and Lomb Spectronic 20 colorimeter is shown in Figure 2. The slope of the graph is −0.1323.

Sample Analysis. Weigh into a 250-ml. beaker 25.0 grams of milk or other dairy product, diluted until its protein concentration is equivalent to that of milk (about 3%). Add 5 ml. of 4% Rivanol and allow the mixture to stand 5 minutes for maximum protein precipitation. Remove the aggregated protein by filtration through folded filter paper. To the filtrate add 1 drop of 10% sodium hydroxide and proceed as directed under "Preparation of Calibration Curve." Determine the concentration of lactose from the calibration curve.

Testing of Method. The method was tested for accuracy and precision by increasing the lactose concentration of a skim milk sample by from 1 to 6% with added lactose. The concentration of lactose was then determined by the described procedure. A single determination was made on each concentration shown in Table II. On the basis of these data the accuracy of the method in the concentration range investigated is within ±0.2%. The largest deviation was 4.7% greater than the true value.

Effect of Sucrose. In some dairy products sucrose is used as a sweetening agent. To determine the effect of added sucrose on the measurement of lactose, 5, 10, 15, and 20% commercial sucrose was added to a skim milk sample and the lactose determined. Allowing for the dilution due to the added sucrose, the amount of lactose found and the amount calculated to be present at each concentration of sucrose are shown in Table III.

The amounts of lactose determined are in good agreement with the calculated amounts. Therefore, the method can be applied to the analyses of dairy products containing sucrose.

Discussion

The linear relationship between the amount of unreacted Fehling solution and the concentration of lactose points to the existence of a similar relationship between the amount of reacted Fehling solution and the concentration of lactose. By the Munson-Walker method, the amount of precipitated cuprous oxide retained on the asbestos mat is used as a measure of the extent of the reaction. That the amount of cuprous oxide retained on the filter from the hot reacting solution is not stoichiometrically related to sugar content was observed in these analyses by the appearance of precipitated cuprous oxide in the cold filtrate. This filtrate has been in contact with borate ion and the reducing action stopped, as found by the measurement of identical absorbance of samples of filtrate taken before and after the appearance of precipitated cuprous oxide. On the basis of these observations, cuprous oxide in a strong alkaline medium in the presence of tartrate has a definite solubility when hot. This fact may explain the need for such careful reproduction of all operations in successfully using the Munson-Walker procedure.

Procedural difficulties which gave technicians the most trouble were in the removal of excess Rivanol after precipitation of the proteins. The amount of Rivanol remaining depends upon the protein content of the product being examined. This Rivanol is precipitated by the addition of a small amount of base. If too much base is added, a cloudy suspension is produced which is not removed by filtration and causes low results. Its nature suggests that it contains some proteins. It is obtained only when dealing with dairy products. If the pH is raised too far, the precipitate reverts to a suspension which reacts with the cupric ion of Fehling solution. The usual appearance of a slight yellow color does not affect the results, as long as the filtrate is clear of suspended material.

This method has the same limitations as the Munson-Walker method, in that it is not specific for lactose. All reducing sugars present in the material analyzed will react with the reagent used.

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FORAGE ESTROGENS

Relative Potencies of Several Estrogen-Like Compounds Found in Forages

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ALFALFA, RED CLOVER, and subterranean clover have been shown to contain at least five compounds (*2, 3, 13, 15*) which can produce an estrogen-like response in the mouse—coumestrol, genistein, biochanin A, formononetin, and daidzein. All, with the exception of biochanin A, have also been found in ladino clover. Each compound has been evaluated at various times by different workers (*5–10, 17, 18*). However, since the bioassay techniques varied considerably between laboratories, it may be difficult or impossible to correlate results.

Bioassays of plants normally measure only total activity. To provide data which will enable us to determine the relative contribution of each compound to the total uterine stimulating activity of a forage, it is necessary to know the relative amounts of each present as well as their relative potencies. The objective of the research reported herein was to provide data on the relative potencies of each of the five estrogen-like compounds, employing diethylstilbestrol and estrone as standards.

Experimental

Preparation of Compounds. The coumestrol and coumestrol acetate were prepared synthetically by a previously published method (*12*). Formononetin was isolated from red clover, and its identity confirmed by comparison of its physical properties with those of an authentic sample. Daidzein was obtained from formononetin by demethylation. Genistein (m.p. 301° C. decomp.) was isolated from subterranean clover. Biochanin A was synthesized according

The relative estrogenic potencies of five compounds found in forages—coumestrol and its diacetate, genistein, biochanin A, formononetin, and daidzein—have been evaluated by a standardized mouse uterine weight bioassay procedure. For comparative purposes, diethylstilbestrol and estrone were also evaluated. Coumestrol was found to be 35 times as potent as genistein. Genistein was slightly more potent than daidzein, about twice as potent as biochanin A, and four times as potent as formononetin.

to the procedure of Yoder *et al.* (*19*). Diethylstilbestrol and estrone were commercially obtained materials.

Bioassay. Immature female mice, 19 to 21 days old and weighing between 8 and 10 grams, were obtained commercially in lots of 50. The groups of mice were, for the most part, randomly divided into lots of 5. In a few cases, an extra mouse or two was obtained and added to the experiment. In some cases, a mouse may have been lost due to accident or death, which would also lead to deviation from multiples of 5. The compounds under test were dissolved in acetone and added to the control diet; the solvent was removed under a current of air with uniform mixing. The details of bioassay were then identical with those previously described for forage extracts (*7*). Briefly, the test consists of feeding the mice for 4 to 6 days until an average of 10 grams of diet is consumed per mouse. The mice are then sacrificed, and the intact uteri are excised and weighed without blotting. The figures reported here represent pooled data and were obtained from a series of assays extending over a 2-year period. The results were examined for time effects and, if such existed, they were not great enough to affect interpretation of the results. The standard errors shown apply to pooled data and were calculated separately for each dose level.

Dose-Response Relationship. The initial plan was to evaluate the relative potencies of the several compounds by means of slope-ratio assays, since for coumestrol, estrone, and diethylstilbestrol, the logarithms of the uterine weights were linear with dosage. However, for the isoflavones this was not true. Instead, a linear relationship existed between the mean uterine weights and the dosage. Therefore, an alternate procedure was chosen for comparing potencies. For each compound, dose response curves were plotted from the data in column 4 of Table I. The respective doses for each compound required to produce a uterine weight of 25 mg. were found from these curves and are presented in column 5 of Table I. Relative potencies, defined as the ratio of the doses shown in column 5, to diethylstilbestrol, which is arbitrarily assigned a value of 100,000, are tabulated for each compound in column 6 of Table I. Slightly different estimates of relative potencies would be obtained if one picked a different response point.

Table I. Dose-Response Data and Relative Potency of Forage Estrogens vs. Diethylstilbestrol and Estrone

Compound	Quantity Fed per Mouse, μg.	Number of Mice	Uterine Weight ± S.E.,[a] Mg.	Quantity to Produce 25 Mg. Uterus, μg.	Relative Potency[b]
Control	0.000	300	9.6 ± 0.3		
Diethylstilbestrol	0.025	16	11.8 ± 0.2	0.083	100,000
	0.050	50	18.1 ± 0.7		
	0.075	40	23.7 ± 0.8		
	0.100	491	29.2 ± 0.4		
	0.200	31	78.1 ± 6.2		
Estrone	0.50	23	14.7 ± 0.9	1.20	6,900
	0.75	15	16.0 ± 1.3		
	1.00	22	23.8 ± 2.5		
	1.50	40	36.1 ± 2.3		
	2.00	5	45.3 ± 6.7		
Coumestrol	100	20	13.8 ± 0.2	240	35
	200	44	24.2 ± 0.2		
	300	126	29.2 ± 1.3		
	400	79	40.7 ± 1.9		
	500	36	76.0 ± 6.0		
Coumestroldiacetate	250	30	19.0 ± 1.2	340	24
	300	60	22.3 ± 1.6		
	400	65	26.7 ± 1.4		
	500	60	42.4 ± 3.3		
	1,000	87	87.0 ± 6.2		
Genistein	5,000	18	19.4 ± 0.9	8,000	1.00
	7,500	15	28.0 ± 5.0		
	8,000	19	27.0 ± 1.6		
	12,000	25	32.4 ± 1.9		
	15,000	35	36.6 ± 0.6		
	20,000	25	52.7 ± 1.9		
Daidzein	5,000	20	17.3 ± 1.3	11,000	0.75
	7,500	15	18.5 ± 2.6		
	10,000	20	24.8 ± 1.3		
	15,000	33	31.2 ± 1.4		
Biochanin A	10,000	30	20.3 ± 2.5	18,000	0.46
	20,000	59	27.9 ± 2.1		
	30,000	20	27.9 ± 2.6		
	40,000	38	45.5 ± 3.8		
Formononetin	15,000	20	16.8 ± 0.8	32,000	0.26
	20,000	15	17.9 ± 1.4		
	25,000	19	23.2 ± 1.0		
	30,000	20	27.5 ± 2.3		
	40,000	15	26.1 ± 2.0		

[a] S.E.—Standard Error mean.
[b] Measured at the dosage required to produce a 25-mg. uterus.

Results

Genistein was the most potent of the isoflavones, while formononetin was the least potent (Table I). Previous studies at this laboratory (*4*) have shown that methylation of coumestrol reduces its estrogen-like potency. This is confirmed again in this study with the isoflavones—i.e., biochanin A (4′-methyl ether of genistein) was about one-half as potent as genistein, while formononetin (4′-methyl ether of daidzein) was one-third as potent as daidzein. The difference between the potencies of genistein and daidzein is apparently due to the presence of the 5-hydroxyl group of genistein. Bradbury and White (*8*) suggested that the presence of the hydroxyl group in the 5- position modifies the properties of the carbonyl group. This type of effect, previously ascribed to hydrogen bonding and chelate-ring formation, has been shown by Hergert and Kurth (*14*) to be due partly to mesomerism which decreases the double-bond character of the carbonyl $\rangle C{=}O$ linkage by partial formation of $\rangle C{=}O^{-}$.

The diacetate of coumestrol was about equal in activity to coumestrol, taking into account the weight of the two acetate groups.

Cheng and coworkers (*11*) under their bioassay conditions found daidzein to be the most potent of the four isoflavones, while biochanin A and genistein had about equal potencies. This apparent variation in results between that of Cheng's laboratory and that included in this study may be due to differences in bioassay procedure. One major difference between their procedure and ours is that in their procedure the excised mouse uteri were fixed in Bouin's fluid, then blotted before weighing.

The authors found formononetin to have definite activity, although less than that of other isoflavones. This is contrary to the findings of at least three other laboratories (*5*, *8*, *18*) which reported formononetin to be inactive by either oral administration or subcutaneous injection at levels of 10 to 50 mg. per mouse. Cheng *et al.* (*11*) reported that the feeding of formononetin to mice at a level of 2.5 mg. per day for 4 days produced a slight increase in uterine weight.

Our results, Table I, show that coumestrol is from 30 to 100 times more potent than the isoflavones. Estrone, the natural animal estrogen, and the synthetic diethylstilbestrol are respectively about 200 and 3000 times more potent than coumestrol under the conditions of our bioassay.

Despite the lower potencies of the forage estrogens, when compared to diethylstilbestrol, they are present in certain forages in sufficient quantities as to be influential in animal nutrition and physiology. Thus, Oldfield *et al.* (*16*) have shown that alfalfa meal, selected for high coumestrol content, can cause increased weight gains in wether lambs. Furthermore, the even less potent isoflavones, notably genistein, have been shown to be present in Australian subterranean clover (*7*) in sufficient quantities to cause reproductive problems in sheep.

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COTTONSEED PROTEINS

Isolation and Chromatographic Characterization of Low Molecular Weight Cottonseed Proteins

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Cottonseed protein is the term assigned to the composite mixture of individual proteins found in the defatted seed. Its value for food or feed is dependent upon the chemical content and physical characteristics of the individual proteins.

Jones and Csonka (*6*), using classical fractionation procedures, isolated six fractions from cottonseed. The α- and β-globulins, glutelin, and pentose protein composed the major fractions. Protamine-type protein was absent.

Spies and coworkers (*12*, *13*) have found the relatively minor water-soluble fraction to consist of a series of proteins varying widely in pentose content.

Since lysine is a major determinant of the nutritive value of cottonseed protein (*9*), the isolation of a lysine-rich fraction in any comprehensive study would be of particular interest. Both the pentose protein of Jones and the water-soluble fraction of Spies were seemingly high in the basic amino acids.

With this carbohydrate content and salt solubility of cottonseed protein in mind, a preliminary fractionation attempt was made using a NaCl–aqueous ethanol solvent system originally designed to dissociate and solubilize nucleoproteins (*2*). This resulted in the isolation of a fraction which was 15% higher than the average protein in lysine content.

This investigation, therefore, is concerned with the isolation and characterization of that portion of glandless cottonseed (*8*) which is soluble in high molarity