Table IV. B Vitamins, Calcium, and Iron in Sesame Flour, Chick Peas, Soybean Flour, and Protein Mixture

	Sesame Flour	Chick Peas	Soybean Flour	Protein Mixture
Thiamine, mg.	2.40–2.56[a]	0.42–0.56	1.10–1.50	1.08–1.45
	2.53	0.46	1.26	1.25
Riboflavin, mg.	0.23–0.30	0.15–0.18	0.40–0.44	0.23–0.24
	0.27	0.16	0.42	0.24
Niacin, mg.	10.6–13.6	1.16–2.85	2.03–2.91	4.93–5.56
	12.6	2.28	2.36	5.13
Pantothenic acid, mg.	2.68–2.80	2.10–3.06	4.70–5.06	3.90–4.83
	2.76	2.53	4.88	4.33
Folic acid, μg.	28–30	79–100	79–86	40–72
	29	87	82	54
Calcium, mg.	124–163	150–161	416–636	220–260
	150	156	526	246
Iron, mg.	14.0–16.0	15.0	11.0–16.0	12.0–7.0
	14.3		12.9	13.5

[a] Per 100 grams.

Finally, the mixture contained considerable quantities of B vitamins, calcium, and iron. In this respect also it appears to be superior to its components.

In preliminary trials the protein mixture was administered to infants in order to study acceptability and tolerance. Water and sugar were added to make it isocaloric and isonitrogenous to cow's milk. After being cooked for 10 minutes it was given to 20 infants who did not suffer from gastrointestinal disorders. The preparation was well accepted and tolerated (*15*).

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CLOVER CONSTITUENTS

Isolation of Phenolic Compounds from Ladino Clover

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Previous studies at this laboratory on the phenolics of ladino clover have resulted in the isolation or detection of five estrogenlike compounds, coumestrol (*1, 2*), genistein, daidzein, formononetin, and biochanin A (*3*). In addition to estrogenic activity, about 33 different physiological and biochemical activities have been reported for 30 of the naturally occurring flavonoids of plants (*5*). The present investigation was a search for new, potentially valuable phenolic compounds in ladino clover.

Extraction and Fractionation

Step 1. Commercially dehydrated, pelleted ladino clover meal was ground, and 227 kg. of the ground meal were mechanically stirred for 24 hours with 190 liters of Skellysolve B at a temperature of 50° to 60° C. The mixture was filtered and the meal was dried under a stream of air, then extracted by heating and stirring with three 190-liter portions of acetone. After each extraction, the mixture was filtered, and the acetone extracts were combined and concentrated to a final volume of 30 liters containing 5.5 kg. of solids. Figure 1 presents a schematic two-dimensional

In a search for physiologically active agents in ladino clover, 17 phenolic compounds have been isolated, and the presence of many more has been indicated by paper chromatography. Procedures for the isolations included acetone extraction, alkali purification, countercurrent solvent distribution, and recrystallization. Partial characterization was accomplished by elemental analysis to indicate empirical formulas, melting point determinations, paper chromatography, and observations of fluorescence under ultraviolet light. Two compounds were identified as the coumarins, coumestrol and daphnoretin, and a third as the isoflavone, formononetin.

paper chromatogram of this extract when viewed under ultraviolet light.

Step 2. The 30 liters of extract were then treated as follows. Two liters of extract, 800 ml. of chloroform, and 700 ml. of 1*N* trisodium phosphate (pH 11.5) were shaken in a separatory funnel. The phases were separated by centrifugation, and the chloroform phase was re-extracted with five 1-liter portions of trisodium phosphate (1*N*). After centrifugation the alkaline phase was acidified to pH 3.5 and extracted with five 1-liter portions of ether. Combined ether phases from the 30-liter extract were evaporated to a viscous sirup containing 400 grams of solids.

Step 3. The sirup from step 2 was added to 6.8 kg. of anhydrous sodium sulfate and the mixture blended. The solids were packed into a chromatographic tube (10 cm. × 48 cm.) and successively washed with Skellysolve B, ether–Skellysolve B (2:8), ether, and acetone (approximately 4 liters of each eluate). The respective eluates were concentrated under vacuum and the solids determined in each fraction. The ether contained 380 grams (from a total input of 400 grams of solids), which included almost all the fluorescent phenolics free of water and of green waxy materials. The other three fractions contained only small amounts of phenolics and were not processed further.

Step 4. Purified solids from the ether fraction were next subjected to a series of countercurrent distributions (CCD).

Two robot-operated CCD instruments (a 100-tube, 200-ml.-per-tube machine and a 200-tube, 20-ml.-per-tube machine) were available for this study. The smaller instrument was employed whenever the quantity of starting material was less than 10 grams.

Forty-four distributions employing seven solvent systems (Table I) were required to separate the 17 compounds from the ether fraction. Figure 2 presents the order of these distributions. The Roman numeral assigned a particular compound depended upon its place in the sequence of purification. Thus, coumestrol is compound I, formononetin, compound II, and daphnoretin, compound VII. No number was assigned a compound unless it was evident that it could be isolated.

Upon completion of each distribution, a one-dimensional paper chromatogram (Figure 3) was prepared by spotting samples from selected tubes. All of the tubes were numbered in the same order as they were collected in the fraction collector. After observing the location of major components on such a chromatogram viewed under ultraviolet light, the contents of tubes having similar components were pooled to form fractions. A two-dimensional paper chromatogram was then made of each fraction to evaluate the separations. The crude concentrate was separated into nine fractions by 30 replicate distributions in solvent system A. Because of the complexity of this crude extract, it was necessary to redistribute certain of these fractions in additional solvent systems.

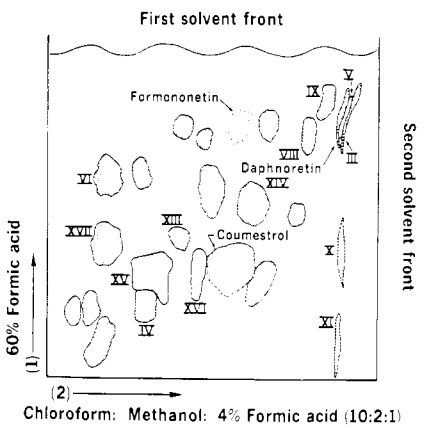

Figure 1. Schematic representation of two-dimensional paper chromatogram of acetone extract

Fraction 1 (tubes 271 to 310) contained the same fluorescent compounds as fractions 2 (tubes 236 to 270) and 3 (tubes 186 to 235) but in different proportions. The three fractions were, therefore, separately distributed in solvent system B and the similar tubes of each run combined to give five fractions (10 to 14). This procedure gave greater purification than if the three fractions had been combined and the CCD runs had been made on this mixture. Fractions 10 (tubes 1 to 91 of runs 31 to 34), 11 (tubes 92 to 117 of CCD runs 31 to 34), and 12 (tubes 118 to 125 of CCD runs 31 to 34) contained a large number of fluorescent compounds but less solids (33 grams) than fractions 13 and 14. Fraction 14 (tubes 169 to 192 of CCD runs 31 to 34) contained principally a compound dull gray-violet under ultraviolet light. Crystallization of the solids from methanol gave colorless crystals of compound V.

Redistribution of fraction 13 (tubes 126 to 268), which contained six major fluorescent compounds in 38 grams of solids, in solvent system C gave fractions 15 to 17. Fraction 15 (tubes 1 to 330 of CCD run 35) contained a gray-violet fluorescent compound (IX), which formed colorless crystals from methanol. Since fraction 17 (tubes 381 to 414) contained very little fluorescent material, no further processing was done on it. Further distribution of fraction 16 (tubes 331 to 380 of CCD run 35) in solvent system E led to the isolation of crystalline compounds XI (tubes 1 to 30 of CCD run 36), XII (tubes 31 to 55 of CCD run 36), XIII (tubes 181 to 220 of CCD run 36),

Table I. Solvent Systems Employed in Countercurrent Distribution of Ladino Phenolics

Solvent System	*Solvent (Proportions by Volume)*	*Starting Material Fraction*[a]	*Solids Employed in Each Distribution, G.*	*Total Transfers Made in Distribution*
A	Acetone-ether-water–Skellysolve B (10:5:5:2)	(Crude solids)	13	400
B	Chloroform–carbon tetrachloride–methanol-water (2:2:3:2)	1, 2, 3	8	288
		34, 35	27	228
C	Methanol-benzene-ether–water (4:4:1:1)	4	29	242
		13	38	510
		39	6	263
E	Acetone–carbon tetrachloride–water (2:1:1)	16	17	610
F	Ethyl acetate–acetone–Skellysolve B–water-ether (2:2:2:2:1)	20	9	303
G	Chloroform-acetone-water (1:1:2)	6	6	305
H	Skellysolve B–water-methanol-ether (2:1:3:5)	29	31	296
		7	17	392

[a] Source of each fraction presented in Figure 2.

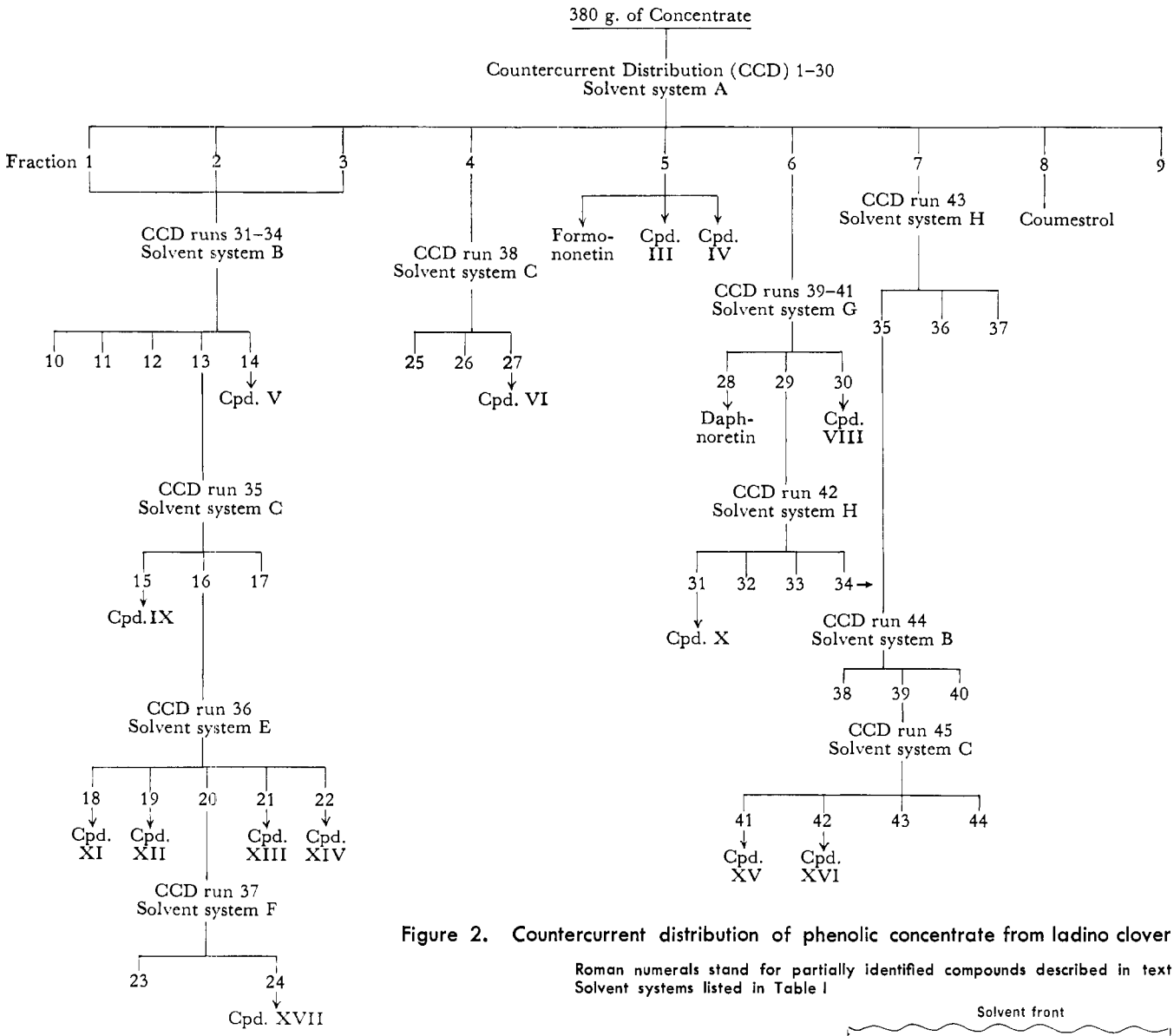

Figure 2. Countercurrent distribution of phenolic concentrate from ladino clover

Roman numerals stand for partially identified compounds described in text
Solvent systems listed in Table I

and XIV (tubes 321 to 520 of CCD run 36). Fraction 20 (tubes 56 to 180 of CCD run 36) contained mainly a gray fluorescent compound in addition to small amounts of three other compounds. Redistribution of this fraction in solvent system F led to the isolation of compound XVII (tubes 151 to 208 of CCD run 36). This compound gave light yellow crystals from acteone.

Fraction 4 (tubes 141 to 185) from the first series of distributions in solvent system A contained three major fluorescent compounds and five or six minor ones. Redistribution of this fraction in solvent system C gave fractions 25, 26, and 27. Fraction 25 (tubes 1 to 15 of CCD run 38) still contained three major fluorescent compounds in addition to several minor components. Fraction 26 (tubes 16 to 65 of CCD run 38) contained yellow and bluish fluorescent compounds in addition to a number of minor components. Because of the complexity of these two fractions, no further purification has been done on them. Fraction 27 (tubes 66 to 145 of CCD run 38) contained a gray-blue fluorescent compound which turned white upon fuming with ammonia. The solids from this fraction were crystallized from acetone to give light yellow crystals of compound VI.

Three pure compounds were obtained by the selection and combining of certain tubes from several of the 30 initial distributions. Together these three are called fraction 5 in Figure 2. Tubes 41 to 48 of distributions 26 to 30 gave a colorless crystalline compound which was identified as formononetin. Tubes 118 to 123 of distributions 27 to 30 gave colorless crystals of a blue fluorescent compound (III). Another blue fluorescent compound (IV) was obtained from three tubes, 34 to 36 of distributions 27 to 30, by recrystallization of the solids from methanol.

Fraction 6 (tubes 61 to 110 of CCD runs 1 to 30) contained three major fluorescent compounds and at least a dozen minor ones. The solubility of the solids in solvent system G was so low that it was necessary to divide the material and make three equal distributions. Crystallization of the solids from fraction 28 (tubes 1 to 50 of CCD runs 39 to 41) gave a pale yellow crystalline compound which was identified as daphnoretin. Fraction 30 (tubes 156 to 207 of CCD runs 39 to 41) contained a small quantity

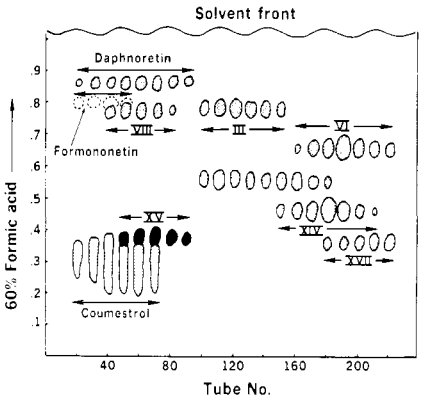

Figure 3. Schematic drawing of typical chromatogram of countercurrent distributions (1 to 30)

Table II. Paper Chromatographic Properties of Phenolic Compounds Isolated from Ladino Clover

Compound	Two-Dimensional Paper Chromatogram, R_f		UV Fluorescence (3660 A.)[a]	
	1st dimension[b]	2nd dimension[c]	Untreated	NH_3 vapor
I (Coumestrol)	0.4	0.65	Violet	White
II (Formononetin)	0.5	1.0	None	Gray
III	0.9	1.0	Blue	Bright blue
IV	0.3	0.3	Blue violet	Gray yellow
V	0.9	0.9	Gray violet	Absorb
VI	0.7	0.2	Gray blue	Bright white
VII (Daphnoretin)	0.9	0.95	Blue	Blue
VIII	0.8	0.8	Blue	Blue white
IX	0.9	0.9	Gray violet	Absorb
X	0.4	1.0	Bright violet white	Bright blue
XI	0.2	1.0	Dull blue gray	Blue gray
XII	0.45	0.45	Gray white	White
XIII	0.85	0.9	Absorb	Dull violet
XIV	0.6	0.7	Blue white	Yellow
XV	0.4	0.4	Yellow	Yellow
XVI	0.4	0.5	Yellow	Yellow
XVII	0.4	0.15	Gray	Yellow

[a] On paper chromatograms.
[b] Solvent system 1; 60% formic acid.
[c] Solvent system 2; chloroform-methanol–4% formic acid (10:1:1, organic phase).

Table III. Physical Properties of Phenolic Compounds Isolated from Ladino Clover

Compound	Weight Isolated, Grams	Melting Point, °C.	Empirical Formula	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
I (Coumestrol)	...	385 (w. dec.)	$C_{15}H_8O_5$	67.1	67.1	3.09	2.98
II (Formononetin)	0.6	255–56	$C_{16}H_{12}O_4$	71.6	71.6	4.48	4.41
III	0.11	255–57	...	...	...	...	...
IV	0.35	250–53	$C_{16}H_{12}O_4$	71.6	71.6	4.51	4.41
V	0.47	287–91	$C_{15}H_{10}O_5$	66.7	66.3	3.70	3.87
VI	0.4	315–16 (w. dec.)	$C_{15}H_{10}O_4$	70.9	70.6	3.94	4.08
VII (Daphnoretin)	3.5	244–45	$C_{19}H_{12}O_7$	64.8	64.9	3.41	3.58
VIII	0.1	224–29	$C_{15}H_{10}O_5$	66.7	66.9	3.70	3.79
IX	0.24	278–80	$C_{15}H_{10}O_5$	66.7	66.1	3.70	3.65
X	...	...	...	...	...	...	...
XI	2.5	327 (w. dec.)	$C_{16}H_{10}O_6$	64.4	64.4	3.36	3.48
XII	1.6	360 >	...	...	...	...	...
XIII	0.05	273–75	...	...	...	...	...
XIV	0.5	292–94 (w. dec.)	...	...	...	...	...
XV	2.4	...	...	...	...	...	...
XVI	0.1	...	...	...	...	...	...
XVII	0.12	318–21 (w. dec.)	$C_{15}H_{10}O_5$	66.7	66.8	3.70	3.65

of colorless crystals which gave a blue fluorescence on paper chromatograms. Fraction 29 (tubes 51 to 155 of CCD runs 39 to 41) contained 31 grams of the original 36 grams of solids. This fraction contained three major fluorescent compounds in addition to at least 25 minor ones. Distribution of this fraction in solvent system H and subsequent separation into four fractions led to the isolation of light tan crystals of compound X from fraction 31 (tubes 1 to 50 of CCD run 42). This compound, which gave a bright blue fluorescence on a paper chromatogram, was difficult to separate from an accompanying impurity. Because of this impurity we have not obtained melting point or elemental analyses for this compound. Fraction 32 (tubes 51 to 90 of CCD run 42) contained about 1 gram of nonfluorescent solids. Fraction 33 (tubes 91 to 155 of CCD run 42) contained a major blue fluorescent compound and smaller quantities of others. Fractions 34 (tubes 156 to 200 of CCD run 42), consisting of 22 of the original 31 grams of solids distributed in this run, contained three major fluorescent compounds. This fraction was part of the material used in distribution 44.

Fraction 7 (tubes 46 to 60 of CCD runs 1 to 30), containing four major fluorescent compounds, in addition to minor amounts of others, was also distributed in solvent H and the distribution divided into fractions 35 to 37. Fraction 35 (tubes 111 to 296 of CCD run 43) contained the same three blue fluorescent compounds as fraction 34. Fraction 36 (tubes 51 to 110 of CCD run 43) contained one major blue fluorescent compound and a large quantity of nonfluorescent impurities. Fraction 37 (tubes 1 to 50 of CCD run 43) contained only nonfluorescent compounds and was not further processed. Fractions 34 and 35 were combined and distributed in solvent system B. Although considerable purification was achieved (fraction 39, tubes 101 to 135), it was necessary to make one additional distribution in solvent system C before the two major fluorescent compounds (XV, tubes 1 to 20 of CCD run 45, and XVI, tubes 21 to 80 of CCD run 45) could be separated. Since these two compounds still contain traces of impurities, they will require further purification before elemental analyses and reliable melting points can be obtained.

Fraction 43 (tubes 81 to 141 of CCD run 45) contained three fluorescent compounds. Fraction 44 (tubes 142 to 166 of CCD run 45) contained two major fluorescent compounds and minor amounts of others. Recrystallization of solids from fraction 8 (tubes 21 to 45 of CCD runs 1 to 30) gave pure coumestrol. Since fraction 9 (tubes 1 to 20 of CCD runs 1 to 30) contained very little fluorescent material, no further processing has been done on it.

Step 5. Following their isolation by CCD the compounds were recrystallized several times from either acetone or aqueous methanol to give constant melting points and analytical samples.

Characterization

Two-dimensional paper chromatograms were prepared and the purity, R_f value, and fluorescence of each compound ascertained (Table II). In addition, melting points (uncorrected) were determined and the compounds subjected to elemental analyses. From the elemental analyses the most probable empirical formulas have been calculated (Table III).

Comparison of compound I from fraction 8 (Figure 2) on the paper chromatograms with and without samples of authentic coumestrol, as well as its distribution in solvent systems A-E, confirmed its identity.

The ultraviolet ($\lambda_{max.}^{EtOH}$248) and infrared spectra of compound II from fraction 5 were identical with those of an authentic sample of formononetin. Likewise there was no depression in melting point when mixed with an authentic specimen.

The isolated formononetin (100 mg.) from above was acetylated in the usual manner with acetic anhydride and fused sodium acetate. The mixture was diluted with ice water and the solids were collected and recrystallized from acetone to give colorless needles (85 mg., m.p.

171°, no depression with an authentic sample of formononetin acetate). The ultraviolet and infrared spectra were also identical with those of an authentic sample.

The paper chromatographic and physical properties of compound VII from fraction 28 agreed with those of authentic daphnoretin, and with that previously isolated from ladino clover by another procedure (*4*). The ultraviolet ($\lambda_{max.}^{EtOH}$ 346 mμ) and infrared spectra were also found to be identical to those of the authentic sample. Acetylation with acetic anhydride and fused sodium acetate gave a monoacetate (m.p. 235–36° C., no depression with an authentic sample). The ultraviolet and infrared spectra were also identical.

Acknowledgment

The authors gratefully acknowledge the receipt of the authentic sample of daphnoretin from R. Tschesche, and the authentic sample of formononetin from E. D. Walter. They are also indebted to Laurence M. White and Geraldine E. Secor for elemental analyses.

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FEED SUPPLEMENTS

Availability of Calcium in a Synthetic Hydrated Calcium Silicate (Micro-Cel)

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The calcium in Micro-Cel, a synthetic hydrated calcium silicate, was found to be available to growing chicks, the degree of availability varying between one half and three quarters of that of the calcium of analytical grade calcium carbonate. The criteria used in this comparison were: body growth, plasma alkaline phosphatase, bone ash, and bone calcium. Bone ash and body growth were the more satisfactory procedures for evaluating calcium availability.

MICRO-CEL is a highly absorptive synthetic hydrated calcium silicate made by the hydrothermal reaction of diatomaceous silica and lime. It is very absorptive and will facilitate a free flow of material when mixed in diets containing a large amount of fat or oil. As much as 2 to 3% Micro-Cel may be used in a feed mixture. Titus *et al.* (*6*) reported that tallow prepared with Micro-Cel was as well utilized in growing chicks as plain tallow, and that the diet supplemented with Micro-Cel supplied more metabolizable energy than the diet which did not contain Micro-Cel. The question arose whether Micro-Cel could serve as a source of calcium in the diet. In the following study, Micro-Cel was compared to $CaCO_3$, which is frequently used as a standard for the comparison of calcium sources in poultry rations. Changes in dietary calcium level have been shown to influence body growth, plasma alkaline phosphatase, bone ash, and the calcium content of bone ash (*3*); therefore these parameters were used as criteria in this comparison.

Experimental Procedure

Day-old Arbor Acre White Rock chicks were distributed into 20 lots on the basis of initial body weight and sex. Two lots, male and female, of 11 birds each, represented each treatment. The birds were housed in electrically heated Wahmann brooders, and at 4 weeks of age they were transferred to larger unheated cages situated in an air-conditioned room maintained at 22° C. The calculated calcium content for the basal diet NR110, shown in Table I, is 0.11% and the total phosphorus content 0.79%, including not less than 0.23% inorganic phosphorus. The basal ration, therefore, contained an adequate supply of phosphorus as recommended by the National Research Council (*4*). $CaCO_3$ or Micro-Cel was added to the ration at the expense of an equal amount of Celluflour. Five levels of both $CaCO_3$ and Micro-Cel were fed (providing 0.2, 0.4, 0.6, 0.8, and 1.0% calcium). The chicks were individually weighed and food consumption data were collected for each treatment level.

At the end of 3 weeks, approximately half of the chicks of each lot were sacrificed and their serum alkaline phosphatase was determined, using the Sigma method (*5*). The ash content of the left tibia was determined using the bone ash procedure of Bliss and Gyorgy (*1*). The calcium content of the ash was determined by an (ethylenedinitrilo)tetraacetic acid (EDTA) titration procedure used by Hurwitz and Griminger (*2*). The remaining birds were sacrificed at 8 weeks of age and plasma alkaline phosphatase, bone ash, and calcium analyses performed.

The Micro-Cel used in the experimental diets contained 17.5% calcium, by analysis. The figure of 40% was used in the calculation of the calcium content of diets supplemented with analytical grade $CaCO_3$.

Table I. Experimental Chick Diet NR110

	% of Diet
Ground yellow corn	42.95
Soybean oil meal, 50% protein	42.00
Celluflour[a]	5.70
Animal fat	3.50
Fish solubles, 50% solids	2.50
KH_2PO_4	1.35
NaH_2PO_4	1.15
NaCl	0.50
Vitamin mix[b]	0.25
Trace mineral mixture[c]	0.10
Choline chloride	0.05
DL-Methionine	0.05

[a] Celluflour, Chicago Dietectic Supply House, Chicago, Ill.

[b] Vitamin mix, mg./kg. of diet: inositol, 62.5; D-α-tocopheryl succinate, 37.5; nicotinamide, 7.5; thiamine NO_3, 5; D-Ca pantothenate, 5; riboflavin, 2.5; menadione, 2.1; vitamin A acetate, 1.29, pyridoxine . HCl, 1.125; folic acid, 0.20; biotin, 0.0375; cyanocobalamine, 0.015; vitamin D_3, 0.010.

[c] Delamix, Limestone Products Corp. of America, Newton, N. J. Contains following trace minerals (as % of Delamix): Mn, 6%; Fe, 2%; Cu, 0.2%; I, 0.12%; Co, 0.02%.

Results

Body Weight. The results in Table II indicate that not less than 1% $CaCO_3$