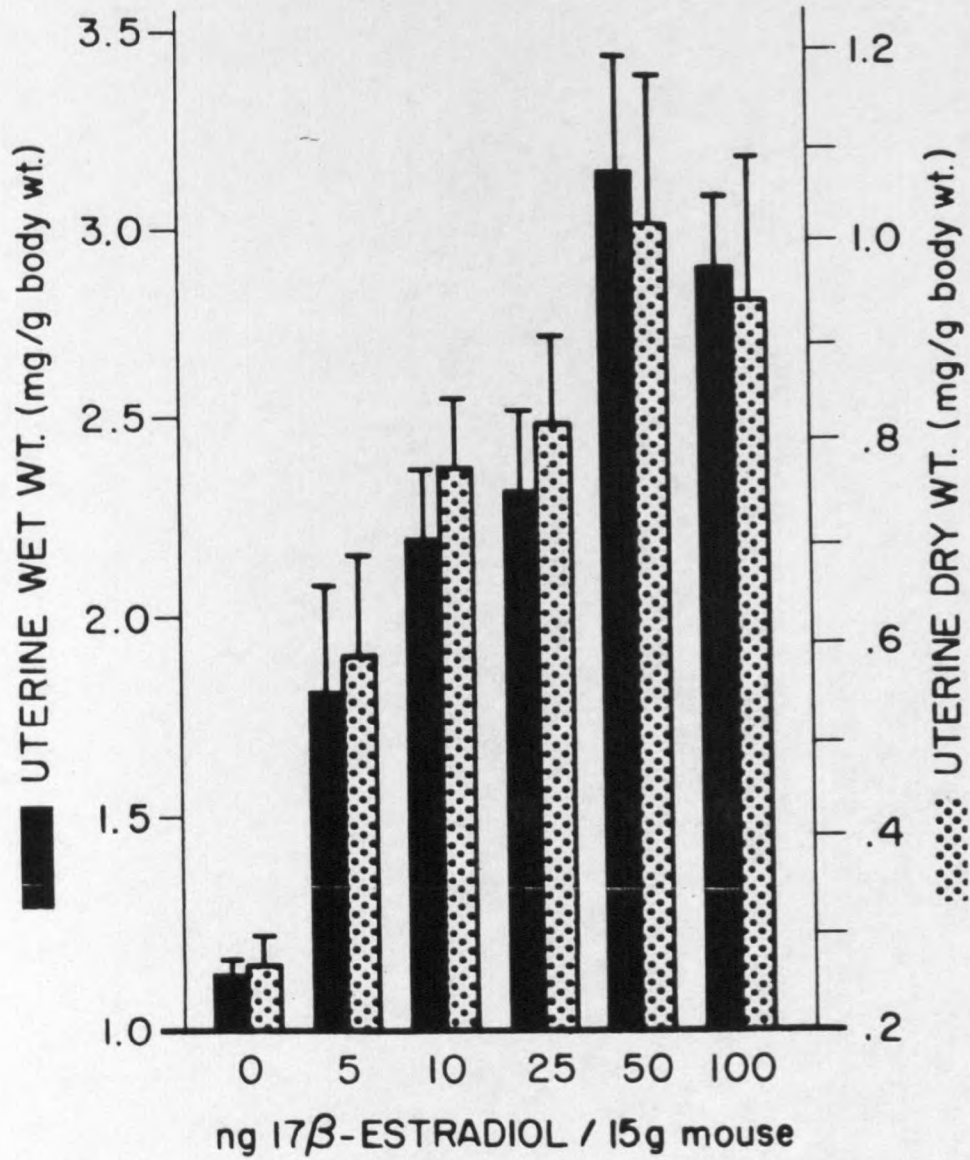


Figure 4. The 24-hour uterine growth response in immature mice to i.p. injection of 17β -estradiol, $n = 6$ except control group where $n = 13$.

weight. Each treatment group consisted of 6 mice each except the control group, where $n = 13$.

The uterotrophic activity of the active phytoestrogen fraction is presented in Figure 5. The dose-response curve is much shallower than that of i.p. injection of 17β -estradiol. The mice were intoxicated at a dose of $350 \mu\text{g}/15 \text{ g}$ body weight. This acute intoxication lasted several hours after injection. These mice typically lost 10% of their body weight or more. The administration of $700 \mu\text{g}$ of the PVP elution fraction represents one gram of pine needle equivalents. All treatment groups consisted of five mice except in the control group, where $n = 13$ and in the $700 \mu\text{g}/15 \text{ g}$ body weight where $n = 6$ (four of ten mice died). Intraperitoneal injection of $62.5 \mu\text{g}/15 \text{ g}$ body weight of the PVP elution fraction did not cause twenty-four hour uterine growth significantly different than controls. The PVP fraction did not elicit the full uterotrophic response that was observed with administration of 17β -estradiol.

Figure 6 compares the relative potency of oral administration of the PVP fraction to intraperitoneal administration. The slope of the dose-response curve for oral administration is approximately one fourth the slope for i.p. injection of the same phytoestrogen sample. Mice displayed acute intoxication for several hours following oral administration of $700 \mu\text{g}/15 \text{ g}$ body weight of the PVP active fraction. Two mice out of seven that were given $1400 \mu\text{g}$ of the phytoestrogenic fraction died within the 24 hour period prior to sacrifice. No controls died as a result of stomach tubing. The $1400 \mu\text{g}$ oral dose represents 2 grams of pine needle equivalents.

The synergism of the uterotrophic activities of the PVP active fraction and 17β -estradiol is summarized with Figure 7. There is a difference in the additivity of the uterotrophic activity of the pine phytoestrogen and 17β -estradiol subject to the *route* of administration of this PVP fraction. *Intraperitoneal* injection of $175 \mu\text{g}$ of the PVP fraction along with a 10 ng dose of 17β -estradiol results in a saturated response in uterine growth. How-

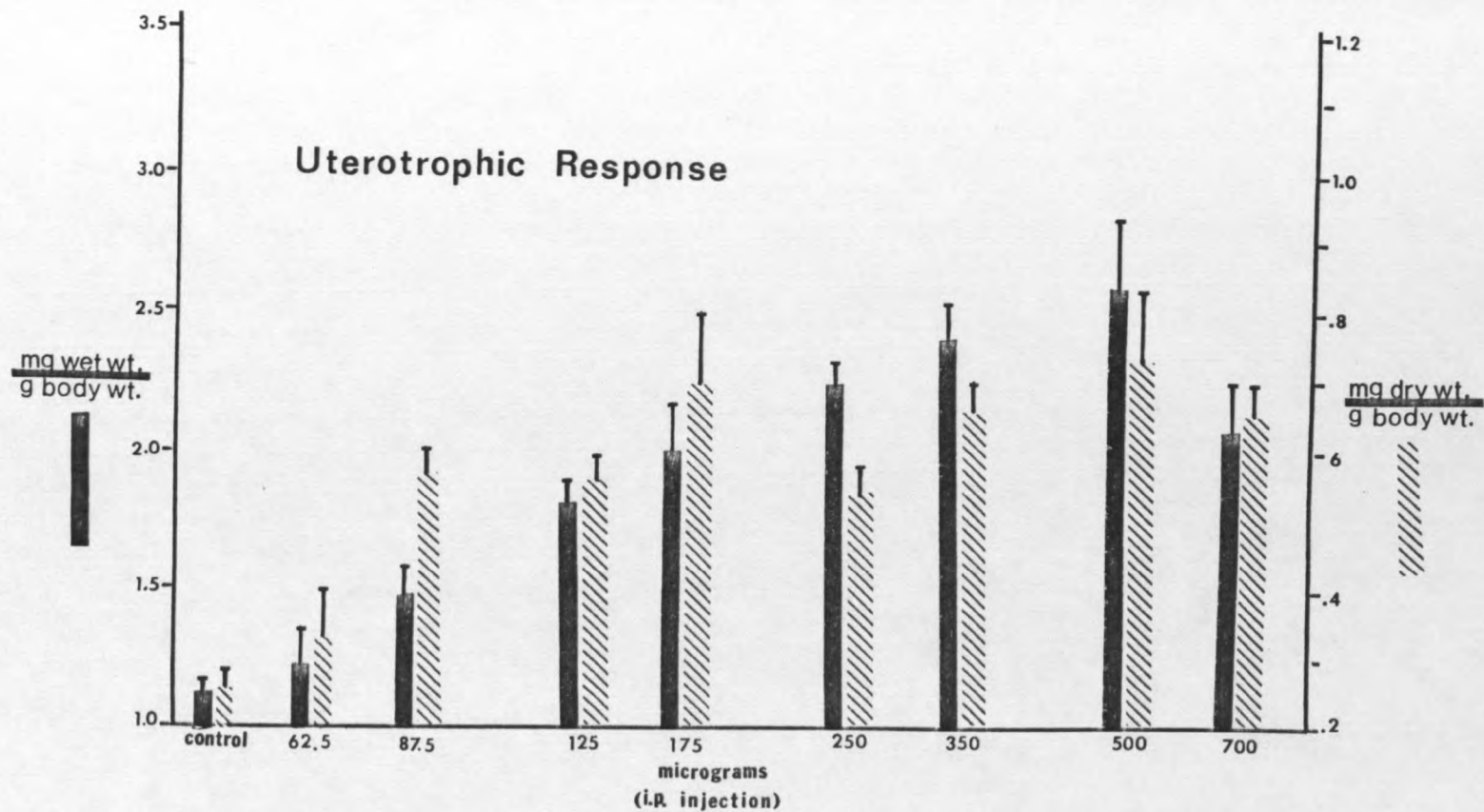


Figure 5. Uterotrophic response 24 hours after i.p. injection of the phytoestrogenic fraction in immature (15 g) mice, n = 5 for all treatment groups except those noted otherwise above bars.

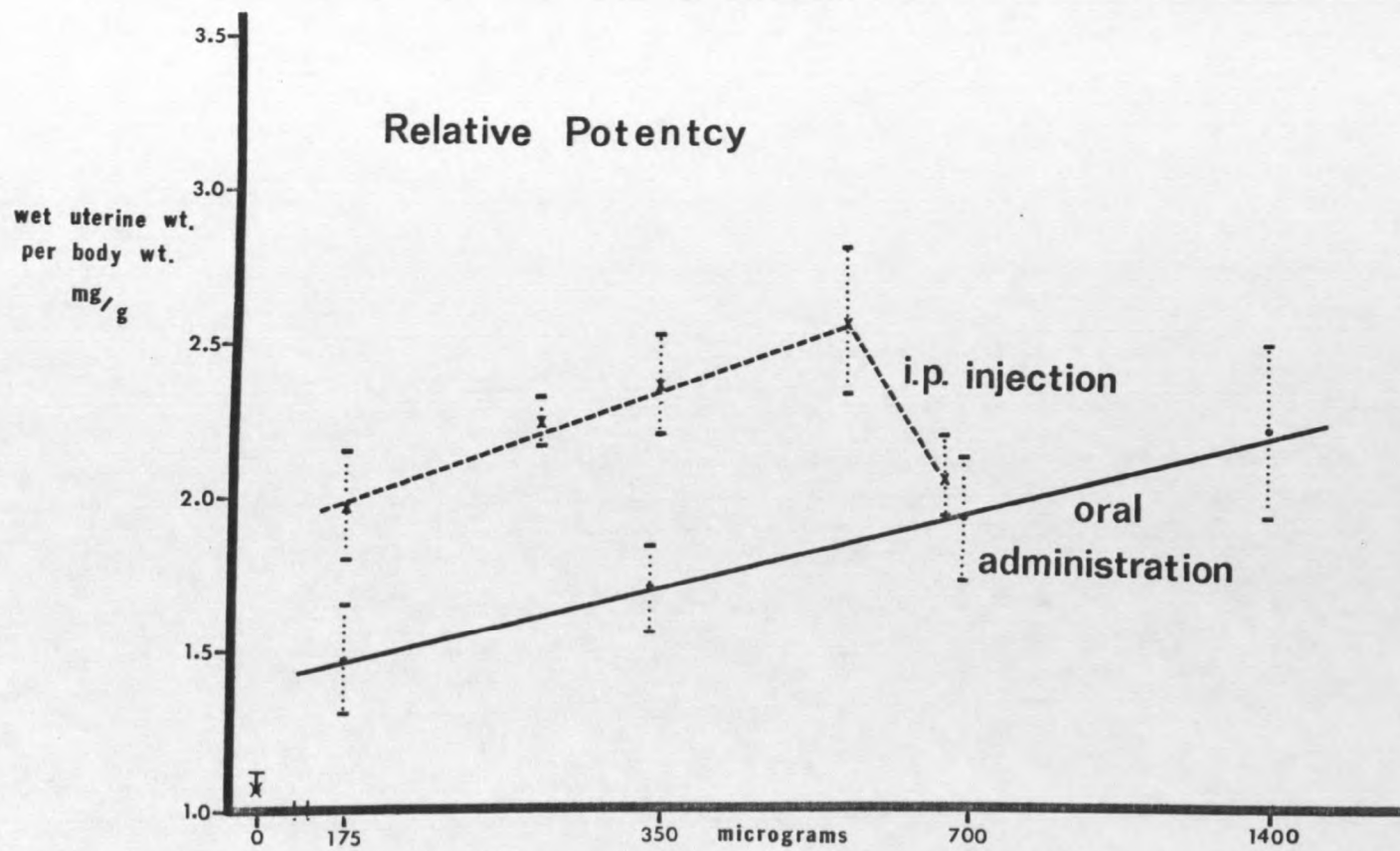


Figure 6. Comparison of interperitoneal to oral administration of the phytoestrogenic fraction
 n = 5 except those listed.

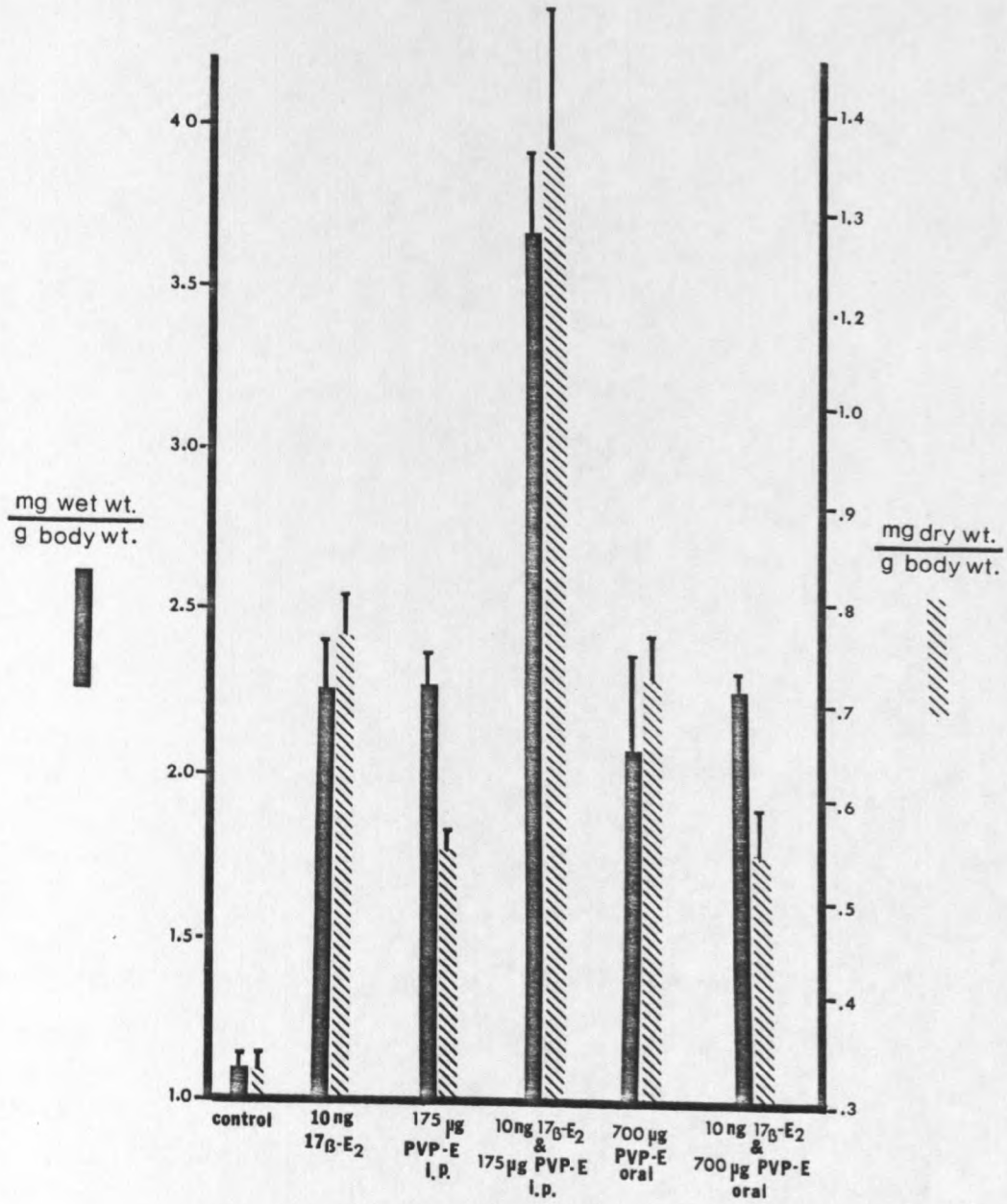


Figure 7. Comparison of i.p. and oral administration of the phytoestrogen in additivity of estrogenic response in immature mouse uteri, $n = 5$ for all groups except those listed.

ever, the *oral* administration of 700 μg of the PVP fraction results in activity which is not additive with that of a 10 ng dose of 17β -estradiol.

Ultraviolet Absorption Spectra

In acidic (pH = 3) aqueous solution the PVP column 16.5-18.7 ml elution fraction had a wavelength maximum absorbance of 208 nm and an absorptivity of 177 l/g \cdot cm. Figure 8 shows the shift to 222 nm with the addition of base (pH = 11). The phytoestrogenic activity described here was always associated with this pH-dependent UV spectra.

Triphenyltetrazolium Determination of Reducing Equivalents

The PVP column elution fraction was found to have 0.204 mg equivalents of glucose/mg and the 90-95% acetone soluble fraction of the aqueous extract had 0.257 mg equivalents of glucose/mg.

Carbohydrate Analysis

The neutral carbohydrate content of the PVP elution used in the uterotrophic assay was found to contain 46.8% carbohydrate by weight based on the absorbance at 490 nm with the phenol-sulfuric acid determination. Figure 9 shows the absorbance spectra for the reaction with glucose and the phytoestrogen sample. The phytoestrogen obtained by elution from the PVP column results in a condensation product with a broad absorption peak between 490 and 540 nm.

The absorbance spectra for the condensation products of anthronol with glucose compared to the phytoestrogen are shown in Figure 10. The wavelength at maximum absorbance for the glucose product is 625 nm compared to the phytoestrogenic fraction which results in an absorbance maximum of 590 nm. Based on the relative absorbance at

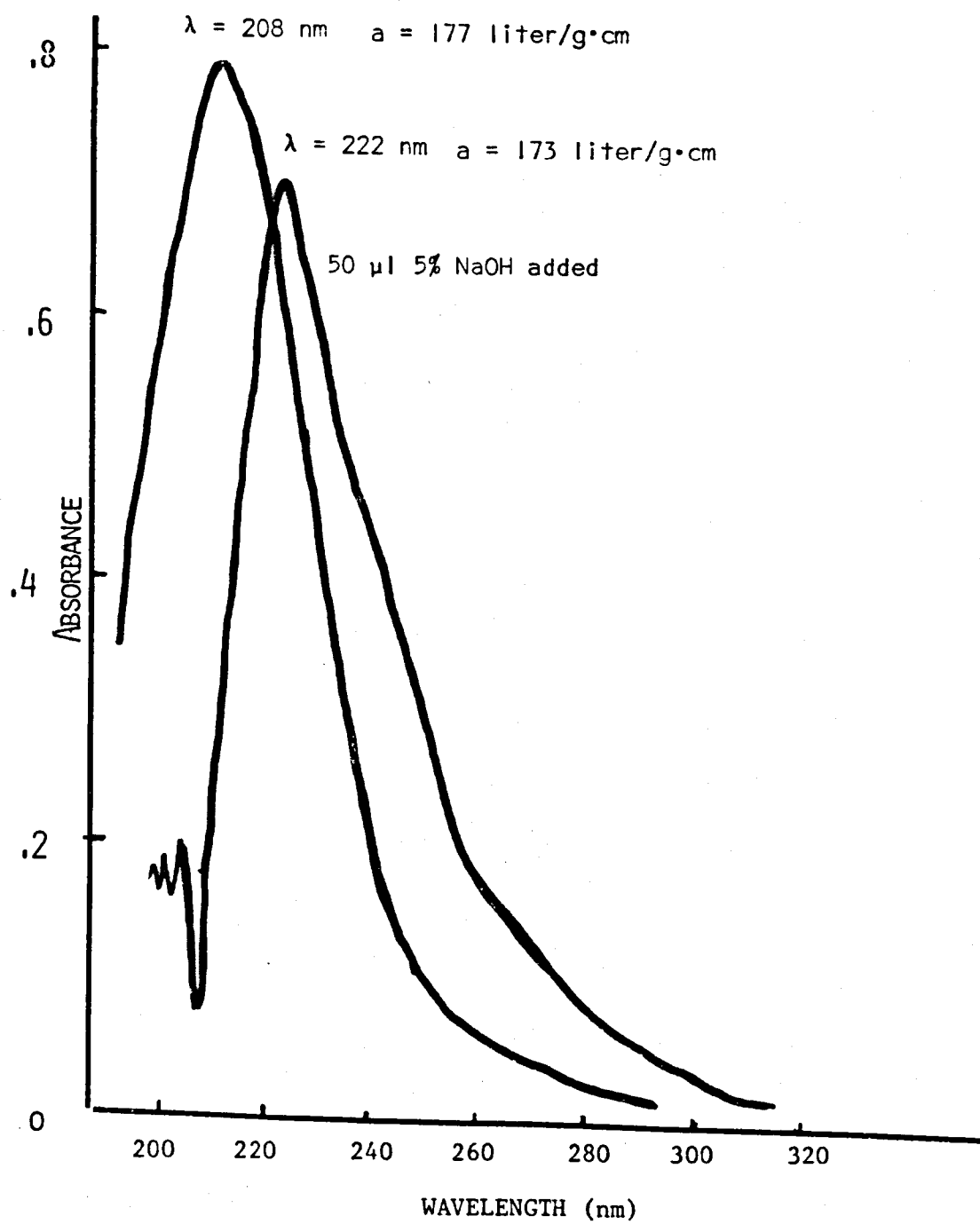


Figure 8. UV absorbance spectrum of the PVP column 16.5-18.7 ml elution fraction.

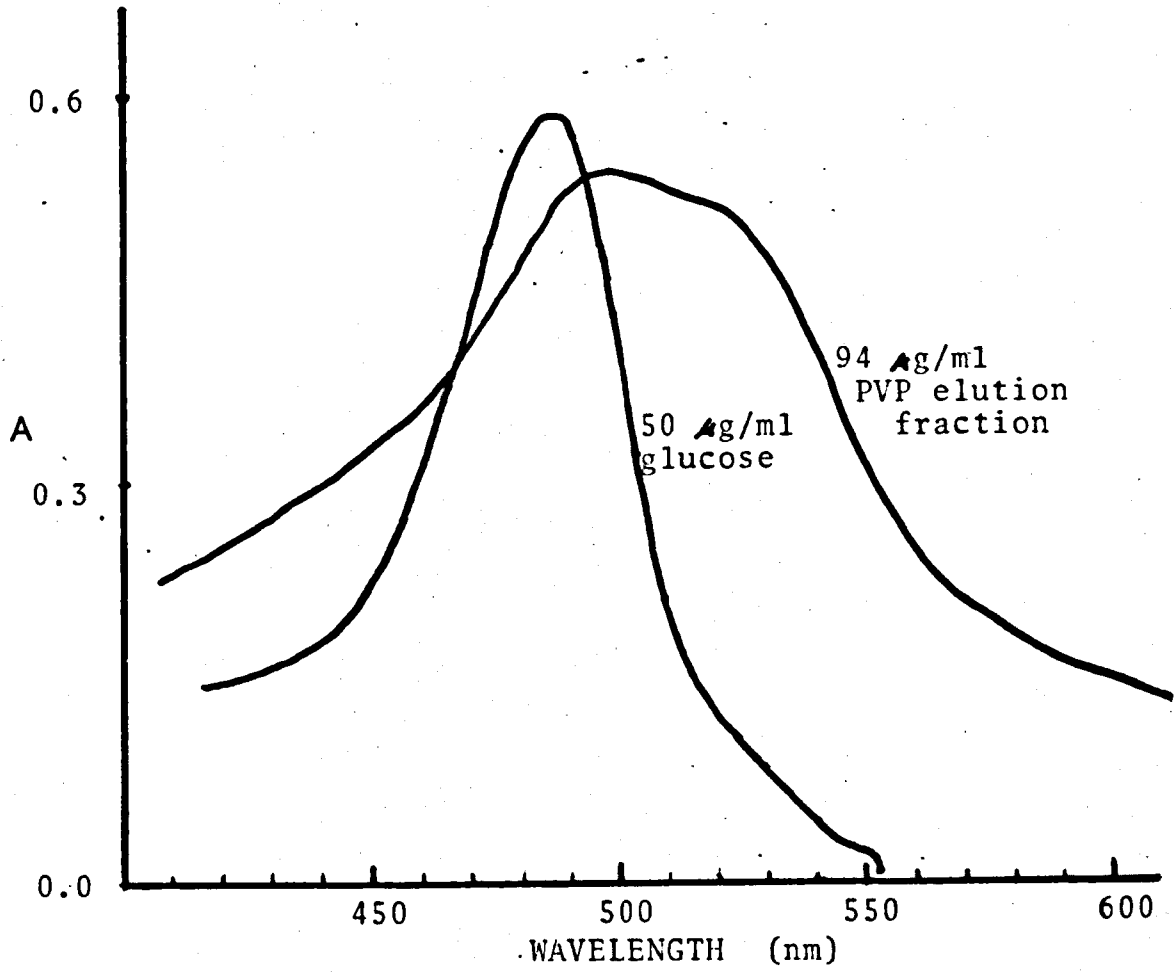


Figure 9. Phenol-sulfuric acid determination for neutral sugars.

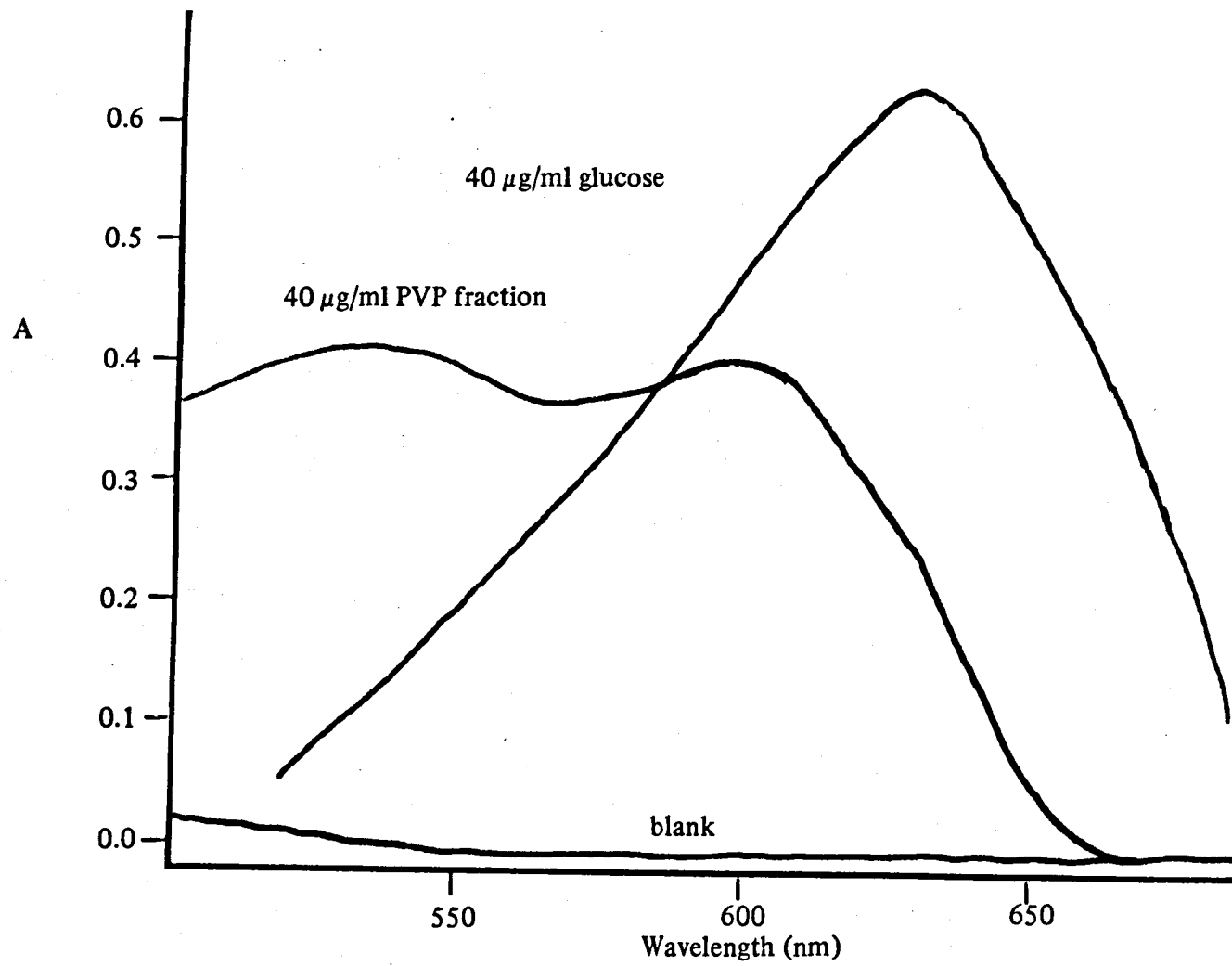


Figure 10. Anthrone determination of hexoses.

630 nm, the PVP elution fraction containing 39% hexose by weight. However, a comparison of condensation products with such different absorbance spectra would not be valid.

Gas-Liquid Chromatography of the Alditol Acetate Derivatives
of the Hydrolysate of the Phytoestrogen Fraction

Figures 11 and 12 show typical chromatographic results of standard sugars and the PVP elution fraction hydrolysate after derivatization to the corresponding alditol acetates. Isothermal chromatography at 200 C with a 30 ml/min carrier gas flow rate resulted in at least 14 different peaks from the alditol acetate derivatives of the acid hydrolysate of the phytoestrogen fraction described in this study. .

There was considerable heterogeneity between different phytoestrogen fractions prepared by elution from the poly-N-vinylpyrrolidone column; however, two peaks were consistently observed in all active phytoestrogen samples. These were the two main peaks with retention times of 6.3 min and 9.2 min in the isothermal run or 13.7 min and 16.8 min in the temperature program chromatograph. The relative retention time to hexa-O-acetylglucitol for these two principal peaks were 0.525 and 0.767 in the isothermal chromatograph and 0.656 and 0.804 with the temperature program from 160-200 C increasing 3 C per min with a 35 ml/min flow rate. These two peaks were consistently 90% of the total peak areas.

No peaks were observed to co-chromatograph with standard alditol acetates in both temperature programs. The standards used in the chromatographic analyses are listed below with retention times. These were the standard alditol acetates and retention times:

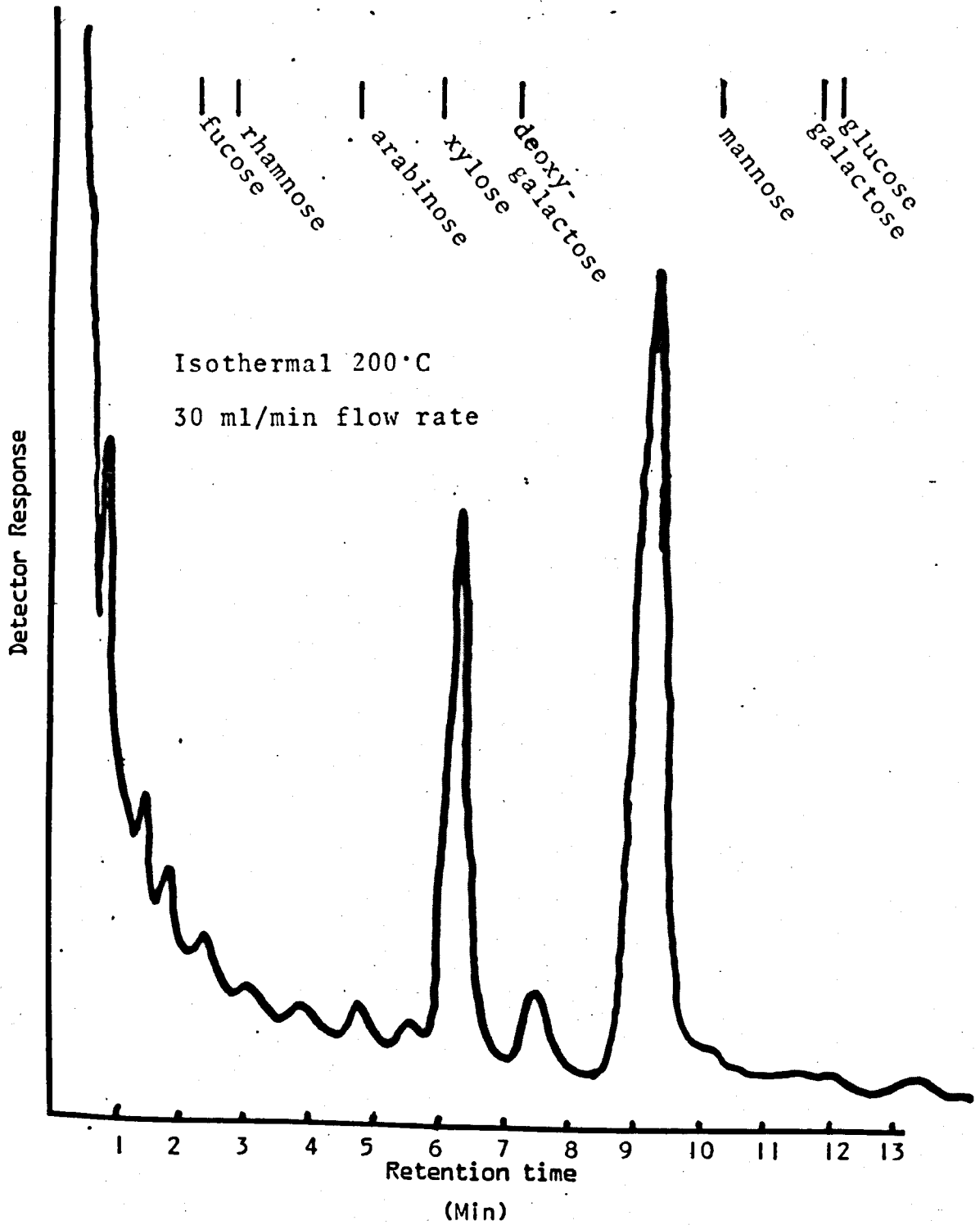


Figure 11. Gas-liquid chromatography of the alditol acetate derivatives of the hydrolysate and standard sugars.

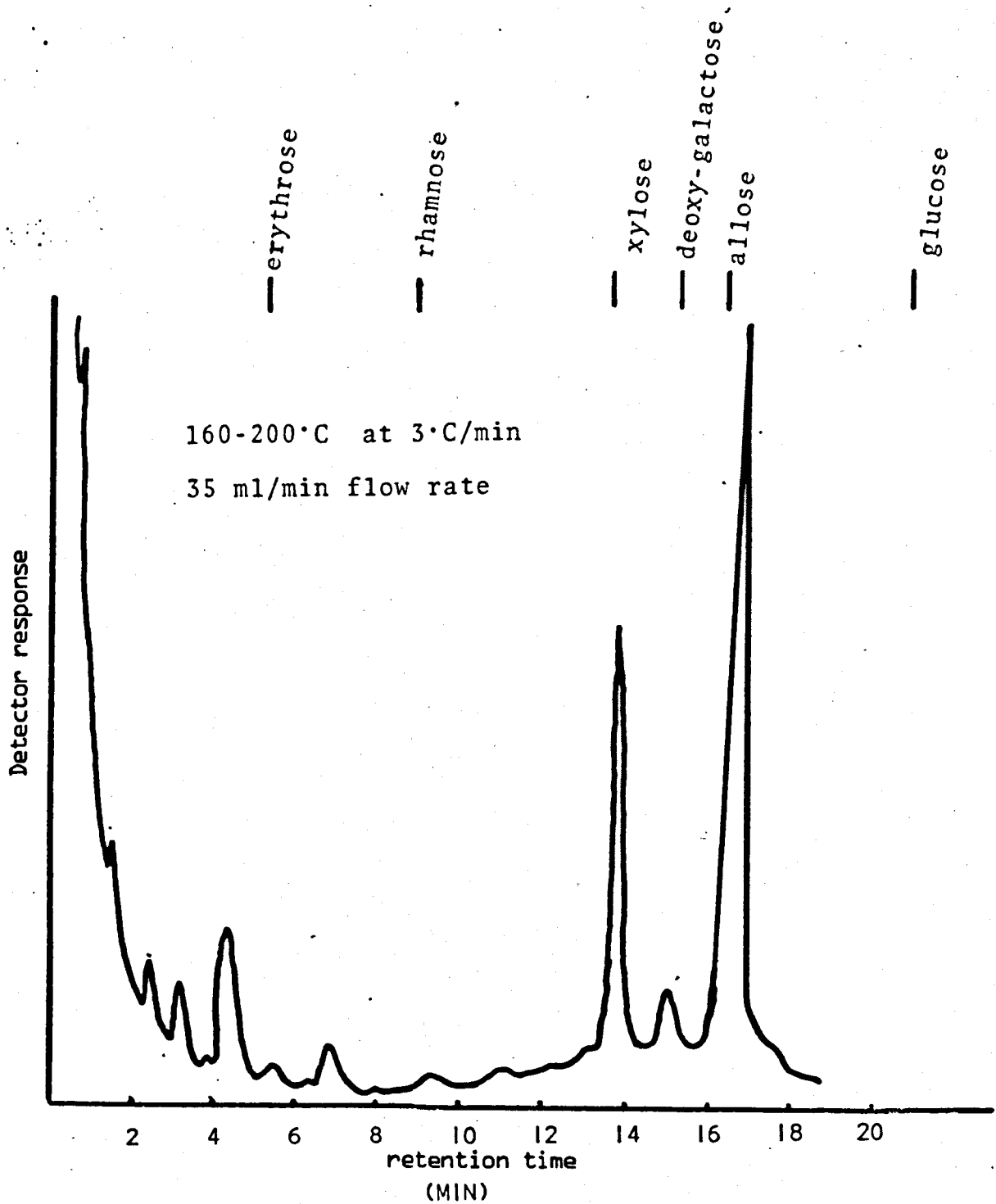


Figure 12. Gas-liquid chromatography of the alditol acetate derivatives of the hydrolysate and standard sugars.

Column temp: 200 C
30 ml/min flow rate

fucitol acetate	2.3 min
rhamnitol acetate	2.8 min
arabitol acetate	4.6 min
deoxy-galactitol acetate	7.0 min
mannitol acetate	10.2 min
galactitol acetate	11.7 min
glucitol acetate	12.0 min
inositol acetate	15.7 min

Column temp: 160-200 C
 increasing 3 C/minute
35 ml/min flow rate

erythritol acetate	5.3 min
rhamnitol acetate	8.8 min
xylitol acetate	13.5 min
allitol acetate	16.4 min
deoxy-glucitol acetate	14.9 min
deoxy-galactitol acetate	15.2 min
glucitol acetate	20.9 min

Ninhydrin Determination of Amino Groups

The phytoestrogen fraction eluting from the poly-N-vinylpyrrolidone column show identical absorbance at 570 nm as the reagent blank.

Additional Column Chromatography of the Phytoestrogen Fraction

The phytoestrogen activity present in the poly-N-vinylpyrrolidone column elution fraction was not recovered from Sephadex or Biogel columns. Those fractions which eluted displayed negligible estradiol displacement activity.

DISCUSSION

This work demonstrates the preparation of a phytoestrogenic fraction from the aqueous extract of Ponderosa pine needles. The yield obtained with the isolation scheme described was 0.7 mg of the phytoestrogenic fraction per gram of ground needles. The estrogenic activity of this fraction was roughly equivalent to 40 ng of 17β -estradiol/gram of needles based on the uterotrophic response in mice 24 hours after intraperitoneal infection. This yield is significant when compared to 11 to 37 ng of estradiol equivalents per gram of lucerne hay, which is reported to cause reproductive failure in cattle. The cattle display similar symptoms of vaginal swelling and udder enlargement after ingestion of lucerne as those observed with pine needle-induced abortion (Moule, Braden, and Lamond, 1963).

The relative activity of this phytoestrogenic fraction is low compared to 17β -estradiol. The relative potency of the fraction was approximately 6×10^{-5} that of estradiol in the 24-hour uterotrophic response assay. This low relative potency is typical of many known phytoestrogens, and is consistent with 3×10^{-5} g estradiol equivalents/gram of genistein (5:7:4'-trihydroxy isoflavone) found in subterranean clover which causes reproductive dysfunction in sheep (Bradbury and White, 1954).

The phytoestrogenic fraction isolated from Ponderosa pine needles acts as an impeded estrogen defined by its shallow dose-response curve and the saturability of uterotrophic response at the higher dose regimens. This impeded estrogenic activity is typical for most non-steroidal phytoestrogens studied (Folman and Pope, 1966; Farnsworth et al., 1975; Labov, 1977). The diminished relative potency resulting with oral administration of the phytoestrogenic fraction versus intraperitoneal injection suggests metabolic inactivation of poor absorption and delivery of the active component(s) of the fraction to the target tissue.

The loss of synergistic uterotrophic activity with 17β -estradiol observed with oral compared to i.p. administration (Fig. 7) of the phytoestrogen may result from metabolic changes in the active components of the fraction.

The toxicity of the phytoestrogenic fraction observed in mice at $350 \mu\text{g}/15 \text{ g}$ body weight i.p. administration or $700 \mu\text{g}/15 \text{ g}$ body weight oral administration is not explained by phytoestrogenic activity alone. The phytoestrogenic fraction eluting from the poly-N-vinylpyrrolidone column resulted in 40% mortality (4 out of 10) with the intraperitoneal administration of $700 \mu\text{g}$ to 15 gram mice. This is the most toxic fraction from Ponderosa pine reported to date. Whether the toxic effect observed here resulted from the same components eliciting the uterotrophic response or other components within this fraction cannot be determined from this study. Further purification of this phytoestrogenic fraction is needed.

Most compounds eliciting estrogenic responses are phenolic compounds (Terenius, 1971; Jordan et al., 1978). The absence of B-band resonance in the 240-300 nm region indicates the phytoestrogenic fraction from Ponderosa pine described is not a flavanoid, coumestan, or resorcylic acid lactone which are the three general classes of phytoestrogenic compounds characterized to date (Scott, 1964). The phytoestrogen from Ponderosa pine described in this work was extremely atypical with characteristics of a high molecular weight complex carbohydrate retained in dialysis tubing with a 14,000 M.W. cutoff.

Some of the few known non-phenolic compounds eliciting estrogenic activity are chlordecone, (KEPONE), and related insecticides and the plant hormone gibberellic acid A_3 . Chlordecone is a polycyclic ketone which is uterotrophic in mice and implicated to cause miscarriage in humans (Bulgar, Muccitelli, and Kupfer, 1978). Gibberellic acid A_3 , a common plant growth hormone, is uterotrophic in mice and acts synergistically with the uterotrophic activity of 17β -estradiol (Gawienowski, Stadnicki, and Stacewicz-Sapuntzakis, 1977; Gawienowski and Chatterjee, 1980).

One advantage of using competitive binding assays to mouse uterine cytosol was the ability to jettison fractions from a resolution step which were void of relative binding activity relative to other fractions. However, negligible competitive binding activity of the non-aqueous extracts of Ponderosa pine needles for 17β -estradiol to mouse uterine cytosol does not *prove* the absence of potential phytoestrogens therein. Many known phytoestrogens are found to be pro-estrogenic, not active initially, but are metabolized by ruminal microorganisms or hepatic oxidation to compounds much more active. The O-demethylation of formononetin to diazein and of biochanin A to genistein are examples of such metabolic activation of pro-estrogenic compounds (Shemesh, Lindner, and Ayalon, 1972; Farnsworth et al., 1975). The competitive binding assay used in this study could easily overlook compounds which do not compete with estradiol for binding in the target tissue of this hormone (Kellie, 1971).

The implications of this phytoestrogenic fraction on the problem of Ponderosa pine needle-induced abortion in range cattle must remain highly speculative since the winter range is a stressful environment for cattle and Corah, Dunn, and Kaltenbach (1975) and Waldhalm et al. (1979) have shown that restricted dietary protein or nutritional stress causes premature parturition and birth of weak, non-viable calves. Phytoestrogenic activity in Ponderosa pine is consistent with reproductive failure in cattle, however.

One of the first bioassays for estrogen was the noted increase in uterine contractibility (Frank, Bonham, and Gustafson, 1925). Diethylstilbestrol, a potent estrogen is well known to induce abortion in cattle (Mackey, 1955; Hill and Pierson, 1958). One symptom associated with diethylstilbestrol-induced abortions and phytoestrogenic effects is the high incidence of retained placenta (Moule et al., 1963).

There is a sharp rise in plasma 17β -estradiol concentrations prior to parturition in cattle (Stellflug, Han, Randel, and Moody, 1978; Adelkoun, Matton, and DuFour, 1978). The significance for this physiological estrogen surge preceding parturition is unclear. It is

suggested that estradiol is luteolytic in cattle (Hoffman, 1979; Currie and Thorburn, 1976; Anderson, Webb, and Turnbull, 1981; Horton and Poyser, 1976). Another possible role of 17β -estradiol during parturition could be the enhanced contractibility of the uterus resulting from the induction of increased oxytocin receptors in the uterus (Soloff, 1975).

One possible role of the estrogen surge immediately prior to parturition is the induction of prostaglandin synthesis. This would result in luteolytic activity by a cascade mechanism (Ramwell, Leovey, and Sintetos, 1977; Thorburn, 1979; Anderson et al., 1981). The action of estradiol on target tissue through prostaglandin synthesis has been substantiated by the inhibition of the uterotrophic response in ovariectomized mice of 10 ng of 17β -estradiol and 1.0 μ g of gibberellic acid A_3 by the concomitant i.p. injection of 200 μ g of indomethacin, an inhibitor of prostaglandin synthesis (Gawienowski and Chatterjee, 1980).

Although the precise relationship between estrogen action in target tissue and its role in Ponderosa pine needle induced abortion is unknown, the phytoestrogen described in this study could interfere with estrogen action at any number of sites. It would be meaningful to observe the effect of the phytoestrogenic fraction described here on the reproduction in cattle. James, Call, and Stevenson (1977) reported that 3.5 kg of dried needles/day resulted in abortion of 2 out of 4 heifers. An appropriate first experiment might be to feed cattle on day 250 of gestation and thereafter one dose/day containing 4.2 g of the phytoestrogen reported here (6 kg equivalents of dried needles) and observe effects on bovine reproduction.

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