Phytochemistry, 1968, Vol. 7, pp. 1751 to 1758. Pergamon Press. Printed in England

THE ROLE OF CHALCONES AND FLAVANONES IN FLAVONOID BIOSYNTHESIS

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(*Received* 10 *April* 1968)

Abstract—Parallel competitive feeding experiments have been carried out in which either (*a*) ^{14}C-isoliquiritigenin (2′,4′,4-trihydroxychalcone) diluted with an equal amount of (−)-liquiritigenin (4′,7-dihydroxyflavanone), or (*b*) ^{14}C-(−)-liquiritigenin diluted similarly with isoliquiritigenin were fed to subterranean clover seedlings and to cell-free extracts of garbanzo beans. The radioactive products, 4′,7-dihydroxyflavone, daidzein, formononetin and garbanzol were isolated and their specific activities determined. The results show that the specific activities for the products from (*a*) are higher than those from (*b*). This is interpreted as indicating that the chalcone, and not the flavanone, is the more immediate precursor for other classes of flavonoid. The possible roles of "oxidized chalcone" intermediates in the biosynthesis of flavonoids are discussed.

INTRODUCTION

THE KEY position of chalcones in the biosynthesis of flavonoids is now well established. Tracer experiments have shown that chalcones are precursors of flavanones,[1] flavanonols,[1,2] flavones,[3] isoflavones,[4] flavonols,[5] anthocyanins,[5] catechins[6] and aurones.[7] Flavanones are isomeric with chalcones and these two classes of compounds are chemically readily interchangeable. On chemical and stereochemical grounds,[8] flavanones very plausibly represent the primary common heterocyclic intermediate for the other flavonoids, and tracer studies again show that flavanones are precursors of flavones,[3] anthocyanins[9] and isoflavones.[9] That flavanones are involved as intermediates in the biosynthesis of these compounds directly, and not via chemical conversion to chalcones, found apparent experimental support when Grisebach and his co-workers[10] showed that the natural (−)-enantiomer of naringenin was incorporated into the anthocyanin, cyanidin, and the isoflavone, biochanin-A, at a much higher rate than the corresponding (+)-enantiomer. The biogenetic interrelationships of the different classes of flavonoid based on these and other published results[11,12] can be summarized as in Fig. 1.

[1] E. WONG, *Biochim. Biophys. Acta* **111**, 358 (1965).
[2] H. GRISEBACH and S. KELLNER, *Z. Naturforsch.* **20b**, 446 (1965).
[3] H. GRISEBACH and W. BILHUBER, *Z. Naturforsch.* **22b**, 746 (1967).
[4] H. GRISEBACH and L. PATSCHKE, *Chem. Ber.* **93**, 2326 (1960); H. GRISEBACH and G. BRANDNER, *Z. Naturforsch.* **16b**, 2 (1951).
[5] H. GRISEBACH and L. PATSCHKE, *Z. Naturforsch.* **16b**, 645 (1961).
[6] L. PATSCHKE and H. GRISEBACH, *Z. Naturforsch.* **20b**, 399 (1965).
[7] E. WONG, *Phytochem.* **5**, 463 (1966).
[8] A. J. BIRCH, in *Chemical Plant Taxonomy* (edited by T. SWAIN), p. 141, Academic Press, London (1963).
[9] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **19b**, 1110 (1964).
[10] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **21b**, 201 (1966).
[11] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **21b**, 45 (1966).
[12] For review, see H. GRISEBACH, in *Chemistry and Biochemistry in Plant Pigments* (edited by T. W. GOODWIN), p. 279, Academic Press, London (1965).

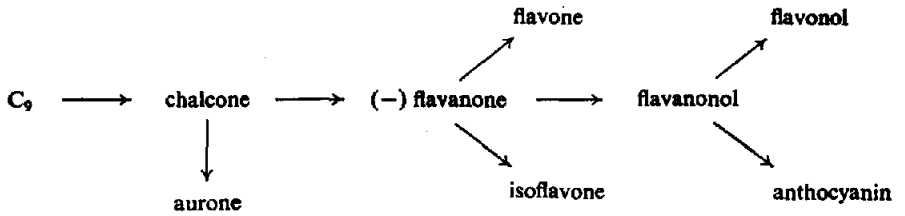
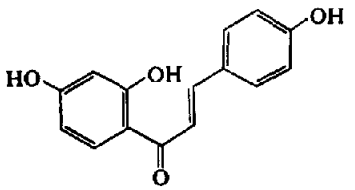
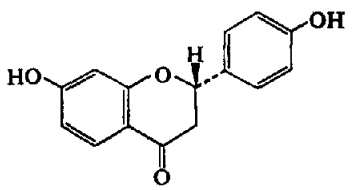


FIG. 1. BIOSYNTHETIC INTERRELATIONSHIPS OF THE DIFFERENT CLASSES OF FLAVONOID, BASED ON PREVIOUS RESULTS.

An enzyme catalysing the isomerization of isoliquiritigenin (I) and other chalcones to (−)-liquiritigenin (II) and the corresponding flavanones was recently isolated from soya bean in this laboratory.[13] This enzyme was found to catalyse also the reverse reaction, converting (−)-liquiritigenin to isoliquiritigenin, although at a much slower rate (unpublished results). In the course of tracer studies using subterranean clover seedlings, it was found that when ^{14}C-(−)-liquiritigenin was fed to the plant, the resulting pattern of labelling of flavonoids was qualitatively very similar to that from parallel experiments using ^{14}C-isoliquiritigenin. In particular, both isoliquiritigenin and liquiritigenin were found to be major radioactive products after the feeding of either ^{14}C-liquiritigenin or ^{14}C-isoliquiritigenin.



(I)



(II)

Both enzymic and *in vivo* feeding experiments thus show that chalcone and (−)-flavanone are biochemically interconvertible. Feeding experiments in which either one is used as precursor would necessarily result in both being formed, providing the isomerase enzyme is present in the particular plant. This raises the question whether both chalcone and flavanone are necessarily involved as direct intermediates in flavonoid biosynthesis.

This question has now been examined by means of parallel competition experiments in which either (*a*) ^{14}C-isoliquiritigenin diluted with (−)-liquiritigenin or (*b*) ^{14}C-(−)-liquiritigenin similarly diluted with isoliquiritigenin was administered to clover seedlings (*Trifolium subterraneum*) or cell-free extracts of garbanzol seedlings (*Cicer arietinum*). The incorporations of radioactivity into some of the flavone, isoflavone and flavanonol constituents in the parallel experiments were compared and results used to indicate whether chalcone or flavanone is the more immediate precursor for other classes of flavonoid.*

RESULTS

Parallel experiments (*a*) and (*b*) were carried out in which (*a*) ^{14}C-isoliquiritigenin is diluted with an equal amount of (−)-liquiritigenin, and (*b*) an equal amount of ^{14}C-(−)-

* A preliminary account of part of this work has appeared in *Chem. Commun.* 395 (1968).

[13] E. WONG and E. MOUSTAFA, *Tetrahedron Letters*, 3021 (1966); E. MOUSTAFA and E. WONG, *Phytochem.* 6, 625 (1967).

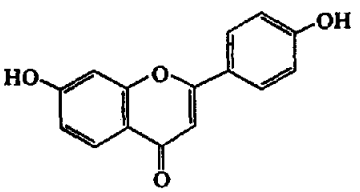
liquiritigenin of the same specific activity is similarly diluted with isoliquiritigenin, are fed as precursors. The specific activity of the chalcone pool in (*a*) during the experiment would be expected to be higher than that of the chalcone pool in (*b*); and conversely, the specific activity of the flavanone pool would be higher in (*b*) than (*a*). This being the case, then depending on whether the product (i.e. flavone, etc.) is formed more directly from the chalcone or the flavanone, the specific activity of the product from (*a*) would be higher than that from (*b*), or vice versa (Fig. 2). Results presented below show that these expected orders of radioactivity for the chalcone and flavanone pools were realized under the experimental conditions used.

(*a*) flavone* ← [chalcone* ⇌ (−)flavanone] → flavone
(*b*) flavone ← [chalcone ⇌ (−)flavanone*] → flavone*

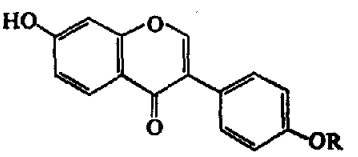
* Indicates the expected compound having the higher specific activity in the two parallel experiments.

FIG. 2. GENERAL PLAN OF PARALLEL EXPERIMENTS AND EXPECTED RESULTS DEPENDING ON WHETHER CHALCONE OR FLAVANONE IS THE MORE IMMEDIATE PRECURSOR.

For the study of the relative roles of isoliquiritigenin and (−)-liquiritigenin in the biosynthesis of 4′,7-dihydroxyflavone (III) and the isoflavones daidzein (IV*a*) and formononetin (IV*b*), parallel experiments were carried out using seeds of subterranean clover (variety Tallarook). After feeding of the mixture (*a*) or (*b*) referred to above, the seeds were

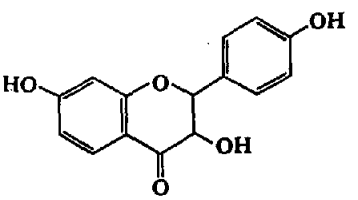


(III)



(IV)

(*a*) R=H
(*b*) R=CH_3



(V)

allowed to germinate for 3–4 days and the flavanoid constituents then extracted and analysed by two-dimensional paper chromatography. Identities of the flavonoid compounds in subterranean clover have previously been reported.[14] The radioactive chalcone and flavanone, and the flavone and isoflavone products were eluted from the chromatograms and their concentrations and specific activities determined. Each compound was then purified several times by means of paper chromatography until constant specific activity was obtained. The results of two such pairs of feeding experiments are given in Table 1.

[14] E. WONG and C. M. FRANCIS, *Phytochem.* in press.

TABLE 1. SPECIFIC ACTIVITIES (cpm/μmole $\times 10^{-3}$) AND AMOUNTS OF FLAVONOID COMPOUNDS FROM CLOVER SEEDLINGS FED ^{14}C-CHALCONE OR FLAVANONE

Precursor*	Liquiriti-genin	Isoliquiriti-genin	4′,7-Di-hydroxy-flavone	Daidzein	Formo-nonetin
Experiment I					
(*a*) ^{14}C-isoliquiritigenin +(−)-liquiritigenin	74 (168)	144 (36)	135 (18)	106 (22)	28 (980)
(*b*) ^{14}C-(−)-liquiritigenin +isoliquiritigenin	134 (89)	54 (40)	66 (18)	51 (15)	13 (950)
Experiment II					
(*a*) ^{14}C-isoliquiritigenin +(−)-liquiritigenin	84 (214)	163 (113)	133 (27)	119 (17)	36 (2,000)
(*b*) ^{14}C-(−)-liquiritigenin +(isoliquiritigenin	151 (162)	64 (86)	67 (20)	52 (11)	25 (1,020)

Values in parentheses are amounts in μg of each compound obtained after the first chromatographic purification.

* Sp. act. 199 cpm/μmole $\times 10^{-3}$.

For the study of the biosynthesis of the flavanonol garbanzol (V), cell-free extracts of 4–5-day-old seedlings of garbanzo beans were used.[1] The extract was incubated with mixture (*a*) or (*b*) for 20 min. Separation and purification of the chalcone and flavanone, and garbanzol, was again by paper chromatography, carried out till constant specific activity for each compound was attained. Again two pairs of experiments were carried out. In the first, the proportions of chalcone and flavanone in the mixture fed were equal, as in the clover experiments; in the second, the amount of flavanone was doubled, giving a chalcone:flavanone ratio of 1:2. This variation was chosen to give a situation where, with a larger pool of flavanone to start with, the difference in the specific activity of the flavanone pool in (*a*) and (*b*) during the experiment would be increased. Results shown in Table 2 show that this was indeed the case.

TABLE 2. SPECIFIC ACTIVITIES (cpm/μmole $\times 10^{-3}$) AND AMOUNTS OF FLAVONOID COMPOUNDS FROM CELL-FREE EXTRACTS OF GARBANZO SEEDLINGS INCUBATED WITH ^{14}C-CHALCONE OR FLAVANONE

Precursor*	Liquiritigenin	Isoliquiritigenin	Garbanzol
Experiment I			
(*a*) ^{14}C-isoliquiritigenin +(−)-liquiritigenin	87 (400)	152 (76)	11·6 (37)
(*b*) ^{14}C-(−)-liquiritigenin +isoliquiritigenin	139 (364)	80 (70)	5·6 (34)
Experiment II†			
(*a*) ^{14}C-isoliquiritigenin +(−)-liquiritigenin	57 (500)	105 (391)	9·3 (84)
(*b*) ^{14}C-(−)-liquiritigenin +isoliquiritigenin	161 (650)	139 (260)	11·9 (84)

Values in parentheses are amounts in μg of each compound obtained after the first chromatographic purification.

* Sp. act. 199 cpm/μmole $\times 10^{-3}$.

† Ratio of flavanone:chalcone fed = 2:1 in this experiment.

In both the clover seedlings and the cell-free extracts from garbanzo, the concentration of indigenous isoliquiritigenin and liquiritigenin is very low. Tables 1 and 2 also show, as expected, that after feeding or incubation, flavanone was present in larger amounts than chalcone.

DISCUSSION

Results presented in this work can be interpreted as indicating that the chalcone is the more immediate precursor for the biosynthesis of the flavone, isoflavone and flavanonol.

In both of the intact feeding experiments (Table 1) the chalcone pool at the beginning and end of the experiment has a higher specific activity in (*a*) than in (*b*) whilst for the flavanone the reverse is true. These orders of activity are as expected. For the products, 4',7-dihydroxyflavone, daidzein and formononetin, the specific activities in (*a*) and (*b*) follow the order of chalcone and not flavanone in being higher in (*a*) than (*b*). Furthermore, within each of the four experiments, the specific activity of the flavone, and to a less extent of the daidzein, follows closely that of the chalcone but not the flavanone. The much lower specific activities for formononetin, which is very likely biosynthesized via daidzein, could be explained by assuming a large pool of indigenous formononetin in the seeds. Quantitatively, formononetin is the most important constituent in the clover, the flavone and daidzein being present in minor amounts only.

In the first cell-free experiment with garbanzo beans, the flavanonol product, garbanzol, is again more active in (*a*) than in (*b*) thus conforming to the pattern already observed for the other two classes of flavonoid compounds in clover. The fact that this same result is obtained independently with a cell-free system gives us greater confidence in interpreting these unexpected results at face value.

The second cell-free experiment was designed to create a much larger differential in flavanone activity in (*a*) and (*b*) so that if garbanzol is really derived directly from the flavanone then garbanzol from (*b*) should have a much higher specific activity than that from (*a*). The garbanzols isolated do have activities in this order but the difference is, however, much smaller than expected. Furthermore it will be noted that the specific activity of the chalcone pool at the end of the experiment is now greater in (*b*) than in (*a*), so that the order of activity observed for the flavanonol is again consistent with it being derived directly from the chalcone. The crossing over of the order of the activity of the chalcone pools can be readily explained in terms of the larger pool of flavanone present in this experiment, resulting in increased contribution of the backward reaction flavanone → chalcone. The high dilution of the radioactivity in garbanzol in these cell-free experiments is accounted for by the presence of a small indigenous pool of garbanzol in the extract. It is also apparent from the results that the *de novo* synthesis of garbanzol by the cell-free system is quite low.

These results lead to the unexpected but inescapable conclusion that chalcone is the more direct precursor of flavone, isoflavone and flavanonol. Since flavanones are more plausibly derivatives rather than precursors of chalcones, the present results would mean that they are not normally involved as intermediates in the biosynthesis of other flavonoids; instead they are products of a minor pathway in flavonoid biosynthesis. The biosynthetic interrelations of the different classes of flavonoid compounds in light of this interpretation can now be summarized as in Fig. 3 (cf. Fig. 1).

This scheme is consistent with the previous results from tracer studies discussed in the Introduction. Because of the reversibility of the chalcone/flavanone pathway, it can readily be seen why flavanones are good precursors for other flavonoids in feeding experiments.

The stereospecific results[10] of Grisebach *et al.* are also readily explicable in this scheme since only the natural (−)-flavanone would be expected to undergo enzymic isomerization to the chalcone.

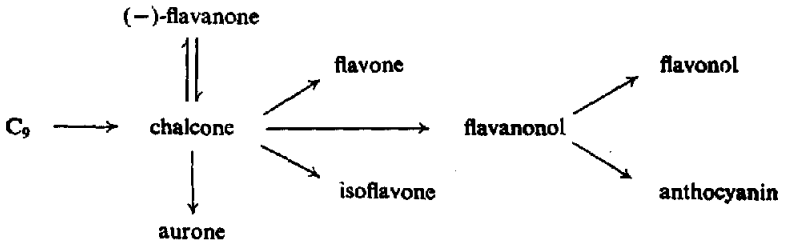
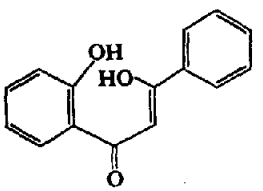
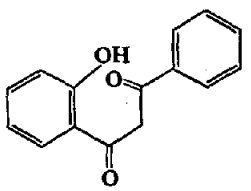


FIG. 3. PROBABLE BIOSYNTHETIC INTERRELATIONSHIPS OF THE DIFFERENT CLASSES OF FLAVONOID.

The fact that a chalcone can be converted biochemically to a flavone, isoflavone and flavanonol without going through preliminary heterocyclic ring formation to the flavanone necessitates a reorientation in thinking on the biosynthetic mechanisms leading to these classes of compounds. For example, the biosynthesis of a flavone in the scheme in Fig. 1, would reasonably be expected to proceed via a dehydrogenation as the last step.[3] The formation of a flavone more directly from chalcone, however, although stoichiometrically requiring also a loss of two hydrogen atoms, is mechanistically more readily postulated as going via an oxidized intermediate such as (VI) or the equivalent (VII), which could then readily lose water to the flavone. The recent finding by Williams[15] of a diketone of structural type (VII) as a natural product is therefore of great interest in this connection.



(VI)



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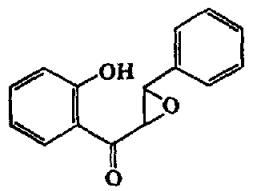

(VIII)

Structures (VI) and (VII) above may be termed an "oxidized chalcone". Another variation of an oxidized chalcone is the 2-α-hydroxybenzylcoumaranone, recently shown by us to be a probable intermediate in aurone biosynthesis.[16] From structural and mechanistic considerations the transformation of a chalcone to a flavanonol or isoflavone is also likely to take place via an oxidized chalcone intermediate. Such a structure as the *trans*-chalcone epoxide (VIII) would seem to be an ideal intermediate for transformation to various modifications of an oxidized chalcone. The roles of chalcone epoxides as chemical or biochemical intermediates to various classes of flavonoids have been postulated by many workers.[3,16,17] In view of the findings in this paper, these hypotheses now take on a higher degree of probability.

[15] A. H. WILLIAMS, *Chem & Ind.* 1526 (1967).
[16] E. WONG, *Phytochem.* 6, 1227 (1967).
[17] F. M. DEAN and V. DODIMUANG, *J. Chem. Soc.* 2978 (1965); F. FISCHER and W. ARLT, *Chem. Ber.* 97, 1910 (1965); A. C. JAIN, *Bull. Nat. Inst. Sci. India* 31, 107 (1965).

EXPERIMENTAL

^{14}C-*Isoliquiritigenin* (*carbonyl-*^{14}C)

This was prepared from sodium acetate-1-^{14}C via the resacetophenone. The method was modified from that previously used,[18] with quantities here scaled down to $\frac{1}{10}$. Labelled acetic anhydride was made by adding 100 μl of acetic anhydride to 1 mc of sodium acetate-1-^{14}C (2·8 mg). The mixture was sealed and allowed to stand for 5 days. Ether (0·3 ml) was then added and the solution carefully pipetted off from the solid sodium acetate. This washing with ether was repeated twice and the combined ether extract was added to 220 mg of resorcinol plus 1·2 ml of boron trifluoride etherate. After standing at 0° for a week the reaction mixture was worked up in a similar manner to that previously described.[18] The yield of chromatographically pure chalcone after recrystallization from 50 per cent ethanol was 98 mg, having a measured specific activity of 727 cpm/μg, equivalent to 199×10^3 cpm/μmole (counting efficiency 23·5 per cent, Beckman Lowbeta II counter).

(−)-*Liquiritigenin*

(−)-Liquiritigenin, with or without ^{14}C-labelling, was prepared from the corresponding isoliquiritigenin by means of the isomerase enzyme.[13] The chalcone (5 mg) dissolved as the disodium salt in 0·4 ml of water, was incubated with 1 mg of the purified isomerase enzyme in 20 ml of 0·05 M Tris buffer, pH 7·5, at 37° for 30 min. After 2 hr at room temperature the mixture was acidified with 4 N HCl and extracted several times with ether. The ether extract was chromatographed as a band on washed 3 MM paper in 30 per cent HOAc which effectively separated the chalcone and flavanone components. The yield of (−)-liquiritigenin after purification was about 50 per cent.

^{14}C-*Incorporation Experiments*

The following descriptions apply identically to treatments (*a*) and (*b*) in each feeding experiment. These treatments differed one from the other in having either the chalcone or the flavanone precursor ^{14}C-labelled (see Introduction).

Subterranean Clover Seedlings—Experiment I

Dry Tallarook clover seeds (2 g) were soaked for 3 hr in 1 ml of water containing 0·95 mg each of isoliquiritigenin and (−)-liquiritigenin, made soluble as the disodium salts. A further 1 ml of water was then added and after 3 hr the seeds were allowed to germinate for a total of 4 days at 25°. The seedlings (*ca.* 10 g) were extracted by grinding in a mortar with sand and a total of 100 ml ethanol. After concentration *in vacuo*, the alcoholic extract was made to 70 per cent ethanol (15 ml) and washed with petrol. ether (3 × 20 ml). The mixture was then hydrolysed by refluxing with 6 ml 4 N HCl for 45 min (final concentration ≑ N HCl in 50 per cent ethanol). The hydrolysate was concentrated *in vacuo* and the aqueous residue (10 ml) extracted repeatedly with ether (4 × 25 ml). The ether-soluble material, after evaporation, was taken up in 1 ml of ethanol. This solution of the phenolic constituents had a total activity of 316×10^3 and 209×10^3 cpm in (*a*) and (*b*) respectively, representing 56 per cent and 30 per cent of total activity fed.

The phenolic compounds isolated were separated by two-dimensional paper chromatography (five papers) in the systems BeAW and 30 per cent HOAc. The chromatograms were radioautographed for 2 weeks to reveal activity in isoliquiritigenin, liquiritigenin, 4′,7-dihydroxyflavone, daidzein and formononetin, together with other spots of uncertain identity. It could be seen by inspection of the radioautographs that the activity of the flavone and isoflavone spots was greater in (*a*) than in (*b*).

From the two-dimensional chromatograms individual spots were cut out and eluted with 85 per cent ethanol. Concentrations were determined spectrophotometrically, using $E^{1\%}_{1cm}$ values of $1{\cdot}13 \times 10^3$, $0{\cdot}483 \times 10^3$ and $1{\cdot}03 \times 10^3$ for isoliquiritigenin, liquiritigenin and 4′,7-dihydroxyflavone, respectively. Values for the isoflavones have previously been reported.[19] Suitable aliquots were plated out on planchettes and counted to pre-set 5000 counts in a Beckman Lowbeta II counter operating at the proportional region. The counting efficiency was 23·5 per cent. The individual compounds were further purified by paper chromatography in various sequences of the systems:[18] BeAW, 30 per cent HOAc, 5 per cent HOAc, 2 N NH_3, and 30 per cent isopropyl alcohol, till specific activity values on repeated purification agreed to within 5 per cent. At least four such chromatographic purifications were carried out for each compound.

Experiment II

This differed from Experiment I only in the amount of precursors fed ; 1·33 mg each of the chalcone and flavanone, and in the shorter time of germination of 3 days instead of 4. The total activity recovered in the ether-soluble phenolic fraction in (*a*) and (*b*) were 347×10^3 and 211×10^3 cpm respectively, representing 36 per cent and 22 per cent of the total activity fed.

[18] E. Wong, P. I. Mortimer and T. A. Geissman, *Phytochem.* **4**, 89 (1965).
[19] E. Wong, *J. Sci. Food Agri.* **13**, 304 (1962).

Cell-Free Extracts of Garbanzo Seedling—Experiment I

Five-day-old garbanzo seedlings (63 g, from 30 g dry seeds) were macerated at 2° with 63 ml of 0·05 M tris-HCl buffer (pH 7·5) in a pestle and mortar. The mixture was centrifuged for 30 min at 27,000*g* and 15 ml of the supernatant (total 60 ml) was incubated at 37° with 1 ml of water containing 0·8 mg each of isoliquiritigenin and (−)-liquiritigenin dissolved as the disodium salts. After 20 min the mixture was boiled with 30 ml of ethanol and filtered. This ethanolic extract was concentrated, washed with petrol. ether, and extracted into ether in a similar manner to that described above. The phenolic constituents were then chromatographed, radioautographed, eluted and purified to constant specific activity, as described above for the clover seedlings.

Cell-Free Experiment II

This differed in the following ways from the above: 4-day-old seedlings were used and the amounts of chalcone and flavanone substrates incubated with 15 ml of extract were 0·915 and 1·83 mg, respectively.