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FLAVONOID CONSTITUENTS OF *CICER ARIETINUM**

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Abstract—Chana seedlings (*Cicer arietinum*) have been found to contain the known compounds isoliquiritigenin, isoliquiritigenin-4′-glucoside, 4′,7-dihydroxyflavonol, daidzein, pratensein, and *p*-coumaric acid; and the hitherto unreported 4′,7-dihydroxyflavanon-3-ol (garbanzol) and biochanin-7-glucoside, both of which have been synthesized. Radioactive garbanzol is metabolized by chana seedlings as shown by its conversion into garbanzol-7-glucoside and 4′,7-dihydroxyflavonol, but formononetin was not labeled.

INTRODUCTION

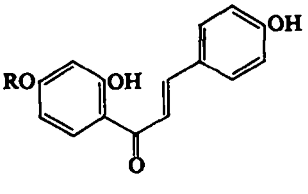
THE leguminous plant *Cicer arietinum* (chana; garbanzo; chick pea) has long been known to contain isoflavones,[1] and has been found to be a convenient plant for the study of the biosynthesis of these compounds.[2] In the course of feeding experiments in which ^{14}C-labeled compounds were administered to germinating chana seeds, it was observed that a number of compounds were labeled in addition to formononetin and biochanin, the most prominent of the seedling constituents. This paper reports the isolation and identification of some of these hitherto unrecognized polyphenols of chana seedlings.

In addition to these studies, experiments have been performed to examine further the course of isoflavone synthesis. Radioactive (4-^{14}C-)3,7,4′-trihydroxyflavanone (garbanzol) has been prepared and fed to germinating chana seeds, and the radioactive flavonoid products studied by chromatographic and radioautographic methods.

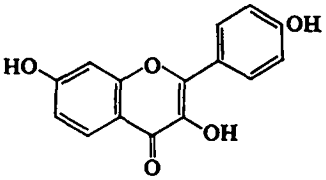
RESULTS

Identification of Flavonoid Compounds

The compounds isoliquiritigenin (2′,4′,4-trihydroxychalcone (I)),[3] isoliquiritigenin-4′-glucoside[3] (II), liquiritigenin (4′7-dihydroxyflavanone), 4′7-dihydroxyflavonol (III),[4]



(I) R = H
(II) R = glucosyl



(III)

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[1] J. L. BOSE, P. R. BHANDARI and S. SIDDIQUI, *J. Sci. Ind. Research* (*India*) **4**, 310 (1945); J. L. BOSE, *J. Sci. Ind. Research* (*India*) **15b**, 325 (1956).

[2] H. GRISEBACH and G. BRANDNER, *Z. Naturforsch.* **16b**, 2 (1961).

[3] H. GRISEBACH and G. BRANDNER, *Biochim. Biophys. Acta* **60**, 51 (1962).

[4] K. S. KIRBY and T. WHITE, *Biochem. J.* **60**, 582 (1955).

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF SOME CHANA CONSTITUENTS

Compound identified as:	R_f*							Color Reaction†			
	BeAW	2 N NH_3	30% HOAc	50% HOAc	5% HOAc	BAW	$BuNH_3$	u.v.	u.v. + NH_3	DSA	Others‡
Garbanzol	0·31	0·75	0·72	0·80	0·47	0·84	...	Y	lB	P-Br	a
Isoliquiritigenin	0·44	0·30	0·26	0·43	—	0·88	—	dk	bO-Y	P-Br	b
Isoliquiritigenin-4′-glucoside	0·11	0·77	0·48	—	—	—	—	dk	bO-Y	-	
Liquiritigenin	0·45	—	0·72	—	—	0·84	—	dk	—	Br	c
4′,7-Dihydroxyflavonol	0·38	—	0·18	0·42	0	0·81	—	bGr-Y	bGr-Y	—	d
Daidzein	0·42	0·55	0·68	...	—	—	—	lB	blB	—	
Pratensein	0·64	0·42	0·63	...	—	—	—	dk	dk	O	
p-Coumaric acid	0·70	0·85	0·76	—	-	—	0·65	Pu	pV-B	P-R	c

* For composition of solvents see Experimental.

† b = bright B = blue O = orange R = red
l = light Br = brown P = pink V = violet
dk = dark Gr = green Pu = purple Y = yellow

‡ (a) Mg/HCl - pink, Zn/HCl - magenta; (b) Visible yellow (c) $NaBH_4$/HCl - magenta; (d) Strong yellow green fluorescence, u.v. + visible light; (e) Acid to methyl red.

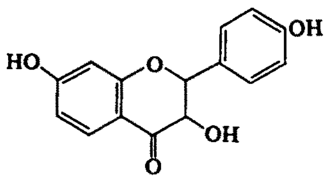
daidzein (4′7-dihydroxyisoflavone), pratensein (3′5,7-trihydroxy-4′-methoxyisoflavone),[5] and *p*-coumaric acid were identified by chromatographic and spectrophotometric methods (Tables 1 and 2). Two-dimensional chromatography along with authentic specimens confirmed the identifications, and, in addition, isoliquiritigenin, liquiritigenin, pratensein and 4′,7-dihydroxyflavonol were isolated by preparative paper chromatography and their identities confirmed by spectrophotometric measurements.

TABLE 2. ULTRAVIOLET ABSORPTION PEAKS FOR CHANA CONSTITUENTS (IN ETHANOL)

Compound identified as:	λ_{max} (mμ) EtOH (85%)	NaOH*
Isoliquiritigenin	242, 370	436
Liquiritigenin	277, 313	249, 336
4′,7-Dihydroxyflavonol	260, 320 358	dec.
Pratensin	263	—
Garbanzol	276, 311	250, 336

* 0·002 N in 85% EtOH.

In addition to these known compounds, two new compounds were found. One of these, isolated by preparative paper chromatography, proved to be 3,4′,7-trihydroxyflavanone (IV), called garbanzol. The compound gave a magenta color with zinc-hydrochloric acid,[6] possessed a flavanone-like absorption spectrum (Fig. 1), which was shifted by sodium acetate (indicating a free 7-hydroxyl group) but not by aluminum chloride (lack of a 5-hydroxyl group). The identity of the compound was established by its synthesis from 2′,4′,4-trihydroxy-chalcone, by way of 4′,7-trihydroxyflavonol; the latter was reduced to garbanzol by sodium dithionite.[7,8] Roux and de Bruyn[8] reported the synthesis of this flavanonol after the present work was completed. They did not, however, characterize the compound fully, establishing its identity by reducing it to the corresponding 3,4-diol. Synthetic garbanzol was identical with the natural substance in chromatographic behavior and in its absorption spectra under the conditions noted above.



(IV)

The presence of appreciable amounts of the previously undescribed biochanin-7-glucoside in chana seedlings appears to have escaped attention prior to our studies. The glucoside was isolated in pure form and identified by comparison with the synthetic material.

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[5] E. WONG, *J. Org. Chem.* **28**. 2336 (1963).

[6] J. C. PEW, *J. Amer. Chem. Soc.* **70**, 3031 (1948).

[7] T. A. GEISSMAN and H. LISCHNER, *J. Amer. Chem. Soc.* **74**, 3001 (1952).

[8] D. G. ROUX and G. C. DE BRUYN, *Biochem. J.* **87**, 439 (1963).

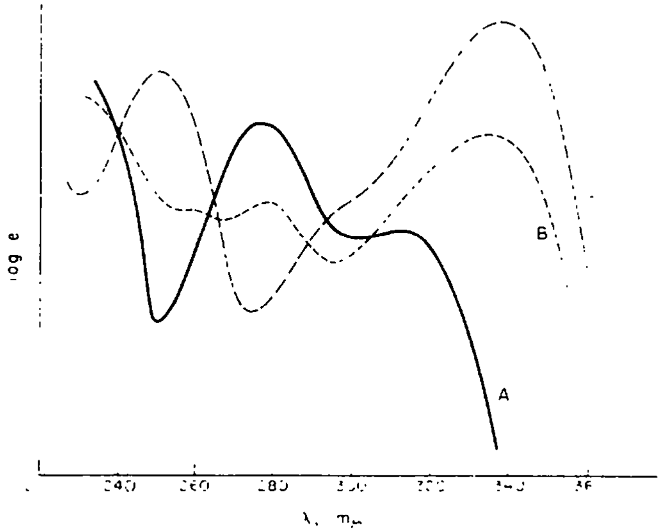

Fig. 1. Ultraviolet absorption spectra of garbanzol.
(A) in 85% ethanol; (B) with added sodium acetate; (C) with added sodium hydroxide (0·002 N). Spectrum is unchanged upon addition of 0·1 % aluminum chloride.

Radioactive Garbanzol; Feeding Experiments

4-^{14}C-Garbanzol was synthesized from 7-^{14}C-resacetophenone by the route described above, and 19·7 mg (0·0206 mc/mM) administered to 20 g of *C. arietinum* seeds. The seeds were allowed to germinate in contact with a solution of the radioactive material and then to grow for 66 hr (in sand). Extraction of the seedlings with methanol, and subsequent paper chromatography followed by radioautography showed the following results: unchanged radioactive garbanzol was present in large amount; a hitherto unrecognized compound that proved to be garbanzol-7-glucoside was found to be radioactive; 4',7-dihydroxyflavonol was found to be labeled; but the chalcone constituents of the seedlings were not radioactive. Formononetin was found to be nonradioactive. A number of radioactive constituents found on the radioautograms were present in minute amounts and have not been identified.

DISCUSSION

The presence in chana seedlings of the chalcone, the flavanonol, the flavonol and isoflavone with the same hydroxylation pattern is further evidence for the close structural relationships in the pathway of biosynthesis in this plant. It is known that 2',4',4-trihydroxy-chalcone-4'-glucoside is converted into formononetin in red clover,[9] and that the chalcone is formed from cinnamic acid[3] in leguminous plants, but the details of the pathway from chalcone to isoflavone (and, indeed, to other flavonoid end-products) remains a matter of conjecture. It is an attractive hypothesis that the pathway followed proceeds from chalcone to flavanonol, perhaps via the chalcone epoxide, and that the rearrangement of the 2-aryl group to the 3-position[10] occurs at the flavanonol stage. The failure to find labeled formononetin in chana seedlings fed with labeled garbanzol does not support this view. That the garbanzol

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[9] H. Grisebach and L. Patschke, *Ber.* **93**, 2326 (1960).

[10] H. Grisebach and N. Doerr, *Z. Naturforsch.* **15b**, 284 (1960).

is incorporated into the metabolic activities of the plant is shown by its conversion into the flavanonol glucoside, the flavonol and several unidentified metabolites. It is clear that the details of the steps in the pathway from cinnamic acid, via chalcone, to isoflavone remain to be established.

EXPERIMENTAL

Plant Material

Commercial chana seeds (garbanzo beans), averaging 190 seeds per 100 g, were germinated in moist sand at room temperature. After 2 days the hypocotyls were 2–3 cm in length and the epicotyls beginning to emerge. Most of the work on isolation was carried out on extracts of seeds germinated for 48 hr.

Extraction

The seedlings (250 g) were extracted three times with hot 95% ethanol in a blendor. A total of about 2 l. of extract was obtained, which was concentrated under reduced pressure to a syrup. This was taken up in 250 ml of 70% ethanol and after the solution was washed with Skellysolve B (5 × 150 ml) to remove fatty and carotenoid substances, it was concentrated under reduced pressure, taken up in 150 ml of water and extracted with five 150 ml portions of ether. Evaporation of the ether extract afforded the mixture of polyphenols which was examined by paper chromatography.

Chromatography

Solvent systems used were (all compositions by volume):
BeAW—benzene: acetic acid: water (125:72:3);[11]
BAW—1-butanol: acetic acid: water (6:1:2);
$BuNH_3$—1-butanol: 5% aq. ammonia (85:15);
2 N ammonia; 5%, 30%, 50% acetic acid.

Whatman No. 1 and No. 3 MM were used for analytical chromatography. For preparative chromatography No. 3 MM paper was washed with 50% acetic acid and 85% ethanol for 2 days with each solvent (downward irrigation). For spectrophotometric measurements spots were separated on paper purified by washing for 5 days. Spectra of compounds eluted from paper were measured against a blank eluted from an exact equivalent of the paper, usually with 80% ethanol.

For two-dimensional chromatography an amount of extract corresponding to 1–2 g of seeds was used; the solvents were usually BeAW and 30% acetic acid, or BeAW and 2 N ammonia.

Table 1 gives chromatographic data for the compounds identified. In Table 2 are given spectral data for compounds identified by this means.

Synthesis of 4′,7-Dihydroxyflavanonol (Garbanzol)

(*a*) *4′,7-Dihydroxyflavonol.* To an ice-cold solution of 2·66 g of 2′,4′,4-trihydroxychalcone[12] in 130 ml of 5% sodium hydroxide was added 6 ml of 30% hydrogen peroxide. The solution was allowed to stand at 4° for 4 days and then acidified with 6 N hydrochloric acid. The light orange–yellow precipitate was collected and washed with 30 ml of hot 40%

[11] E. Wong and A. O. Taylor, *J. Chromatog.* **9**, 449 (1962).

[12] T. A. Geissman and R. O. Clinton, *J. Am. Chem. Soc.* **68**, 697 (1946).

ethanol, leaving the flavonol as a pale yellow solid. Recrystallized from aqueous ethanol the flavonol (1·11 g) formed pale yellow needles, m.p. 304–306° (lit.[13] m.p. 310°).

(*b*) *4′,7-Dihydroxyflavanonol.* To a vigorously stirred, refluxing solution of 670 mg of 4′,7-dihydroxyflavonol in 35 ml of ethanol was added 4 g of sodium dithionite during 7 min, and refluxing was continued for an additional 8 min. The cooled solution was filtered and the filtrate concentrated to dryness under reduced pressure. The residue was extracted by stirring with 30 ml of water and the filtered solution concentrated under reduced pressure. The flavanonol separated as an off-white solid (93 mg). Recrystallized from water it gave white needles, m.p. 207–208° (turning yellow on melting). (Found: C, 66·29, 66·10; H, 5·07, 4·53. Calc. for $C_{15}H_{12}O_5$: C, 66·17; H, 4·44%.)

The flavanonol gave the typical color reactions with magnesium-hydrochloric acid and zinc-hydrochloric acid, and its absorption (Fig. 1) was typical of the flavanone chromophore. The synthetic material proved to be identical with the compound isolated from chana seedlings by chromatographic and spectrophotometric comparisons.

Radioactive (4-^{14}C) *Garbanzol*

This was prepared exactly as described above. The radioactive resacetophenone was prepared as follows:

To 2 mc of sodium acetate-1-^{14}C (161 mg), cooled in ice, was added 0·3 ml of acetyl chloride and the mixture allowed to stand for 30 min. Five ml of dry ether was added and the mixture refluxed for 30 min, cooled, and filtered from inorganic material. The ether filtrate (the salts were washed with 8 ml of dry ether) was added to a mixture of 3 ml of acetic anhydride, 7·33 g of purified resorcinol and 10 ml of boron trifluoride etherate, and the solution stoppered and allowed to stand in the refrigerator for 4 days. The solution was concentrated on the steam bath and 25 ml of water was added, followed by heating until all of the ether was removed. The resulting aqueous solution upon cooling in ice deposited a light yellow solid (7·27 g) which was recrystallized twice from water to yield 6·30 g (63%) of light yellow needles of 4-^{14}C-resacetophenone, m.p. 142–144°. The radioactivity was measured by means of a Dynacon Electrometer, and was found to be 0·0205 mc/m-mole. The maximum activity for conversion of all of the available acetyl radical into ketone would have been 0·0333 mc/m-mole.

Conversion of the resacetophenone into the chalcone yielded 3·08 g of the latter, m.p. 200–202°, having a (measured) specific activity of 0·0206 mc/m-mole. The succeeding steps were carried out as described above to yield 4-^{14}C-4′,7-dihydroxyflavanonol, m.p. 205–206°, chromatographically pure.

After administration of the labeled garbanzol to chana seeds, the extract of the seedlings was examined by two-dimensional chromatography and radioautography on Kodak No-screen X-ray film. The paper chromatograms were examined visually and with the aid of spray reagents and matched with the developed radioautograms. A prominent radioactive component, different from garbanzol, was isolated by preparative paper chromatography. Its u.v. absorption spectrum was closely similar to that of garbanzol and was unaltered by the addition of sodium acetate or dilute sodium hydroxide solution. The compound gave the characteristic flavanonol color test with zinc-hydrochloric acid. Confirmation of its structure was provided by acid hydrolysis to garbanzol and glucose, identified by chromatographic comparison with the authentic compounds.

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[13] K. V. RAO and T. R. SESHADRI, *Proc. Indian Acad. Sci.* **28A**, 96 (1948).

Biochanin-7-glucoside

(*a*) *From* C. arietinum *seedlings.* When chana seeds are permitted to grow with the cotyledons attached, both the seedlings and the cotyledons are found to contain large amounts of a compound that has been isolated and shown to be biochanin-7-glucoside. The seedlings (33·7 g) from 50 g of seed (allowed to germinate and grow for 4 days with the cotyledons attached) were extracted by blending with hot methanol. The extract was concentrated in vacuum to an aqueous residue, diluted somewhat with methanol and the solution treated with neutral aqueous lead acetate, the precipitate removed, and the filtrate treated with lead acetate and ammonia until precipitation was complete. The precipitate was washed with methanol and decomposed in methanol suspension with hydrogen sulfide. The clear de-leaded filtrate was concentrated and cooled. Biochanin-7-glucoside crystallized as prisms, m.p. 198–203°, and after recrystallization from aqueous acetone formed colorless flakes, m.p. 213–214°.

The compound was identified by comparison with a synthetic specimen (see below), and by the following properties: an alcoholic solution gave a purple color with ferric chloride. Hydrolysis with dilute hydrochloric acid gave biochanin, identified by comparison with an authentic specimen, and glucose, identified chromatographically.

(*b*) *By synthesis.* (i) *Biochanin-7-tetra-O-acetyl glucoside.* The procedure of Zemplen, Farkas and Bien[14] for the synthesis of formononetin-7-glucoside (ononin) was followed, with the substitution of biochanin (0·6 g) for formononetin. The glucoside tetra-acetate was crystallized from methanol; there was obtained 0·35 g of fine white needles, m.p. 178–179°. (Found: C, 58·46; H, 4·94. Calc. for $C_{30}H_{30}O_{14}$: C, 58·65; H, 4·92%.)

(ii) *Biochanin-7-glucoside.* The glucoside tetra-acetate (0·3 g) was hydrolyzed as described by Zemplen *et al.*,[13] for ononin tetra-acetate, and yielded 0·20 g of biochanin-7-glucoside as white needles, m.p. 216–216·5° after recrystallization from aqueous methanol. (Found: C, 59·14, 59·25; H, 5·06, 5·08. Calc. for $C_{22}H_{22}O_{10}$: C, 59·19; H, 4·97%.)

The synthetic and natural materials were shown to be identical by mixed melting point and by their identical behavior on paper chromatograms.

Ungerminated Seeds

Extraction of 50 g of ungerminated *C. arietinum* seeds yielded fractions which were examined for isoflavones. No formononetin or biochanin could be detected; addition of a known amount of formononetin and examination of the resulting solution showed that formononetin could have been detected if it were present in an amount of over 0·1 mg per 50 g of seeds. It therefore appears that substantially all of the isoflavones present in the seedlings were synthesized *de novo* during germination and growth.

Acknowledgements—We are grateful for support provided by the U.S. Public Health Service under Research Grant GM-03667; and to Miss Heather King for elemental analyses.

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[14] G. Zemplen, L. Farkas and A. Bien, *Ber.* 77, 452 (1944).