it is difficult or inconvenient to use a taste panel. This is particularly true with large studies where panel fatigue and costs become important factors.

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Structural Determination of Two Basal Metabolic Rate-Stimulating Flavones from Grass Silage

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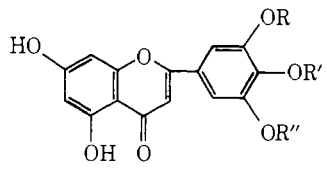
Two chemicals from dried grass silage, previously shown to be basal metabolic rate-stimulating when fed to male rats, were shown by ultraviolet spectral analysis and paper chromatography to be tricin and probably 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone. The basal metabolic rate-stimulating activity of these compounds is greater than certain other flavonoids, probably because they are resistant to degradation by intestinal flora.

McLaren *et al.* (1964) have shown that dried grass silage (DGS), made from approximately equal parts of wheat, vetch, orchard grass, and alfalfa, stimulated basal metabolic rates (BMR) when fed to male rats. McLaren *et al.* (1964, 1966) also demonstrated that an 80% ethanol extract of DGS and various other flavonoid-containing extracts were BMR-stimulating to the rat. More recently (Qasim, 1970), DGS was extracted according to the method of McLaren *et al.* (1964) and subjected to extensive fractionation with solvent extractions and paper chromatography. Several of the fractions, including compounds I and II of this paper, were shown to cause elevated BMR when incorporated into the diet of male rats. The present report offers proof that one of these fractions is the flavone tricin (I) and there is strong evidence that the other is 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone (II).



I, R = R″ = CH_3; R′ = H
II, R = R′ = R″ = CH_3
III, R = R′ = R″ = H

EXPERIMENTAL SECTION

Spectral Analyses. Ultraviolet spectra were measured in 95% ethanol as well as in 95% ethanol saturated with sodium acetate or containing 5% $AlCl_3$ or 2 *N* NaOH (Jurd, 1962).

Paper Chromatography. All chromatography was one-dimensional on 56-cm sheets of Whatman no. 1 filter paper. The solvents were of analytical reagent quality and were used without further purification except for phenol, which was distilled over zinc dust according to the method of Gage *et al.* (1951). The solvents employed were 1-butanol–acetic acid–water (4:1:5, v/v) (BAW), acetic acid–concentrated HCl–water (30:3:10, v/v) (Forestal solvent), 73% (w/w) phenol in water, 30% (w/v) acetic acid in water and double-distilled water (Seikel, 1962).

Compounds separated by chromatography were routinely located under ultraviolet light after fuming the papers with ammonia. When studying the color properties of the separated chemicals, the chromatograms were sprayed with 5% ethanolic $AlCl_3$, 5% aqueous neutral or basic lead acetate, or Folin reagent followed by exposure to ammonia fumes.

Derivatizations. Compound II was demethylated by refluxing in benzene containing $AlCl_3$ according to the method of Seshadri and Varadarajan (1953). Another sample of compound II was hydrolyzed by refluxing in 2 *N* HCl for 2 hr, as described by Harborne and Hall (1964).

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Table I. Selected Absorption Maxima (nm) of Various Flavonoids in 95% Ethanol and 95% Ethanol Containing the Indicated Chemical

Flavonoid	95% Ethanol				Sodium acetate,
	Band I	Band II	$AlCl_3$, Band I	NaOH, Band I	Band II
Compound I	352	244, 269	359, 386	424	272
Compound II	331	272	338	363	277
Tricin	353	244, 270	359, 386	424	272
5,7-Dihydroxy-3′,4′,5′-trimethoxyflavone	329	271	335	366	274
Hydrolyzed compound II	352	245, 270	359, 389	420	272
Demethylated compound II	353	250, 269	362, 395	Decomposed	266
Tricetin[a]	355	248, 269	398	Decomposed	270

[a] From Harborne (1967).

Table II. R_f Values of Various Flavonoids on Whatman No. 1 Paper in Five Solvents[a]

Flavonoid	BAW	H_2O	Forestal	Phenol	Acetic acid
Compound I	0.74	0.00	0.76	0.93	0.32
Compound II	0.88	0.04	0.89	0.94	0.56
Tricin	0.74	0.00	0.76	0.93	0.32
Hydrolyzed compound II	0.74	0.00	0.76	0.95	0.32
Demethylated compound II[b]	0.67		0.47	0.30	0.15
Tricetin[c]	0.56		0.37	0.28	
5,7-Dihydroxy-3′,4′,5′-trimethoxyflavone[d]	0.85		0.86		

[a] Each value is the average of three chromatograms, except as indicated. [b] Value of a single chromatogram. [c] From Harborne (1967). [d] From Griffiths and Smith (1972).

RESULTS

The ultraviolet absorption maxima of compounds I and II, derivatives of compound II, authentic tricin, authentic 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone, and tricetin (III) are listed in Table I.

The R_f values and color properties of compounds I and II, derivatives of compound II, and authentic tricin are given in Tables II and III, respectively. In all instances only a single spot could be seen for each chromatographed sample.

DISCUSSION

Preliminary observations of color tests suggested that compounds I and II were both flavonoids. The appearance of a green color with alcoholic ferric chloride indicated polyhydroxy flavonoids. Yellow precipitates in the presence of basic lead acetate suggested flavones or flavonols. Bright yellow-green colors with concentrated sulfuric acid or alkali substantiated the flavone or flavonol nature of compounds I and II (Geissman, 1955).

The ultraviolet spectrum of compound I in 95% ethanol indicated that it was a flavone. The reported spectrum of tricin as well as reported chromatographic data (Harborne, 1967) suggested that compound I might be tricin. An authentic sample of this flavone was obtained. Comparative spectral analyses (Table I), paper chromatography (Table II), and color properties (Table III) showed that compound I and tricin were identical.

The presence of tricin in DGS is not surprising since glycosides of this flavone are common in grasses (Harborne and Hall, 1964), including alfalfa (Bickoff *et al.*, 1964).

The ultraviolet spectrum of compound II in 95% ethanol showed that it was also a flavone. Table I lists the absorption maxima of compound II in 95% ethanol and 95% ethanol containing various chemicals. A bathochromic shift of Band II from 272 to 277 nm and a higher intensity of this peak in 95% ethanol containing saturated sodium acetate indicated a free hydroxyl group at position 7. A bathochromic shift of Band I toward the visible in 95% ethanol containing 5% aluminum chloride demonstrated a free hydroxyl group on carbon-5. A pronounced bathochromic shift and a reduced intensity of Band I in 2 *N* NaOH demonstrated a substituted 4′-hydroxyl group (Jurd, 1962).

A sample of compound II was acid hydrolyzed and then shown to be identical with compound I and authentic tricin (Tables I-III). That compound II was some 4′ derivative of tricin was further supported by the fact that the demethylated derivative of compound II had spectral properties identical to those reported for tricetin (Table I).

Compound II was suspected to be a 4′-glycoside of tricin. However, no carbohydrate could be detected in an acid hydrolysate. Additional evidence that compound II is not a glycoside is that compound II had a higher R_f in BAW than its hydrolytic product, tricin. Bate-Smith (1950) reported that flavonoid glycosides have lower R_f values in BAW than the corresponding glycosides. This conclusion is substantiated by the fact that the R_f values of compound II in BAW, Forestal solvent, and phenol are higher than the value of any reported flavonoid glycoside in these solvents (Harborne, 1967).

The conditions used to hydrolyze the group from the 4′ position of compound II would not normally be sufficient to remove a methyl ether group. However, since tricin should be substantially more stable than a flavone containing a 3′,4′,5′-trimethoxy structure, it was felt that compound II could be 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone. An authentic sample of this flavone was obtained and it was shown that the spectra of the authentic sample and compound II were identical (Table I).

Unfortunately the authentic sample of 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone was not paper chromatographed and an additional sample was not available. Nonetheless, on the basis that demethylation and hydrolysis of compound II yielded tricetin and tricin, respectively, that compound II and the authentic sample had identical ultraviolet spectra, and that the R_f values of compound II were nearly identical to literature values for 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone (Table II), it is almost certain that compound II is 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone. This compound has been chemically synthesized (Mentzer and Pillon, 1953), but has apparently never before been isolated from a biological source.

Compounds I and II were both BMR-stimulating at low concentrations when fed to male rats. Silage fractions containing substantial amounts of other flavonoids either did not cause elevated BMR or were less active than either of the compounds reported here (Qasim, 1970). The commercial flavonoids quercetin, rutin, and hesperidin were shown to be BMR-stimulating (McLaren *et al.*, 1966)

Table III. Colors of Various Flavonoids on Paper under Visible (V) and Ultraviolet (UV) Light in the Presence of Various Chemicals[a]

Flavonoid	Untreated		NH_3		Alcoholic $AlCl_3$		Basic lead acetate,	Neutral lead acetate,	Folin reagent,
	V	UV	V	UV	V	UV	V	V	V
Compound I	PY	DB	Y	BYG	Y	YG	Y	Y	Bl
Compound II	PY	RB	Y	RB	Y	LYG	Y		Bl
Tricin	PY	DB	Y	BYG	Y	YG	Y	Y	Bl
5,7-Dihydroxy-3′,4′,5′-trimethoxyflavone	PY		Y	RB					
Hydrolyzed compound II	PY	DB	Y	BYG	Y	YG	Y	Y	Bl
Demethylated compound II	PY	DB	Y	BGoY	Y	LY	Y	Y	Bl

[a] Abbreviations: PY, pale yellow; DB, dark brown; Y, yellow; BYG, bright yellow green; YG, yellow green; Bl, blue; RB, red brown; LYG, light yellow green; BGoY, bright golden yellow; LY, light yellow.

but were less active than tricin or 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone.

Bickoff *et al.* (1964) and Stelzig and Ribeiro (1972) were unable to detect phenolic degradation products in the urine of rats fed low levels of tricin and Stelzig and Ribeiro (1972) showed that less than half of the ingested tricin was excreted in the feces. Griffiths and Smith (1972) were able to detect only small amounts of 3,5-dihydroxyphenylpropionic acid in the urine of rats that were given a single 100-mg feeding of tricin or 5,7-dihydroxy-3′,4′,5′-trimethoxyflavone. Stelzig and Ribeiro (1972) demonstrated that substantially greater quantities of phenol were excreted in the urine of tricin-fed rats than in the urine of quercetin-fed rats.

The mechanism of BMR-stimulation that results when flavonoids are fed to rats is not understood. However, the greater activity of the flavonoids reported in this paper compared to certain other flavonoids may simply be due to the fact that these two compounds are not easily degraded by the intestinal flora of the rat.

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