Table 1. Uterine response to orally administered estrogen.

Test material and total amount fed per mouse	No. of mice	Av. uterine wt. (mg)
None (control)	5	10
Alcohol extract from 3.5 of dried ladino clover meal	5	41
Crystalline ladino clover estrogen (0.5 mg)	5	34
Crystalline ladino clover estrogen (0.75 mg)	5	61
Genistein (15 mg)	4	28
Diethylstilbestrol (0.2 μg)	5	47
Mentzer's M84* (1.0 mg)	5	101

* 3-(*p*-hydroxyphenyl)-4-*m*-propyl, 7-hydroxycoumarin (*9*).

the presence of genistein, although it has also been reported that the treatment of clover "chloroplast" with alkali yielded a small amount of a second estrogen with at least 10 times the activity of genistein (*8*). No information on the chemical nature of this second estrogen was presented, however.

A crystalline compound possessing estrogenic activity has recently been isolated at this laboratory from ladino clover. We have also found estrogenic activity in several alfalfa samples as well as in a sample of fresh strawberry clover; the activity in these samples appears to be attributable to the same estrogen. The compound is the predominant estrogen in strawberry clover, ladino clover and alfalfa, and it appears to be a coumarin derivative rather than an isoflavone. Because of the coumarin structure of the molecule, we propose the name *coumestrol* for the estrogen. The effectiveness of coumestrol as an estrogen has been demonstrated by feeding it to immature female mice and measuring the effect on uterine weight increase. The results of one such assay are presented in Table 1. Genistein and diethylstilbestrol were also included in this study for purposes of comparison. From the data in Table 1 it can readily be seen that coumestrol is considerably more potent than the estrogenic isoflavone, genistein, although it is much less active than diethylstilbestrol.

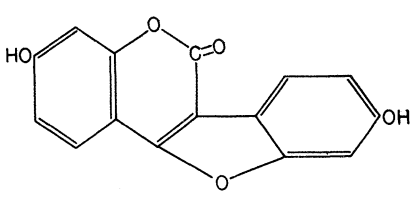

Fig. 1. Proposed structure of coumestrol.

For the bioassay, the estrogen, incorporated in the stock diet of the animals, was fed to 18-day-old immature female mice for a period of 7 days. The mice were killed and the weights of the freshly excised uteri were determined.

The estrogenic compound was isolated by means of solvent extraction of the dried meal, followed by several countercurrent distributions, with final recrystallization from methanol.

Coumestrol has a bright blue fluorescence in neutral or acid solution, turning to a greenish yellow in strong alkali. This characteristic greatly facilitated its isolation. It melts with slight decomposition at 385°C (Kofler block). Its ultraviolet absorption spectrum (measured in methanol) shows maxima at 208, 243, and 343 mμ. Alkali fusion of the compound yielded resorcinol and beta-resorcylic acid but no other identifiable phenols or phenolic acids.

Coumestrol has the empirical formula, $C_{15}H_8O_5$. Among the derivatives that have been prepared are the diacetate, $C_{19}H_{12}O_7$ (melting point, 234°C) and the dimethyl ether derivative, $C_{17}H_{12}O_5$ (melting point, 198°C), indicating that two free hydroxyl groups are present. There are no methoxyl groups in the compound.

Treatment of coumestrol with dimethyl sulfate under strongly alkaline conditions gave a trimethyl ether-monomethyl ester, $C_{19}H_{18}O_6$ (melting point, 98°C). Mild alkaline hydrolysis of this compound gave the trimethyl ether acid, $C_{18}H_{16}O_6$ (melting point, 178°C). The formation of an acid by this means confirms the presence of a coumarinlike structure in the molecule. Titration of this acid indicated a minimum molecular weight of 331. Decarboxylation of this acid derivative of coumestrol yielded a compound having the empirical formula $C_{17}H_{16}O_4$ (melting point, 82°C). Ozonolysis of this decarboxylated product yielded a number of degradation products, two of which have been identified as 2-hydroxy-4-methoxybenzoic acid and 2,4-dimethoxyzenzoic acid. The close agreement between the analytical data obtained and the theoretical data expected from the above derivatives has led us to propose the structure shown in Fig. 1 for coumestrol.

Mentzer *et al.* (*9*), on purely theoretical grounds, have synthesized a number of coumarin derivatives that showed estrogenic activity. Their most active estrogen was 3-(*p*-hydroxyphenyl)-4-propyl-7-hydroxycoumarin. Except for the alkyl side chain at position 4, a striking similarity exists between our proposed structure for coumestrol and the synthetic coumarin derivative. A recent article (*10*) reports the isolation of a lactone from *Wedelia calendulacea* with basic structure similar to our proposed structure, differing however, in number, position and type of substituent groups. Therefore, it would seem that if the proposed structure proves to be correct, coumestrol represents an estrogenic compound not previously reported in the literature.

Note added in proof: We have confirmed the proposed structure of coumestrol and also the estrogenic effectiveness of the synthetic material.

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9 August 1957

Wet Freeze-Drying of Muscle

It was found that acetone, at Dry-Ice temperature, does not denature proteins but still is capable of dissolving water to some extent. It was also found that chlorated paraffins do not harm contractile proteins at room temperature. This allows one to dehydrate muscle without destroying its molecular structure and contractility.

Fresh frog sartorius muscles or thin strips (1 to 2 mm) of freshly isolated rabbit's psoas were tied to applicator sticks at rest length and immersed in acetone cooled in Dry Ice. The frozen muscle is kept at this low temperature in acetone for a week, after which time the acetone is exchanged with fresh precooled anhydrous acetone in which the muscle is left for a fortnight. We have used 50-ml test tubes as containers and have kept these test tubes in thermos bottles filled with Dry Ice. The acetone slowly dehydrates the frozen muscle. Instead of exchanging the acetone, one can also bind the water extracted from the muscle with granulated $CaCl_2$.

After the water has thus been extracted, the dehydrated muscle is transferred into precooled ethyl chloride and

left in this solvent for a week or so at Dry Ice temperature. The ethyl chloride extracts the acetone. After this extraction has been completed, the muscle preparation is simply taken out of the solvent and is allowed to warm up to room temperature, whereupon the ethyl chloride evaporates. In order to avoid condensation of water, it is advisable to let the preparation warm up in a desiccator or in a small tube closed by a loosely fitting cork, or to allow the ethyl chloride to evaporate in the Deep-Freeze.

The preparation thus obtained is white but has the shape and appearance of the original muscle and is feather-light. On immersion into water, the muscle contracts violently to one-third to one-fifth of its original length. If the water does not penetrate uniformly, the muscle bends towards the more hydrated side, and may double up and break. Contraction can be demonstrated best by cutting up the preparation with a razor blade into thin strips and immersing these in water. The preparation can be kept for several weeks in the desiccator without loss of its contractility. When it is kept in an open vessel, it loses contractility in a few days.

One of us (A. S.-G.) introduced previously the method of glycerination (*1*). The glycerol-extracted muscle demands adenosine triphosphate and ions for its contraction, these having been washed out. The above "wet freeze-dried" muscle contains these constituents and so demands only the replacement of the extracted water (*2*).

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References and Notes

1. A. Szent-Györgyi, *Biol. Bull.* 96, 140 (1949).
2. This research has been sponsored by the National Heart Institute (grant H-2042 R), the National Science Foundation, the American Heart Association, the Muscular Dystrophy Associations, and Association for the Aid of Crippled Children, and the United Cerebral Palsy Foundation.

9 August 1957

Detection of Manganese-54 in Radioactive Fallout

During the 1956 nuclear test series at the Eniwetok Proving Grounds, fallout samples were collected and returned to this laboratory, where they were routinely submitted to gamma spectral analysis. A sample analyzed approximately 300 days after detonation revealed the presence of a gamma emitter with an energy of 0.84 Mev (*1*). Subsequent to this observation other fallout samples collected from the same operation also exhibited gamma

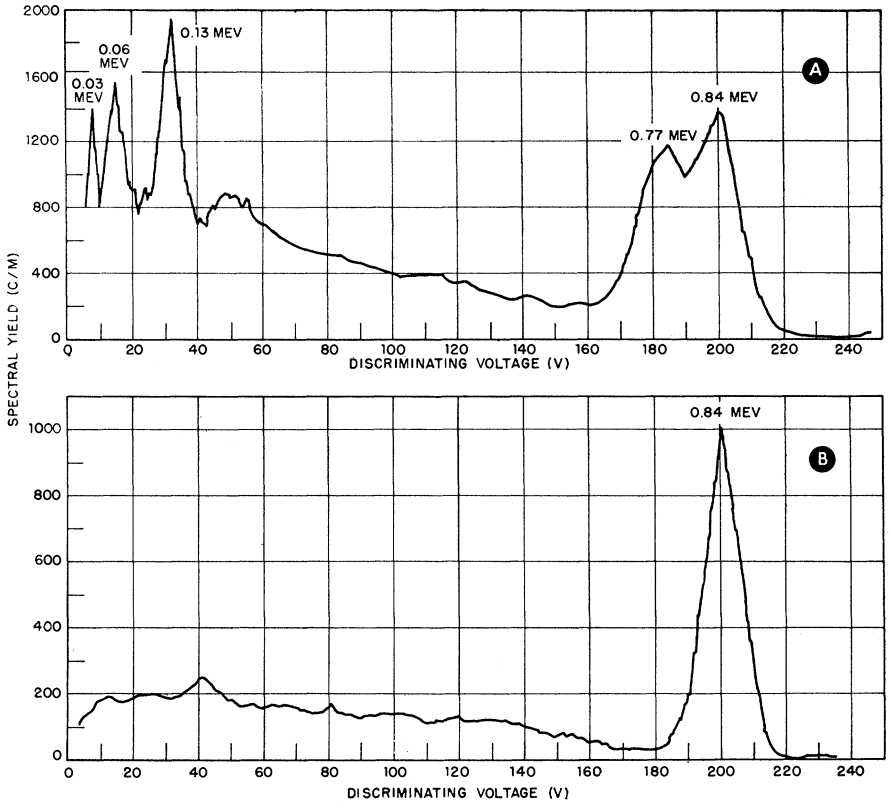

Fig. 1. *A*, Gamma spectrum of the gross activity in the fallout sample. *B*, Gamma spectrum of the activity in the isolated sample.

spectral peaks at this energy (*2*). Since gamma photons with energy of 0.84 Mev are not known to exist in fission products of this age, further study was undertaken to identify the gamma-emitter.

Since Mn^{54} has a relatively long half-life (291 days) (*3*) and a single gamma energy of 0.84 Mev (*4*), an analytical scheme was adapted for its isolation. The chemical method consisted of the following steps. Manganese carrier, together with cerium and zirconium hold-back carriers, was added to the dissolved fallout sample, and the whole was oxidized with sodium chlorate. The insoluble manganese dioxide was reduced and solubilized with sodium bisulfite and hydrochloric acid. The solution was scavenged with the aid of basic ferrous acetate (*5*) to remove interfering nuclides. Manganese was precipitated as the ammonium phosphate and ignited to the pyrophosphate for evaluation of chemical recovery. The resulting precipitate was gamma-counted with a sodium iodide-thallium activated crystal detector and resubmitted to gamma spectral analysis.

Figure 1 shows the gamma spectrum of the activity in the gross sample as well as the gamma spectrum of the activity in the sample isolated by chemical separation. The reliability of the analytical procedure was evident, and the presence of Mn^{54} was confirmed. Its radiations persisted and were more sharply defined after chemical separation, by which the energy peaks characteristic of fission products were completely eliminated. Furthermore, the gamma spectrum of the isolated sample activity was exactly superimposed on the spectrum of an authentic sample of Mn^{54}. Aluminum and beryllium absorption curves were taken to establish the identity of the isotope unequivocally. The absorption characteristics were the same as those of the Mn^{54} standard.

Manganese-54 gamma activity (in counts per minute), corrected for chemical recovery, was compared with the gross gamma activity (in counts per minute) of the fallout sample. This radionuclide represented about 40 percent of the total gamma activity. Moreover, calculations indicate that megacurie quantities of this nuclide were produced at the time of detonation.

Stable manganese and iron are possible precursors of Mn^{54} in the presence of high-energy neutrons. The probable nuclear reactions are

$$Mn^{55}\ (n, 2n)\ Mn^{54}$$

and

$$Fe^{54}\ (n, p)\ Mn^{54}$$

The appearance of readily detectable quantities of Mn^{54} again emphasizes the importance of considering induced radioactivities in fallout (*6*). Because of the biological importance of manganese as a trace element, we contemplate an in-