

RECOVERY OF KNOWN QUANTITIES OF DIETHYLSTILBESTROL  
FROM THE BILE OF TREATED CATTLE

by

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## INTRODUCTION

The estrogenic activity of the synthetic compound, 4, 4'-dihydroxy-alpha, beta-diethylstilbene was discovered by Dodds et al. (1938a). They suggested that since 4, 4'-dihydroxystilbene is the mother substance of a series of estrogenic agents it be termed stilbestrol. Later in the same year (1938b) they found that diethylstilbestrol has an action similar to estrone on the uterus of ovariectomized rats, and on the uterus of immature rats and rabbits. By using the vaginal-smear assay they demonstrated that diethylstilbestrol was approximately two and one-half times as active as estrone.

It was established by Morrell and Hart (1941) that diethylstilbestrol, more commonly called stilbestrol, has in general the qualitative effects of the natural estrogens. They found no effect of the natural estrogens on the uterus of immature female rats that was not duplicated by stilbestrol and vice versa.

The three common natural estrogens are alpha-estradiol, estrone, and estriol. Alpha-estradiol is the most active of the natural estrogens and estrone the least active.

Stilbestrol has been used in many commercial animal feeds for fattening purposes and it has been used to stimulate lactation in virgin heifers. Folley et al. (1940) showed that copious lactation was produced in goats by the administration of stilbestrol. In a later experiment, Folley et al. (1941) observed the same results when working with virgin heifers.

According to Levin (1945) cows apparently excrete little

estrogen in the urine. He investigated the possibility of fecal excretion of estrogen in this species and found that during the last two weeks of pregnancy, cows excrete 5,000 to 10,000 rat units of estrogenic substance per kilo of dry feces.

Pearlman et al. (1947) were able to isolate estrone from the bile of pregnant cows. They found that while the total estrogen content of the bile seemed to be low, it was considerable by comparison with estrogenic levels in the blood.

In an attempt to determine if milk produced by stilbestrol treated cows contained estrogenic substances, Baron (1956) in this laboratory assayed milk extracts from treated and normal cows. He found that estrogens were not present in detectable amounts in the milk from stilbestrol treated cows. As a result of this work it was decided to conduct further experiments to determine the metabolic pathways of the stilbestrol. Since the liver functions in inactivation of natural estrogens it was considered as a possible pathway. Studies were undertaken to determine whether substances showing estrogenic activity could be obtained from the bile of normal male dairy calves after oral administration of stilbestrol.

Because one of the first experiments (experiment II) indicated that there were estrogenic substances present in the bile a quantitative determination was undertaken. This was done by comparing the results of bioassays of bile extracts (using ovariectomized mice) with previously derived stilbestrol standards.

#### LITERATURE REVIEW

It is well known that the liver plays an important role in

the inactivation of estrogens in most mammals. In vitro studies by Zondek et al. (1943) demonstrated that rat liver pulp will inactivate estrone and stilbestrol; however, about twice as much liver pulp is required to inactivate stilbestrol. It was suggested that this might be the reason for the greater oral efficiency of stilbestrol as compared with a comparatively low oral efficiency of estrone. They also found that estrone when administered orally exerts only 20 per cent of its efficiency as manifested in subcutaneous administration, whereas stilbestrol given orally shows 50 per cent of the efficiency it manifests in subcutaneous administration.

Heller (1940) found that estradiol was inactivated by the liver and kidneys of rats and rabbits, but not by any other tissues studied. He believed that the inactivation was not due to a conjugation or conversion to a less active nature, but rather due to an enzymatic destruction of an oxidative nature. Heller and Heller (1943) believed that the liver and kidneys have a definite threshold capacity for oxidizing alpha-estradiol and any amount above the threshold will escape oxidation.

However, Cantarow et al. (1943) were not able to find any difference in ability to inactivate estradiol between normal rat liver tissue and liver from animals fed with  $C Cl_4$ . Their findings suggest that some mechanism other than enzyme inactivation may be responsible for the enhanced estrogenic activity in vivo following administration of  $C Cl_4$ .

Many in vivo studies concerning the role of the liver in estrogen metabolism have also been carried on. Hanahan et al.

(1953) studied the possible routes of absorption of stilbestrol from the intestinal tract of the rat and found that the main pathway of absorption was the portal system rather than the lymph system. They were able to recover about half of the injected stilbestrol from the bile, and evidence indicated that it was present mainly in the conjugated form probably to be reabsorbed and to undergo an enterohepatic circulation. They believed that perhaps the stilbestrol conjugate represents a pooled storage form from which the free estrogen can be liberated enzymatically in small amounts as required for the maintenance of ovarian activity and other necessary functions.

Rakoff et al. (1944) found that estrogenic activity disappears rapidly from the peripheral blood of normal dogs and humans after injection of alpha-estradiol or stilbestrol. This lends support to the hypothesis of an enterohepatic circulation of estrogens, similar to that of bile acids. Cantarow et al. (1942) found that following intravenous injection of estrone or estradiol in dogs a substance could be recovered from the bile excreted during the subsequent 48 to 72 hours which had about 90 to 95 per cent of the activity of the injected estrogens. However, after the administration of diethylstilbestrol, only about half as much estrogen could be recovered from the bile as after the administration of natural estrogens. They believed that probably some of the estrogen is stored in the portal field to be excreted during the next few days. According to Cantarow et al. (1943) estrogen is probably removed from the blood by the liver cells and converted to a substance possessing little or no estrogenic activity, which is

in part stored temporarily in the liver and is "reactivated" during the process of its excretion by the hepatic cells. They thought that the estrogen is probably reabsorbed in the intestine and returned to the liver, being gradually destroyed or converted to a permanently inactive substance. These studies place serious doubt on the validity of the hypothesis that estrogens are rapidly inactivated or destroyed by the liver in the intact animal.

According to Kirgis and Rothchild (1952) estradiol absorbed into the hepatic portal system in women exercises little estrogenic effect while that absorbed directly into the systemic circulation is associated with definite evidence of estrogenic activity. In this respect the human liver is essentially similar to the rat's, rabbit's, guinea pig's and dog's, but is entirely different from the monkey's. Wagenen and Gardner (1950) demonstrated that the liver of the monkey, Macaca mulatta, does not inactivate estrogens.

Studies in vivo comparing the activity of ovaries or pellets of estrogen implanted in the portal field with that of ovaries or pellets having venous drainage into the systemic circulation, have also demonstrated that the liver is a major site of estrogen inactivation in the mouse, (Bernstorf 1951), in the rat, (Biskind 1941) and in the rabbit, (Engel 1944). However, Bernstorf (1951) and Werthessen and Field (1950) found that the inactivation was not complete after intrasplenic ovarian transplantation in the mouse and the immature rat.

According to Segaloff (1943) intrasplenic injections of estrogens have shown that the liver is a site of extensive inactivation and that the degree of inactivation is governed, in

part at least, by the nature of the specific estrogen. Stilbestrol is also inactivated in the liver, but to a lesser extent than estrone or alpha-estradiol, (Segaloff 1944a b). He also found that the inactivation of stilbestrol probably proceeds through the hydroxyl groups since esterification or methylation affords some protection against inactivation.

It has been demonstrated that any impairment of normal liver functions results in an enhanced effect of both endogenous and exogenous estrogens. Such impairment has been obtained by liver poisoning, partial hepatectomy, and vitamin B complex deficiency. Talbot (1939) observed that after severe poisoning, the liver was no longer capable of inactivating estrogen efficiently. As a consequence, the concentration of estrogen in the circulation of the rat rises and the characteristic uterine response becomes evident.

Schiller and Pincus (1944) found that partial hepatectomy of the male rat leads to increased estrogenic activity in all phenolic urine fractions after estrone injection.

Biskind and Biskind (1942) found that after implantation of estrone pellets in the spleen of adult castrate female rats, the animals remained anestrus; however, when the rats were placed on a vitamin B complex-free diet, protracted estrus occurred. Segaloff and Segaloff (1944) demonstrated that both inanition and vitamin B-complex deficiency greatly decrease the ability of the rat's liver to inactivate stilbestrol. Others have demonstrated that it is not a deficiency of the B vitamins but the concomitant inanition which impairs the degradation mechanism for estrogens in the



rat liver (Drill and Pfeiffer 1946; and Jailer 1948).

Another possible route of estrogen inactivation is by way of the kidneys. After administration of stilbestrol to rabbits, Dodgson et al. (1948) reported that much of the estrogenic activity can be recovered in the urine within the next few hours. The estrogen excreted in the urine is found mainly in the form of a glucuronide, while a quite small amount is excreted in the free state (Wilder Smith and Williams 1948; and Mazur and Shorr 1942).

The elimination mechanism of estrogens in the dog may differ from that of some other animals. Dingemans and Tyslowitz (1941) found that after administration of stilbestrol a substance could be recovered from the urine which had from 8 to 25 per cent of the estrogenic activity of the administered material. Since a considerable concentration was found in the bile, and the liver showed the highest concentration of all organs, possibly the biliary system is the most important route of elimination in the dog.

Mazur and Shorr (1942) found that stilbestrol differs from the naturally occurring estrogens in that its ratio of oral to parenteral effectiveness is much higher and it is excreted in the urine in much larger amounts after administration. Both of these phenomena suggest a less drastic degradation of the synthetic estrogen in the body than is undergone by the natural estrogens.

Bioassays of estrogenic materials date back to Allen and Doisy (1923) who discovered that a typical estrus condition could be produced in ovariectomized mice and rats by injecting them with oily solutions of ovarian extracts.

It was demonstrated by Bulbring and Burn (1935) that solutions of estrone in oil can be estimated with reasonable accuracy by the

vaginal smear method, and that they produce a growth of the uterus of immature rats which is proportional to the dose. They were able to estimate the strength of seven dilutions of estrone with an average error of only 13 per cent.

Lauson et al. (1939) described a method of estrogen assay based on an increase in weight of the immature rat uterus proportional to an increasing dosage. They found that the method was especially adapted to the assay of clinical materials, where the quantity of estrogen is very limited, because relatively great accuracy was obtained consistently when as few as one to four rats were used for each assay.

A six-hour assay for the quantitative determination of estrogen was described by Astwood (1938). He observed that during the first few hours following an injection of estrogen the uterus of the immature rat undergoes a rapid change, accompanied by an increase in weight, due almost entirely to an accumulation of water. This response was calibrated with sufficient precision to permit its use as a simple method for the accurate assay of estrogenic substances.

Hartman and Littrell (1945) employed 16 and 21 day old female rats as recipients for small injections of estrogens and noticed that the vaginae were open within four days. They found the test to be almost unbelievably sensitive to blood estrogens.

Koch (1942) devised a method for the assay of the phenol fraction for urine extracts in which the concentration of estrogenic material was too low to assay by the usual spayed rat method. A priming of pure theelin about 48 hours before injecting the un-

known made the rats more sensitive. He compared the results of unknowns and standards run at the same time and found the method to be reliable.

Levin and Tyndale (1937) attempted to assay "follicle stimulating" material using immature mice on the basis of follicular enlargement, increase in ovarian weight, vaginal canalization and uterine weight. Their results showed that the weight of the uterus is a most sensitive indicator of the potency of the preparation being tested and that by using this as a criterion of induced ovarian activity much more accurate assays can be obtained than by the use of any combination of the other three responses studied.

Evans et al. (1941) reported that the sensitivity and relative consistency of uterine weight responses in immature mice makes this assay method a very reliable one providing all assays are made in parallel with controls.

In the bioassay of estrogens Rubin et al. (1951) used a method based on the straight line relationship between the logarithm of the dose and the uterine weight response of the immature mouse. They found this method sensitive, precise, convenient, and inexpensive.

A comparative assay of gonadotropic substances on rats, mice and chicks was made by Evans et al. (1940). They showed that in the assay of unfractionated pituitary extracts, pregnant mare serum preparation, normal male urine preparation and menopause urine preparation, the mouse uterus was much more sensitive than either the rat ovary or uterus.

Assays of the same preparations of anterior pituitary-like

substance were run on both immature mice and rats by Katzman and Doisy (1932). They found that approximately four times as much material was required to produce the same effects in the rat as in the mouse.

## MATERIALS AND METHODS

### Source of Bile

Calves used in these experiments as sources of bile were obtained from the Department of Dairy Husbandry, Kansas State College. The untreated or control animal used to obtain bile for experiment I was a normal Holstein bull calf (H-1) about 10 weeks old and weighing about 160 pounds. The stilbestrol treated animal used to obtain bile for group Two of experiment II (Table 1) was a normal Holstein bull calf (H-2) about 10-11 weeks old and weighing 185 pounds. The treated animal used to obtain bile for experiments II (except for group Two), III, IV and V was a normal Ayrshire bull calf (A) about 12 weeks old and weighing about 200 pounds. While in the laboratory the calves were fed chopped alfalfa hay and a regular calf grain ration.

Four days before the bile collection was to begin, a gelatin capsule containing 10 mg stilbestrol in 4.5 gr of "Stilbosol"<sup>1</sup> was administered orally to the treated calf. This treatment was continued daily until the collection of bile was stopped. The "Stilbosol" was obtained through the Department of Dairy Husbandry of

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<sup>1</sup> "Stilbosol" is Eli Lilly and Company's trademark for Diethylstilbestrol Premix.

Table 1. Source and dilutions of bile extracts administered to groups of mice.

Group of Mice	No.	Oil Added : to Original : (cc)	Secondary : of Bile : Dilution :	Source : (calves)	Collection :	Test Sample
<u>Experiment I</u>						
One	9*	--	--	--	--	--
Two	9*	6	--	H-1	50 cc	--
<u>Experiment II</u>						
One	9	--	--	--	--	--
Two	6	5	--	H-2	100 cc	--
Three	6	3	--	A	First 3-day	a-1
Four	6	6	--	A	" "	b-1
Five	6	12	--	A	" "	c-1
<u>Experiment III</u>						
One	6	--	--	--	--	--
Two	7	12	1 to 2	A	First 3-day	c-1
Three	7	12	1 to 4	A	" "	c-1
Four	7	12	1 to 8	A	" "	c-1
<u>Experiment IV</u>						
One	10*	--	--	--	--	--
Two	11	12	1 to 8	A	First 3-day	c-1
Three	11	12	1 to 16	A	" "	c-1
Four	10*	12	1 to 4	A	Sec. 3-day	a-2
Five	11	12	1 to 8	A	" "	a-2
Six	11	12	1 to 16	A	" "	a-2
Seven	11	12	1 to 32	A	" "	a-2
<u>Experiment V</u>						
One	9*	--	--	--	--	--
Two	10	12	1 to 32	A	Sec. 3-day	a-2
Three	10	12	1 to 64	A	" "	a-2
Four	10	12	1 to 128	A	" "	a-2
Five	10	12	1 to 128	A	" "	b-2

\* One or more mice not used because of incomplete ovariectomy.

Kansas State College.

### Collecting the Bile

In order to obtain collections of bile from the animals over a period of several days, it was necessary to put in a biliary fistula. A preliminary unsuccessful attempt to place a fistula directly into the common bile duct indicated that perhaps an easier method would be to place the fistula into the gall bladder and then to tie off the common bile duct.

The fistula was made by placing a 16 gauge hypodermic needle in each end of a small rubber tube approximately two feet long. One needle was to be placed in the gall bladder and the other in a collecting bladder. A small amount of solder was placed near the distal end of each needle to prevent its detachment from the bladder wall.

In preparing for the operation, the animal was given an intravenous injection of 10 cc of Nembutal Sodium as a general anesthetic. Later an additional injection was given until a state of anesthesia was reached. The animal was then placed on an operating table and fastened securely. The hair was trimmed from the lumbar region on the right side and the area was closely shaven. At this time approximately 10-12 cc of a local anesthetic, procaine hydrochloride 2 per cent, was injected in the region where the incision was to be made. A transverse incision was made through the skin of the lumbar region. The incision was completed through the musculature of the body wall and care was taken not to cut any

of the larger blood vessels. Usually there was little bleeding and it was not necessary to tie off any of the blood vessels.

The gall bladder was emptied by means of a hypodermic syringe. A small incision was made in the bladder wall and the fistula placed directly in the lumen. The bladder wall was sutured to the end of the fistula leaving it firmly attached in the wall. The common bile duct was then tied off.

The peritoneum and muscle layers were sutured together and then the skin was sutured leaving the free end of the fistula protruding. Aseptic conditions were followed throughout the operation and a small amount of sulfadiazine was placed on the incision to prevent infection. The protruding end of the fistula was placed in a rubber collecting bladder which was attached beneath the abdomen and between the hind legs of the animal.

After the completion of the operation, the calves were usually able to get back on their feet within a few hours. They were kept in the laboratory at all times while the collection of bile was being made. During this period the appetite of the animal appeared to be normal and no abnormal behavior was noticed.

The biliary fistula was not successful in the control calf (H-1) used in experiment I; therefore, the only bile obtained was approximately 50 cc which had been removed directly from the gall bladder with a hypodermic syringe. Bile was removed from the collecting bladder of the treated calf (H-2) at irregular intervals; consequently, none of the extract of this bile was used in attempting a quantitative determination. The bile was removed from the

collecting bladder of the treated calf (A) after one 24 hour and two 72 hour intervals. The two 72 hour collections, which were extracted and used in experiments II through V, contained approximately equal volumes of bile. The calves were killed upon the completion of the bile collection.

The bile was placed immediately in polyethylene cartons and frozen. It was stored in a frozen condition until ready for extraction.

#### Extraction of the Bile

The bile extract administered to group Two of experiment II was prepared by the Department of Dairy Husbandry, Kansas State College, using in general the method described by Pope and Roy (1953) for the extraction of estrogens in bovine colostrum.

All of the other groups of treated mice were given bile extracts which were prepared by the Department of Chemistry, Kansas State College. The extraction was accomplished by use of an ordinary liquid-liquid extractor employing diethylether as the solvent.

#### Preparation of Extract Dilutions

Each of the three-day collections of bile from the stilbestrol treated animal, A, used in experiments II through V was extracted by the chemistry department and was obtained from them in a diethylether solution. These solutions, each representing the extract of a three-day bile collection, were divided into three equal portions and then the ether allowed to evaporate.

After evaporation, there remained only a small quantity of



solid material which was immediately dissolved in olive oil as follows. Each of the three equal portions of the extract residue, test samples a-1, b-1, and c-1, was dissolved in a different amount of olive oil for use in experiment II. The test samples were later diluted further with olive oil, and used in the succeeding experiments. In the bioassay with ovariectomized mice, the different dilutions were used. Test samples in 12 cc were diluted further in olive oil in ratios ranging from 1:2 to 1:128 as indicated in Table 1.

Groups of mice were injected subcutaneously with 0.1 cc of the dilutants once daily for four days making a total dosage of 0.4 cc for each treated mouse. The uterine weight was used as the bioassay to determine the amount of estrogenic substances present in the different bile extract dilutions.

#### Bioassay of the Extract

Female albino mice were obtained at about six weeks of age and were bilaterally ovariectomized when they were eight weeks old. In this laboratory the mice were fed Purina Laboratory Chow which was assayed by the Ralston Purina Company, St. Louis, Missouri, for estrogenic activity and found to be essentially estrogen free.

The operative procedure during ovariectomy was as follows: Ether was used as the anesthetic and was placed in a test tube with cotton in the bottom. The test tube was then placed over the head of the mouse. Extreme care was needed to keep the mouse in a light state of anesthesia during the operation as a slight

excess would be fatal. After the mouse was in a state of anesthesia, the hair was trimmed from the general region where the incisions were to be made. A transverse incision was made in the skin of the lumbar region on the right side. The skin was shifted so the incision was over the right ovary, which was usually visible through the abdominal wall embedded in a small piece of perovarian fat. A small incision was made through the musculature of the body wall to admit the tip of a pair of fine forceps. The fat lobule was grasped with a pair of forceps and pulled out through the incision in the body wall. With another pair of forceps, the tip of the uterine horn was held and the ovary, intact in its capsule, together with the Fallopian tube, was excised. The horn of the uterus was released and allowed to slip back into the abdominal cavity. After the right ovary had been removed, the same procedure was followed in removing the left ovary.

Aseptic conditions were not necessary; however, a small amount of sulfadiazine was placed in the incision to prevent infection. Healing was rapid therefore it was not necessary to suture the abdominal wall.

One week after the ovariectomy, the assay was started. The mice were injected subcutaneously with 0.1 cc of extract in olive oil once daily for four days. They were killed 48 hours after the final injection and the individual body weights were determined. The uterus was dissected free from adhering tissues and fat. It was then trimmed by cutting through the utero-tubal junction and through the junction of the cervix and the uterus, and freed from fluid by blotting against paper toweling. The uterus was then

weighed on a Fisher Gram-atic Balance.

The criterion used to detect estrogenic activity was an increase in uterine weight of the treated animals in comparison to the controls. All the results were expressed as uterine ratios, which were calculated as 100 times the uterine weight in milligrams divided by the body weight in grams.

A total of 202 mice in 23 groups were used in the assay of different dilutions of bile extracts. Five experiments were performed; each of them having one group of mice serving as the control group and from one to six groups being treated with bile extract dilutions.

In Experiment I, the mice were used in the assay of an extract from the control calf (H-1) that had not been treated. Two groups of mice were used: one control group of nine mice and one treated group of nine mice. Group One, the control group, was not treated while group Two was injected with bile extract residue dissolved in six cc olive oil.

Experiment II. Bile extract solutions from the stilbestrol treated animals were administered to four groups of mice in experiment II. Five groups of mice were used: a control group of nine mice and four treated groups of six mice each. Group One, the controls, was not injected. Group Two received the bile extract prepared by the dairy department and dissolved in about five cc olive oil. Groups Three through Five each received dilutions of test samples a-1, b-1, and c-1, which had originally come from the same three-day bile collection. Group Three received test sample a-1 which had been dissolved in three cc of olive oil; group Four re-

ceived test sample b-1 in six cc olive oil; and group Five received test sample c-1 in 12 cc olive oil.

In Experiment III, the ovariectomized mice were used in the bioassay of bile extracts from the same stilbestrol treated calf as was used in most of experiment II. Four groups of mice were used; three treated groups of seven mice each and one control group of six mice. Group One was the control group and was not treated. The three treated groups were injected with 0.4 cc, per mouse, of bile extract in olive oil and each group received a different dilution of the extract administered to group Five of the previous experiment. This was test sample c-1 and it had been dissolved in 12 cc olive oil. Group Two received a one to two dilution of this extract; group Three received a one to four dilution; and group Four received a one to eight dilution of the same extract.

Experiment IV. A total of 75 ovariectomized mice were used in experiment IV in the bioassay of further dilutions of bile extracts from the stilbestrol treated calf used in the previous experiments. The mice were divided into seven groups; a control group of 10 mice and six treated groups of 11 mice each, except for group Four which also contained 10 mice. Group One served as the control group and was not treated. Groups Two and Three were administered 0.4 cc, per mouse, of different olive oil dilutions of test sample c-1 which was one-third of the extract of a three-day collection that had been dissolved in 12 cc olive oil. Group Two received a one to eight dilution of this extract and group Three received a one to sixteen dilution. Groups Four through Seven were given different dilutions of an extract from a second three-day collection

of bile. This extract was also divided into three equal portions and each of the three portions, test samples a-2, b-2, and c-2, was dissolved in 12 cc olive oil. Group Four received a one to four dilution of test sample a-2; group Five received a one to eight dilution; group Six received a one to sixteen dilution; and group Seven received a one to thirty-two dilution of the same extract.

In Experiment V, ovariectomized mice were used in the bioassay of further dilutions of bile extracts from the stilbestrol treated calf (A) used in the three previous experiments. Five groups of mice were used: four treated groups of 10 mice each and one control group of nine mice. Group One was the control group and was not treated. Groups Two, Three and Four were injected with dilutions of test sample a-2 while group Five was injected with a dilution of test sample b-2 that had been dissolved in 12 cc olive oil. This extract was originally a portion of the same extract of a three-day collection as the extract given to groups Two, Three and Four of this experiment. Group Two received a 1 to 32 dilution; group Three received a 1 to 64 dilution and group Four received a 1 to 128 dilution of the extract. Group Five was injected with a 1 to 128 dilution from test sample b-2.

## RESULTS

Experiment I was conducted in order to determine whether bile from a nontreated or control calf contained any substances which would produce estrogenic activity. Two groups of mice were used; group One serving as the controls and group Two as

the recipient of the extract. The results, summarized in Table 2, were all expressed as uterine ratios, which are defined as the weight of the uterus (in milligrams) per gram of body weight times 100. Group One had an average uterine ratio of 36.1, (individual uterine ratios ranged from 26.3 to 56.5) while group Two had an average uterine ratio of 36.8 (individual uterine ratios ranged from 29.0 to 46.7).

There was no apparent difference between the uterine ratios of the two groups; therefore, it was assumed that the bile from the normal non-treated calf contained little or no estrogenic substances detectable by this procedure.

Experiment II was conducted with four different dilutions of bile extracts from stilbestrol treated calves. Group One, the control group, had an average uterine ratio of 42. Group Two, which received a bile extract solution from calf H-1, had an average uterine ratio of 313. Groups Three, Four, and Five received different dilutions of extracts from calf A. The extract given to group Three (test sample a-1) was dissolved in three cc olive oil and the average uterine ratio of the group was 200. Group Four received the extract (test sample b-1) in six cc oil and the average uterine ratio of this group was 271. Group Five was injected with a portion of a 12 cc solution of test sample c-1 and the average uterine ratio was 262.

There seemed to be no gradation of the responses from the various dilutions. This indicated that apparently each of the dilutions, when injected, was causing a maximum response.

Table 2. Results of all experiments.

Test Group	No.:	Oil Added : to Original: Extract (cc)	:Secondary: Dilution	Average: Body Weight (gm)	Average: Uterine Weight (mg)	Average Ratio (mg/gm x 100)
<u>Experiment I</u>						
One	9*	--	--	29.8	9.7	36.1
Two	9*	6	--	30.2	11.0	36.8
<u>Experiment II</u>						
One	9	--	--	26.5	11	42
Two	6	5	--	26.8	8 $\frac{1}{2}$	313
Three	6	3	--	27.5	55	200
Four	6	6	--	26.6	72	271
Five	6	12	--	26.3	69	262
<u>Experiment III</u>						
One	6	--	--	27.9	15	57
Two	7	12	1 to 2	30.3	73	241
Three	7	12	1 to 4	29.2	62	212
Four	7	12	1 to 8	28.6	44	154
<u>Experiment IV</u>						
One	10*	--	--	26.2	17.3	66.7
Two	11	12	1 to 8	26.0	32.4	125.3
Three	11	12	1 to 16	25.4	19.5	78.5
Four	10*	12	1 to 4	24.7	53.8	223.8
Five	11	12	1 to 8	26.3	59.7	225.2
Six	11	12	1 to 16	26.3	49.0	185.7
Seven	11	12	1 to 32	26.1	27.9	107.5
<u>Experiment V</u>						
One	9*	--	--	32.3	23.4	72.4
Two	10	12	1 to 32	25.6	29.4	111.8
Three	10	12	1 to 64	26.1	24.7	94.8
Four	10	12	1 to 128	28.0	22.4	80.2
Five	10	12	1 to 128	31.9	26.2	83.1

\* One or more mice not used because of incomplete ovariectomy.

The results indicated that there was a relatively large amount of estrogenic substance in each of the extract solutions; that is, a large amount in relation to the physiological response produced. In order to get a graded response it was apparent that it would be necessary to dilute the extract solutions further before administration to the mice.

The bile extract solution of test sample c-1 which was given to group Five of the previous experiment was diluted further and then administered to three groups of mice in experiment III. Group One, the controls, had an average uterine ratio of 57. Group Two was given a one to two dilution; group Three, a one to four; and group Four, a one to eight dilution of extract originally in 12 cc olive oil. Group Two had an average uterine ratio of 241, group Three an average uterine ratio of 212, and group Four an average uterine ratio of 154.

These results indicate a somewhat graded response to the extract dilutions. In order to get a lower level response it was necessary to further dilute the extract solutions.

Experiment IV was conducted using one group of control mice and six groups of treated mice. Group One, the controls, had an average uterine ratio of 66.7. Group Two received a one to eight dilution and group Three received a one to sixteen dilution of the 12 cc extract solution of test sample c-1 which was given to group Four of Experiment III. Group Two had an average uterine ratio of 125.3 while group Three had an average uterine ratio of 78.5.



Groups Four, Five, Six, and Seven received dilutions of a 12 cc extract solution of test sample a-2 from the second three-day collection. Group Four which received a one to four dilution had an average uterine ratio of 223.8. Group Five received a one to eight dilution and had an average uterine ratio of 225.2. Group Six was given a 1 to 16 dilution and had an average uterine ratio of 185.7. Group Seven which received a 1 to 32 dilution had an average uterine ratio of 107.5.

There apparently was a graded response only among the groups receiving the lowest concentrations of extract; therefore, it seemed necessary to repeat the test with greater dilutions of extract.

It was observed that the average uterine ratios of groups receiving dilutions of test sample c-1 were lower than those of groups receiving similar dilutions of a-2. Therefore, only those groups which received dilutions of a-2 were used in attempting a quantitative determination of estrogenic substances present. In order to make a quantitative determination, it was necessary to compare the results of these experiments with stilbestrol standards previously derived in this laboratory. These standards were derived by injecting ovariectomized mice with three levels of stilbestrol dilutions ranging from a total dosage of 0.048 micrograms to a total dosage of 3.12 micrograms.

Since the lowest assay level of stilbestrol standard caused a relatively low level response it seemed necessary to conduct bioassays of greater dilutions of bile extract than used in experiment IV.

Experiment V was conducted using four groups of treated mice along with a control group. The control group, group One, had an average uterine ratio of 72.4. Groups Two, Three and Four received dilutions of test sample a-2 while group Five received a dilution of test sample b-2. Group Two was given a 1 to 32 dilution of extract originally in 12 cc olive oil and was found to have an average uterine ratio of 114.8. Group Three received a 1 to 64 dilution and had an average uterine ratio of 94.8. A 1 to 128 dilution was given to group Four and the average uterine ratio was 80.2. Group Five received a 1 to 128 dilution from a second sample (b-2) of the same three-day collection as previously used in this experiment. This group had an average uterine ratio of 83.1.

It was assumed that the 1 to 128 dilutions caused a minimum response; therefore, it was unnecessary to repeat the test with greater dilutions.

#### DISCUSSION

Since the metabolic pathways of stilbestrol in the bovine are unknown, an attempt was made to recover estrogenic substances from the bile of normal male dairy calves after oral treatment with 10 mg of stilbestrol per day. However, before attempting to determine whether bile extracts from a treated calf contained any estrogenic substances, it was necessary to determine whether bile extracts from an untreated calf contained any natural estrogenic materials.

In experiment I, one group of mice was injected with a bile extract obtained from a normal untreated animal. The extract

solution apparently caused no estrogenic stimulation as there was very little difference between the average uterine ratios of the control and the treated groups. It was assumed that the bile of a non-treated calf contained no estrogenic substances which could be detected by these procedures. Therefore, if any estrogenic substance could be detected in the bile extracts from a treated calf, this substance probably could be attributed to the administered stilbestrol.

Experiment II was conducted in order to determine whether estrogenic substances could be recovered from the bile of stilbestrol treated calves. Four groups of mice were treated with bile extracts from two treated calves. Each of the extract solutions apparently caused a near maximum response as there was no gradation of the responses even though the test samples were dissolved in varying amounts of olive oil. This indicated that there was an estrogenic substance or substances present in each of the test samples of extract.

Each of the test samples was one-third of the extract of a three-day collection or the amount of extract which theoretically could be obtained from a one-day collection of bile.

Since experiment II indicated that there were estrogenic substances present in the bile of stilbestrol treated calves, an attempt was made to determine the quantities present. In order to do this it was necessary to obtain results which could be compared with stilbestrol standards previously derived in this laboratory by Baron (1956).

These standards were derived by injecting ovariectomized mice

with three known levels of stilbestrol dilutions. The three levels were 3.12 micrograms, 0.388 micrograms and 0.048 micrograms. The control mice used in the stilbestrol standard determination had an average uterine ratio of 53.26. The mice which received a total dosage of 3.12 micrograms had an average uterine ratio of 263.0; those receiving 0.388 micrograms had an average ratio of 201.3; and those that received 0.048 micrograms had an average uterine ratio of 89.6.

In order to compare the experimental results with these standards it was necessary to dilute the test samples so that a relatively low level physiological response might be obtained. Therefore, test samples c-1 and a-2 (both in 12 cc olive oil) were diluted to various levels and administered to groups of mice in experiments III and IV. The dilutions of test sample c-1 ranged from a 1 to 2 to a 1 to 16 dilution and those of a-2 ranged from a 1 to 4 to a 1 to 32 dilution. Each of the dilutions was then injected into a group of mice. The results indicated that the groups which received dilutions of test sample c-1 did not respond as much as those receiving similar dilutions of test sample a-2. Since the treated calves were given equal quantities of stilbestrol, per day, and the three-day collections contained equal volumes of bile, it might be assumed that equal amounts of estrogenic substance were excreted in the bile each day. It seemed probable that some of the estrogenic substance present in test sample c-1 had been lost during some part of the procedure. Therefore, the results of groups receiving dilutions of c-1 were not used in the quantitative determination.

Since test sample a-2 apparently contained more estrogenic substance than c-1, the average uterine ratios of groups receiving dilutions of a-2 were compared to the standards. It was observed that the proportion of the average uterine ratios of the treated group (receiving the 1 to 32 dilution) to the control group appeared to correspond closely with the stilbestrol standard of 0.048 micrograms. A comparison between the standards and the experimentals is shown in Fig. 1.

Experiment V was conducted with four groups of mice receiving extract dilutions of test samples a-2 and b-2 which included 1 to 32, 1 to 64 and 1 to 128 dilutions. The greatest dilutions apparently caused near minimum responses as the difference between the average uterine ratios of the control and the treated groups was relatively small. The ranges of the uterine ratios within each group were also nearly alike. The results indicated that again the 1 to 32 dilution caused a response similar to that obtained with the stilbestrol standard of 0.048 micrograms (also shown in Fig. 1).

It was assumed that the mice, which were injected with the 1 to 32 dilution, each received a substance or substances with an estrogenic potency about equal to that of 0.048 micrograms of stilbestrol. If this is true, then one cc of the dilutant contained the equivalent of 0.120 micrograms since each mouse received 0.4 cc of extract solution. Correspondingly, 32 cc contained about the equivalent of 3.84 micrograms and the whole test sample contained 12 times this amount or about the equivalent of 46 micrograms, since test sample a-2 was originally dissolved in

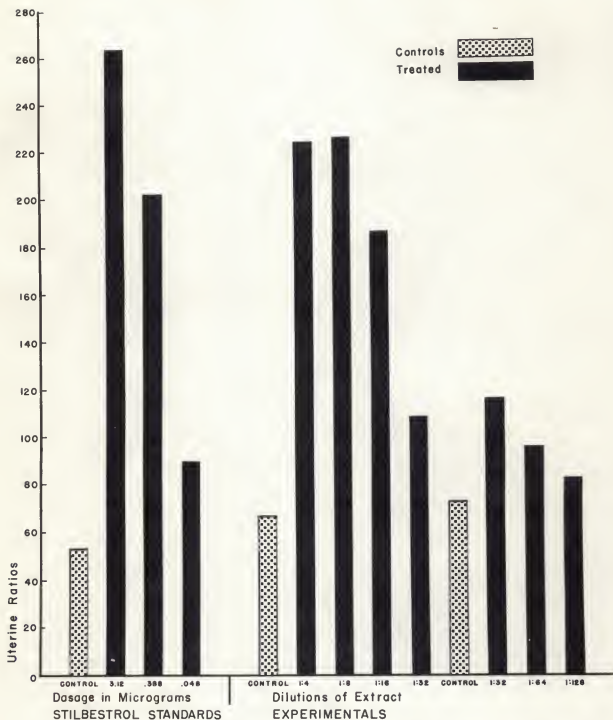


FIG. 1. A comparison between uterine ratios of standards and experimentals

12 cc olive oil. In this study no attempt was made to identify the estrogenic substances present.

Each test sample was one-third of the extract of a three-day collection, or each was equal to the extract of a one-day collection. Therefore, it was assumed that bile from the treated calf (A) contained, per day, a substance or substances which had an estrogenic potency at least as great as that of 46 micrograms of stilbestrol.

However, there may have been more than the equivalent of 46 micrograms of stilbestrol, per day, present in the original bile. It is possible that some of the estrogenic substances could have been lost during the extraction procedure. In the extraction by the chemistry department, diethylether was used as the solvent. The ether soluble fractions were then recovered, but any water soluble substances present were lost. If any of the stilbestrol was conjugated (that is, combined with a substance such as sulfuric or glucuronic acid), then this portion would probably have been lost since the most common conjugated forms are water soluble. However, many investigators seem to agree that most of the estrogenic substances, when present in the bile, are found in the "free" or unconjugated form.

The objectives of these experiments were to determine whether estrogenic substances could be recovered in the bile of stilbestrol-treated dairy calves, and, if present, to recover quantitative amounts of this material. It has been shown that bile extract dilutions from the treated calves produced marked estrogenic responses in the ovariectomized mice. The quantities of estrogenic

material in the bile were measured by comparing the results of experiments using various ranges of extract dilutions with stilbestrol standards. The results appeared to indicate that the bile contained, per day, a substance with an estrogenic potency as great as that of 46 micrograms of stilbestrol.

There are several factors which should be considered in future work with this method. First of all, it would be advisable to improve the stilbestrol standard for use in a quantitative determination. This could be done by administering more known amounts of stilbestrol to groups of mice in order to establish a better response curve. It then would be possible to plot experimental results directly on the established curve and thus obtain a fairly accurate quantitative determination. Possibly this method then could be used for the determination of estrogen in other substances such as blood or urine.

Another factor which should be considered is that it might be valuable to use other extraction procedures which would recover both "free" and conjugated estrogenic substances. This would be especially true when working with a material such as urine which probably contains estrogens mainly in the conjugated form.

Finally, it would be advisable to use larger groups of mice in the bioassay in an attempt to reduce the variance between groups.

#### SUMMARY

After ligation of the common bile duct, fistulas were placed in the gall bladders of three normal male dairy calves (one control



and two treated) in order to recover all of the daily output of bile. A dosage of 10 mg of diethylstilbestrol, per day, was given orally to each of the treated animals.

Five experiments were conducted using ovariectomized mice in the bioassay of bile extract dilutions. The criterion used to detect estrogenic activity was a growth of the uteri of the treated mice in comparison to the controls.

Bile obtained from one normal male dairy calf (the control) contained no detectable amounts of natural estrogenic substances.

Bile obtained from two treated calves contained a substance or substances which stimulated uterine growth in ovariectomized mice.

A quantitative determination was attempted using a bile extract from one treated calf and comparing the uterine stimulation caused by this extract with previously derived stilbestrol standards. The results appeared to indicate that the bile contained, per day, a substance with a potency as great as that of 46 micrograms of diethylstilbestrol.

Bile extracts used in the quantitative determination were prepared using a liquid-liquid extractor with diethylether as the solvent; therefore, only the ether soluble substances were recovered.

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RECOVERY OF KNOWN QUANTITIES OF DIETHYLSTILBESTROL  
FROM THE BILE OF TREATED CATTLE

by

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B. A., Concordia College,  
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Diethylstilbestrol has been used in many commercial animal feeds for fattening purposes and it has been used to stimulate lactation in virgin heifers. Because of the possibility that estrogenic substances might be found in the milk of treated cows, it was necessary to learn more about the metabolism of this synthetic estrogen.

Earlier work in this laboratory indicated that estrogens were not present in detectable amounts in the milk from stilbestrol-treated cows. As a result of this work, further experiments were conducted in an effort to determine the metabolic pathways of the stilbestrol. Since the liver functions in inactivation of natural estrogens it was considered as a possible pathway.

Studies were undertaken to determine whether substances showing estrogenic activity could be obtained from the bile of normal male dairy calves after oral administration of stilbestrol. It was shown that bile extracts from two treated calves contained estrogenic substances; therefore, a quantitative determination was attempted. This was done by comparing the results of bioassays of bile extract dilutions with stilbestrol standards.

In order to recover all of the calves' daily output of bile, a fistula was placed in the gall bladder and the common bile duct ligatured. Three calves (one control and two treated) were used in this study. Each of the treated animals was given an oral dosage of 10 mg of diethylstilbestrol, per day, beginning about four days before and continuing throughout the collection period.

Bile extracts used in the quantitative determination were prepared using a liquid-liquid extractor with diethylether as the



solvent; therefore, only the ether soluble substances were recovered.

Five experiments were conducted using ovariectomized mice in the bioassay of bile extract dilutions. The criterion used to detect estrogenic activity was an increase in the uterine weight of the treated mice in comparison to the controls. All the results were expressed as uterine ratios, which are defined as the weight of the uterus (in milligrams) per gram of body weight times 100.

A bile extract obtained from the untreated or control calf contained no estrogenic substance detectable by these procedures. However, bile extracts from the two treated calves produced a marked estrogenic response in the ovariectomized mice.

Since an estrogenic substance could be demonstrated in the bile extracts of the stilbestrol treated calves, an attempt was made to determine the quantities present. The results of experiments using various ranges of bile extract dilutions were compared with stilbestrol standards previously derived in this laboratory. The results appeared to indicate that the bile contained, per day, a substance with an estrogenic potency as great as that of 46 micrograms of stilbestrol.