# Tricin from Alfalfa—Isolation and Physiological Activity

By E. M. BICKOFF, A. L. LIVINGSTON, and A. N. BOOTH

**Tricin was isolated from the press juice of fresh alfalfa (*Medicago sativa*) and identified by comparing its physical and chemical properties to authentic material. Tricin caused smooth muscle relaxation when in contact with isolated guinea pig intestinal strips but did not produce bloat in sheep. It exhibited only slight activity as an antioxidant, measured by the protection of epinephrine from oxidation. The activity of tricin as an estrogen was slight.**

THE FLAVONE, tricin, recently reported in the seed of *Phoelipaea ramosa* (1), was originally isolated from wheat (2) and subsequently reported in alfalfa (3). The latter report stated that it was isolated by ether extraction of clarified juice and purified by high-vacuum sublimation but gave no further details. In this laboratory, extraction of dehydrated alfalfa with 95% alcohol, using the procedure of Anderson (2), gave a low yield of tricin. Furthermore, a crude product, including much extraneous material, was extracted with ether from alfalfa juice by the procedure of Ferguson *et al.* (3). The isolation procedure finally developed was a modification of these two procedures.

Ferguson's observation that tricin relaxed and partially paralyzed excised rabbit intestine led him to postulate that the substance might cause bloat (4–6). However, he was not able to obtain sufficient tricin to test this theory.

The subject of estrogen-like substances in forages has been of continuing interest since 1948, when Curnow *et al.* (7) prepared estrogenic extracts from subterranean clover and showed their relation to reproductive problems of sheep grazing this forage. To date, five estrogen-like substances have been isolated from alfalfa. These include the coumarin-like compound, coumestrol (8), and the four isoflavones, genistein, daidzein, formononetin, and biochanin A (9). No estrogenic flavones have been isolated yet from forage, although Wenzel and Rosenberg (10) in a study on the estrogenic activity of flavones reported that 4′-6-dihydroxyflavone was active.

## EXPERIMENTAL

**Preparation of Tricin from Alfalfa.**—Fresh alfalfa was passed through a double-roller cane crusher and the juice collected. The bagasse was extracted twice with fresh portions of water and the juice expressed. The combined juices were heated to 60° for 5 minutes, then rapidly cooled with ice. Following removal of the green sediment by centrifugation, the clear brown juice was concentrated about tenfold under vacuum. Centrifugation was followed by extraction of the juice in a separator with an equal volume of ether. Small quantities of alcohol were added to the mixture to prevent the formation of emulsions. Greenish-yellow crystals formed as the ether layer was slowly evaporated on a steam bath. Before permitting the solution to go down to dryness, the crystals were collected on a filter and recrystallized from ethanol and dimethylformamide.

A total of about 4000 lb. of fresh alfalfa was processed to collect about 20 Gm. of tricin. Following this procedure, in numerous individual isolations from 100- to 200-lb. portions of fresh alfalfa, the quantity isolated varied greatly, ranging from almost none to about 0.02% of the dry weight of the alfalfa.

**Identification of Tricin.**—The analytical sample was recrystallized from ethanol and dimethylformamide, m.p. 291–294° dec.; no depression in melting point with an authentic sample.

*Anal.*—Calcd. for $C_{17}H_{14}O_7$: C, 61.8; H, 4.24. Found: C, 61.3; H, 4.57.

**5,7,4′-Triacetoxy-3′,5′-dimethoxyflavone.**—One-hundred milligrams of tricin was acetylated in the usual way with acetic anhydride and fused sodium acetate. Recrystallization from chloroform gave 95 mg. of colorless needles of the triacetate, m.p. 251–254°; no depression in melting point with an authentic sample.

*Anal.*—Calcd. for $C_{23}H_{20}O_{10}$: C, 60.5; H, 4.38; $OCH_3$, 13.56; $CH_3CO$, 28.2. Found: C, 60.5; H, 4.43; $OCH_3$, 13.3; $CH_3CO$, 27.5.

Identical X-ray diffraction patterns, infrared, and ultraviolet spectra were obtained with the isolated tricin and an authentic sample as well as with the acetate derivative and an authentic sample of the acetate.

### Pharmacological Studies with Tricin

**Effect on Guinea Pig Intestine.**—The direct action of tricin on smooth muscle strips was compared to the action of the flavonoid, quercetin. Two milligrams of tricin in the 50-ml. tissue bath was required to produce a relaxation equal in magnitude to that produced by 0.5 mg. of quercetin. Wilson and DeEds (11) have reported the correlation between the antioxidant properties of various flavonoids and their ability to prolong the epinephrine relaxation time of excised guinea pig colon segments. Using their technique, Fig. 1 shows that the epinephrine relaxation time was only slightly prolonged by tricin, in contrast to the striking effect of an equal weight of quercetin.

**Effect on Bloat in Sheep.**—The procedure for the administration of test materials to sheep has been previously described (12). Ten grams of tricin was administered in this same manner but failed to reduce ruminal motility or cause bloat in the sheep.

A water suspension of 25 Gm. of quercetin administered to five intact sheep also failed to produce

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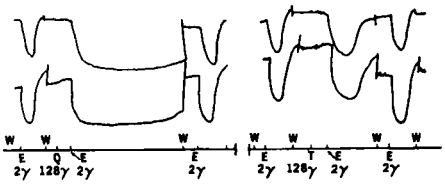


Fig. 1.—Prolongation of epinephrine (E) relaxation time of guinea pig colon segments in the presence of quercetin (Q) and tricin (T). W = wash out and replacement of bath fluid (50 ml.) with fresh Tyrode solution.

bloat symptoms. On the basis of muscle relaxing power, the quercetin administered was equivalent to 100 Gm. of tricin (12).

In another study (13) each of three cows were given 2 Gm. of quercetin on 1 day, followed by 10 Gm. each on 2 successive days. The cows showed no indication of bloat. If we assume a tricin content of 0.02% in alfalfa, it would be impossible for cows to consume enough alfalfa to ingest 10 Gm. of tricin in 1 day.

**Mouse Uterine Weight Bioassay Procedure.**—In this previously described report (14), weanling mice (five per cage) were fed diets containing the test materials. At the end of 4 days, the mice were sacrificed, and the uteri were excised and weighed. The results are presented in Table I. For comparative purposes, coumestrol was included in the study. The data indicate that tricin was weakly estrogenic ($p$ = <0.01).

**Metabolic Fate Studies.**—That quercetin is absorbed from the intestinal tract is shown by the appearance of three split products in the urine when the compound is fed to rats by mouth (15). However, when tricin was fed under similar conditions, no split products like syringic acid were detected. Thus, it is doubtful that tricin is absorbed from the intestinal tract.

TABLE I.—EFFECT OF TRICIN, COUMESTROL, AND DIETHYLSTILBESTROL ON THE IMMATURE MOUSE UTERUS

| Group[a] | Compd. | Quantity Fed per Mouse, mg. | Mean Uterine Wt., mg. |
|---|---|---|---|
| 1 | Control | 0 | 9.6 ± 0.25 |
| 2 | Tricin | 20 | 11.2 ± 0.57 |
| 3 | Tricin | 40 | 11.4 ± 0.75[b] |
| 4 | Tricin | 40 | 12.4 ± 1.02[b] |
| 5 | Coumestrol | 0.6 | 32.4 ± 2.63 |
| 6 | Diethylstilbestrol | 0.0001 | 29.2 ± 0.40 |

[a] Five weanling female mice per group. Supplements incorporated into control diet and fed 4 days (10 Gm. diet/mouse). [b] $p$ = <0.01.

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# Effects of Gibberellic Acid on the Fixed Oils of Four Plants

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**The influence of gibberellic acid on the quality of fixed oils from four plants has been studied. Sesame, castor bean, sunflower, and flax plants were treated with aqueous solutions of gibberellic acid when the plants reached blooming size and at 2-week intervals until harvest. The iodine values and the quantity of unsaponifiable matter varied between samples and between oils from different species of plants. Significant variations from normal were produced in castor and sesame oils. The differences consisted of higher saponification values in both oils and a lower acid value in the latter. A higher saponification value indicating shorter chain fatty acids was the only characteristic common to all oils from treated plants. Some of the changes obtained were from plants that had insignificant or no visible morphological differences.**

THE GIBBERELLINS have been known in the United States and Europe for only about 12 years, and they have been available for extensive experimental use for less than a decade (1). The number of research papers involving the gibberellins runs into the thousands; there have been a number of reviews which give detailed information regarding such aspects as the physiology, chemistry, and recommended economic applications (1–5).

Sciuchetti (6), in a recent review, has included the influence of gibberellic acid (GA) on volatile oil, glycoside, and alkaloid producing plants. Only a few of the long list of references deal with changes in the active components, and no references are listed relative to changes in fixed oils. The percentages of certain terpenes in volatile oils have been changed by the use of GA. A slight increase

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