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SYNTHESIS OF C^{14}-LABELLED ISOFLAVONES

G. TANG

SYNTHESIS OF C^{14}-LABELLED ISOFLAVONES

A Thesis

by

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GENERAL INTRODUCTION

The presence in a plant of factors which are capable of inducing estrus in animals was first reported by Loewe et al. (1927) in 1926. This fact, however, did not attract much attention until during World War II, when sheep in Australia, grazing on pastures of subterranean clover (Trifolium subterraneum), showed severe breeding disorders (Bennetts et al., 1946). In 1951, Bradbury and White (1951) demonstrated that the infertility was due to the presence in this clover species of genistein (4',5,7-trihydroxy-isoflavone). The early reports from Australia on reproductive disorders in grazing animals were soon followed by others from Germany (Schoop and Klette, 1952), New Zealand (The New Zealand Department of Agriculture Report, 1950; Ch'ang, 1958) and U. S. A. (Bickoff, 1958; Sanger and Bell, 1959).

As well as being found in subterranean clover, genistein was also found in red clover (Pope and Wright, 1954), ladino clover and alfalfa (Guggolz et al., 1961). Other isoflavones present in forages include biochanin A (5,7-dihydroxy-4'-methoxy-isoflavone) (Pope et al., 1953; Guggolz et al., 1961), formononetin (7-hydroxy-4'-methoxy-isoflavone) (Bate-Smith et al., 1953; Guggolz et al., 1961) and daidzein (4',7-dihydroxy-isoflavone) (Guggolz et al., 1961). All

these compounds produce varying degrees of estrogenic effects in animals, resembling those induced by animal estrogens. Among the foregoing, genistein was found to be the most active estrogen. In general, however, the estrogenic activities of the isoflavones are much lower than the activities of the naturally-occurring steroid estrogens or the more active stilbene derivatives. The estrogenic activity of genistein, for example, is only about 10^{-5} times that of oestradiol or diethylstilbestrol.

Considerable attention has been given to the possibility that a moderate ingestion of forages rich in estrogenic compounds might well have beneficial effects on the grazing animals. The so-called "spring flush" of milk in dairy cows, associated with qualitative and quantitative improvement of yield, is probably due to the presence of estrogens in the spring pasture (Bartlett et al., 1948). It has also been demonstrated that feeding on plants rich in natural estrogens may affect the growth rate and carcass quality of cattle and sheep (Bickoff, 1958).

Because of the many important aspects of forage estrogens in animal husbandry, intensive studies of their estrogenic activities were made in the last decade in many countries, such as England (Jennings and Daw, 1959), U. S. A. (Bickoff et al., 1962), Sweden (Nilsson, 1962b) and Australia

(Lloyd-Davies and Bennett, 1962). The metabolic transformations of these compounds, however, are very imperfectly known. It was suggested that formononetin or daidzein might give equol (4',7-dihydroxy isoflavan), by the reduction of a 2:3 double bond and a 4-oxo group. This suggestion was made by Klyne and Wright (1957) in connection with their isolation of equol from the urine of the goat. Equol was subsequently detected in both the urine and the feces of hens fed on a diet containing dried alfalfa meal (MacRae et al., 1960). This work was extended by Ainsworth (1961), who succeeded in isolating crystalline equol from the urine of hens fed on a similar diet. Finally, Cayen (1964) successfully demonstrated the conversion of genistein to equol in the fowl. Running parallel with the foregoing research in this laboratory, Nilsson, working in Sweden, was able to demonstrate the demethylation of biochanin A to genistein in vivo (1961c). Nilsson also found that biochanin A and formononetin could be demethylated in vitro to genistein and daidzein, respectively, in rumen fluid by the influence of micro-organisms (1961b; 1962a), and by liver enzyme systems present in the microsomal fraction of liver homogenates (1963a).

In order to study more closely the transformations in vitro and the conversions in vivo of the estrogenic isoflavones commonly found in forages, a supply of these

compounds tagged with isotopes is most fundamental and absolute. The present thesis is concerned mainly with the syntheses and characterizations of C^{14}-labelled biochanin A, formononetin, genistein and daidzein. With all these isotopic isoflavones at hand, the subsequent steps in tracing their transformation patterns and demonstrating their metabolic products are likely to be facilitated appreciably.

I. HISTORICAL REVIEW

I.1. The Discovery of Natural Isoflavones:

The natural isoflavones (3-phenylchromone) were first discovered by workers interested in the pigments of plants. It is probable that the first isoflavone to be described was the glycoside ononin, which was isolated in 1855 by Hlasiwetz (Dean, 1963) in the course of an investigation of the constituents of the roots of Ononis spinosa L., although at the time of its first isolation the structure of ononin was unknown. Forty-four years later, Perkin and Newbery (1899) isolated two yellow colouring materials from the dyer's broom, Genista tinctoria L.. One of these colouring materials was the well known flavone, luteolin (3',4',5,7-tetrahydroxy flavone); the other was a glycoside called genistin, which afforded the aglycone genistein when hydrolysed by acid. Perkin and Horsfall (1900) gave the molecular formula of genistein as $C_{14}H_{10}O_5$. This formula was subsequently shown to be incorrect, the correct formula being $C_{15}H_{10}O_5$ (Finnemore, 1910). However, the incorrect formula led to the ascription of an incorrect structure. The incorrect structure is to be seen in the first edition of "The Natural Organic Colouring Matters" by Perkin and Everest (1918), which appeared as late as 1918.

In 1910 Finnemore (1910) examined the bark of an unspecified species of Prunus and isolated therefrom a glycoside which could be hydrolysed to yield a substance which he named prunetin, $C_{16}H_{12}O_5$. Demethylation of prunetin yielded a substance which Finnemore named prunetol, $C_{15}H_{10}O_5$. Fusion of prunetol with caustic potash yielded p-hydroxyphenyl acetic acid and phloroglucinol and on the basis of this degradation Finnemore suggested an isoflavonoid structure for prunetol and prunetin. This would seem to be the first occasion on which an isoflavonoid structure was suggested for a natural product.

In 1925, Perkin (Baker and Robinson, 1925) suggested the possibility that prunetol was identical with genistein. His suggestion was based on the following comparisons:

(a) Genistein: long, colourless needles from aqueous alcohol, m.p. 291-293°; prunetol: colourless needles from aqueous alcohol, m.p. 290°;

(b) Triacetylgenistein: m.p. 197-201°; triacetylprunetol: m.p. 205°;

(c) Genistein dimethyl ether: colourless leaflets from alcohol, m.p. 137-139°; prunetin monomethyl ether: flat needles, m.p. 145°;

(d) Acetyl derivative of genistein dimethyl ether: colourless needles, m.p. 202-204°; acetyl derivative

of prunetin monomethyl ether: colourless needles, m.p. 202°.

In addition, the two compounds gave identical colour reactions with a variety of reagents. Both compounds gave a pale yellow solution in alkalis; a violet colouration with ferric chloride in alcoholic solution, becoming brownish-green with excess of the reagent; and a pale yellow solution in concentrated sulphuric acid which exhibited a weak bluish-green fluorescence in the light of an iron arc. It was found, moreover, that genistein dimethyl ether yielded p-methoxyphenyl-acetic acid by hydrolysis with alcoholic potassium hydroxide.

In 1925, Bargellini (1925) suggested an alternative structural formula for genistein. The suggested formula had an α-pyrone structure instead of the γ-pyrone structure proposed by Finnemore. The behaviour of genistein and prunetol, in most respects, could be interpreted equally well by either formula, (I or II Chart I). However, the nature of the products obtained on methylation of genistein and prunetin afforded strong evidence for the superiority of Finnemore's supposition (1910). Only two of the three hydroxyl groups of genistein and only one of the two in prunetin could be methylated by means of methyl iodide and alcoholic potassium hydroxide, whilst a by-product in the former case was a methylgenistein dimethyl ether that still

contained a free phenolic group. Such a protection of a hydroxyl group from the action of the methylating agent was not explicable on the basis of the structural formula suggested by Bargellini, but it was explicable on the basis of Finnemore's formulation, because the 5-hydroxy group was protected by hydrogen bonding.

I.2. The Syntheses of Natural Isoflavones:

In order to have a solid proof of the identity of genistein and prunetol, and in order to supply a conclusive evidence of their constitutions, a synthesis had now become absolutely necessary. In 1928, Baker and Robinson (1926, 1938) eventually succeeded in the synthesis of genistein, a synthesis which proved to be very difficult and gave them a great deal of trouble. Nevertheless, they were able thereby to supply unequivocal evidence that genistein and prunetol were in fact identical and that they had the structure proposed by Finnemore. The method involved a vigorous acylation of a deoxybenzoin (I Chart II) with sodium cinnamate and cinnamic anhydride. The resulting 2-styrylpyrone (II) was oxidized by potassium permanganate to the isoflavone-2-carboxylic acid (III) which was decarboxylated pyrolytically just above its melting point to the corresponding isoflavone

(IV). The method was very clumsy in practice and the only satisfactory step was the decarboxylation. However, it was the first successful synthesis of isoflavone and it is of historical interest because it proved the isoflavone structure.

The main reason for the interest taken in isoflavones up to that time was simply that they constituted a group of naturally-occurring plant pigments. It was found that chromone itself was colourless, but visible colour appeared when the pyrone ring carried a benzene ring and hydroxyl groups were simultaneously introduced. The orientation of the hydroxyl groups had a regular effect in the sense that hydroxyls in the 5- and 7- positions exerted only a minor action, whilst those in the 3'- and 4'- positions produced deep yellow shades (Mayer, 1943).

I.3. The Discovery of the Estrogenicity of Certain Isoflavones:

A very significant development in plant estrogen research came about during the period from 1941 to 1951, when an outbreak of infertility in sheep appeared with spectacular suddenness over an area of 8,000 square miles of Western Australia. The symptoms included failure of ewes to conceive, stillbirth or early death of lambs, and various disorders of the female reproductive system. Rams were not affected.

Because of the importance of the sheep industry to the economy of Australia and because of the vast amount of capital involved, intensive large-scale research on the problem was begun at once. In the first place, it was discovered that the sheep were being grazed on improved pastures that contained a high proportion of subterranean clover, Trifolium subterraneum, and that had given excellent results over periods of as long as fifteen years. A closer study, however, revealed that the affected animals had been consuming unusually large amounts of the clover-rich pasture. This unusually high intake of clover was traced to a combination of wartime shortages of fertilizer and bulk feeds and exceptional climate conditions of drought (Curnow et al., 1948; Robinson, 1949). The symptoms exhibited by the affected sheep were such as might be expected from excessive estrogen activity. Extensive tests on the herbage of the pastures revealed the presence of water-soluble estrogenic materials in subterranean clover, and eventually this activity was traced to certain isoflavones present in the clover (Bradbury and White, 1951). Most of the potency was due to genistein, which was about 10^{-5} times as active as diethylstilbestrol. Biochanin A, formononetin (Pope and Wright, 1954) and daidzein (Guggolz et al., 1961) were also found in the clover. The relative estrogenic activities of these compounds as compared to diethylstilbestrol were determined by Cheng et al. (1953a; 1953b). The activities of genistein, biochanin A and daidzein

were approximately the same, whereas formononetin had little, if any, potency. However, the discovery that at least some isoflavones were estrogenic led to a much wider interest in these substances.

The four estrogenic isoflavones, i.e., genistein, biochanin A, formononetin and daidzein have so far been isolated from subterranean clover, red clover and alfalfa. (Bate-Smith et al., 1953; Pope and Wright, 1954; Guggolz et al., 1961). All, with the exception of biochanin A, have also been found in ladino clover (Bickoff et al., 1962). Although the estrogenic activity of these isoflavones as compared with that of diethylstilbestrol or the natural steroid estrogens is very low, large amounts of isoflavone-rich forage may be consumed by farm animals. Sufficient estrogenic substances may be ingested, therefore, to exert an important influence on the endocrine state of the animals. Recently Nilsson (1962b) made an assay of the estrogenic activity of these isoflavones by the mouse-uterine-weight method under different administration routes. It was found that daidzein, in contrast to the three other isoflavones, was not absorbed when injected subcutaneously and that its estrogenic activity was in consequence negligible. By oral or intraperitonial administration, the estrogenic activities of daidzein and genistein were approximately 10^{-5} times that of diethylstilbestrol.

Biochanin A was about half as active as daidzein or genistein. Formononetin was inactive. A free para-hydroxy group in the phenyl nucleus of the molecule is present in daidzein and genistein but is lacking in biochanin A and formononetin. The presence of a free para-hydroxy group seems, therefore, to be of importance for the appearance of estrogenic activity. The differences in potencies between genistein and daidzein, as well as between biochanin A and formononetin, may be associated with the presence of the 5-hydroxy group.

I.4. The Syntheses of Estrogenic Isoflavones:

Once Baker and Robinson (1928) had achieved the first synthesis of an isoflavone, it was natural that efforts would be directed to the synthesis of other isoflavones, and this even before the discovery of the estrogenicity of some of these substances provided a powerful stimulus to work in this field.

First of all, Baker, Robinson and Simpson (1933; 1937) themselves made some modification of the original method of Baker and Robinson (1928). Without having recourse to cinnamoylation, they achieved acylation of the deoxybenzoin (I Chart III) with acetic anhydride and then condensed the resulting 2-methyl pyrone (II) with benzaldehyde and a base so as

to obtain the desired 2-styrylisoflavone (III), which was then oxidized and finally decarboxylated to the corresponding isoflavone (V). However, after genistein, daidzein and pseudobaptigenin had been obtained thus, the method was abandoned entirely.

In 1930, Späth and Lederer (1930), following an ancient tradition of organic chemists faced by a recalcitrant reaction, heated deoxybenzoins with ethyl formate and sodium in a sealed tube at 100°C (Equation I, Chart IV). Pseudobaptigenin was prepared by this method but in very poor yield; daidzein and formononetin were made similarly in 1933 (Wessely et al., 1933). Later on, the reaction was first modified by conducting it at 0°C, and the yields were then acceptable, provided that there were no free hydroxyl groups other than the one needed for cyclization (Joski and Venkataraman, 1934; Mahal et al., 1934). In 1949, Sathe and Venkataraman (1949) introduced further modifications and thus overcame most of the difficulties encountered by Späth and Lederer. Their principal modifications included the use of ethyl orthoformate as the ester, pyridine as the solvent and piperidine as the base. Under these conditions, deoxybenzoins were converted directly into isoflavones.

The most important single consideration here seemed to be the reactivity of the methylene group of the deoxybenzoin,

for hydroxyl groups in ring B had to be protected to avoid depression of the reactivity of the methylene group by phenoxide formation, whereas those in ring A needed no protection at all. Conversely, a nitro-group in ring B (Equation II, Chart IV) aided the reaction considerably, and as the nitro-group could be transformed into a hydroxyl group at a later stage, its incorporation allowed a certain useful flexibility.

Years later, Baker and Ollis et al., (1952a; 1952b; 1953) introduced a very powerful technique, which relied on the ease with which isoflavone-2-carboxylic acids could be decarboxylated. They treated a deoxybenzoin (I Chart V) with ethoxalyl chloride (the acid chloride from ethyl hydrogen oxalate) in pyridine, and an ∝-oxoester (II) was formed which readily cyclized to the isoflavone-2-carboxylate (IV). At the same time, any free hydroxyl groups were automatically protected as oxalic esters, which, moreover, were very easily hydrolysed, so that the hydroxyl groups could be regenerated later with minimal inconvenience.

In the synthesis of genistein (VI), there was tangible evidence for all the stages depicted. Even the 2-hydroxy-isoflavanones (III) could sometimes be isolated, despite the readiness with which they split out the elements of water to form the isoflavone. The many other compounds synthesized

by this route included prunetin, pseudobaptigenin, daidzein, formononetin and biochanin A.

In 1958, Farkas et al. (1958) found that a deoxybenzoin, of which the free hydroxyl groups needed no protection, could be attacked by zinc cyanide and hydrogen chloride to give an isoflavone in good yield (Equation I Chart VI). To date, only formononetin and pseudobaptigenin have been prepared by this method, and it remains to be seen whether deoxybenzoins derived from phloroglucinol will suffer nuclear substitution instead of forming isoflavones. The presence of aluminium chloride in the reaction mixture has been known to favour nuclear substitution even in resorcinol-based deoxybenzoin, the reaction then affording a derivative of resorcyl aldehyde (Equation II), (Kawase et al., 1958a; 1958b).

All the foregoing syntheses patently require a deoxybenzoin as starting material. Such compounds are available without much difficulty (Karmarkar et al., 1955; Kawase et al., 1958a; 1958b), since phenols may be condensed with derivatives of aryl-acetic acids in various ways, e.g. Friedel-Crafts reactions, Fries rearrangements, and Hoesch condensations, especially the last. The route investigated by Yoder et al. (1954) in 1954 for their syntheses of four estrogenic isoflavones, genistein, biochanin A, formononetin

and daidzein is worthy of mention. The synthesis involved a condensation of two intermediate aromatic phenolic derivatives to a corresponding deoxybenzoin. Of these, only the substituted benzyl cyanides were not available commercially. However, the preparation of these by Julian and Sturgis (1935) through the Gränacher (1922; 1923) synthesis was most promising as to yields, variety and availability of the starting materials. For the synthesis of biochanin A, it involved the condensation of anisaldehyde (I Chart VII) with rhodanine (II), alkaline cleavage of the condensation product to p-methoxyphenyl thiopyruvic acid (III), oximation, dehydratization and decarboxylation of the oximino-p-methoxy-phenyl pyruvic acid (IV) to p-methoxybenzyl cyanide (V). The latter might then be condensed with phloroglucinol or resorcinol to give biochanin A or formononetin respectively as the final product.

Isoflavones may also be obtained without recourse to deoxybenzoins by two methods. One of these was devised by Baker, Pollard and Robinson (1929) in 1929 and applied by Späth and Lederer (1930) to the preparation of pseudobaptigenin, but it has lain in disuse since. In the other method, which was too difficult to control to be of practical value, a flavanone was oxidized by lead tetra-acetate to a mixture of a 3-acetoxy flavanone, a flavone and an isoflavone. This

method was devised in 1958 by Cavill et al. (1958).

I.5. The Syntheses of Labelled Estrogenic Isoflavones:

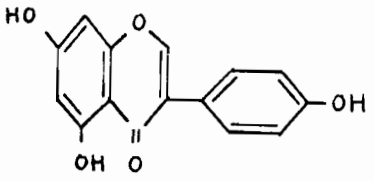
Although a considerable amount of knowledge has been accumulated concerning the isolation of isoflavones in various forage species, the levels of estrogenic activity of these isolated isoflavones and the different methods of syntheses, little is known so far about the metabolism of these compounds in the animal body. Labelled isoflavones are synthesized, therefore, to enable the metabolic pattern of the estrogens to be followed.

Yoder's method cannot be used to prepare C^{14}-labelled p-methoxybenzyl cyanide when it is desired to introduce the isotopic carbon into the cyano group by means of $KC^{14}N$. In 1961, Nilsson et al. (1961a) in Stockholm were successful in the synthesis of biochanin A-4-C^{14}. As p-methoxybenzyl alcohol required for the preparation of p-methoxybenzyl chloride was not available, they investigated the reduction of anisaldehyde (I Chart VIII) to the alcohol with formaldehyde, adapting the crossed Cannizzaro reaction. The p-methoxybenzyl alcohol (II) was then converted to p-methoxybenzyl cyanide (IV) through p-methoxybenzyl chloride (III). The isotopic carbon was introduced at this stage by

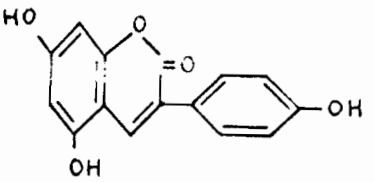
means of $KC^{14}N$.

Through the Hoesch synthesis, phloroglucinol and labelled p-methoxybenzyl cyanide condensed together to give the desired deoxybenzoin, 2,4,6-trihydroxyphenyl p-methoxybenzyl ketone-C^{14} (VI). The further steps for the synthesis of labelled estrogenic isoflavones comprised a slight modification of the method of Baker and Ollis et al. (1952a; 1952b; 1953) that has been described above.

CHART I. The Structural Formulae for Genistein as given by Finnemore and by Bargellini.

(I) 

Finnemore's supposition

(II) 

Bargellini's supposition

CHART II. Synthesis of Isoflavones: Method of Baker and Robinson.

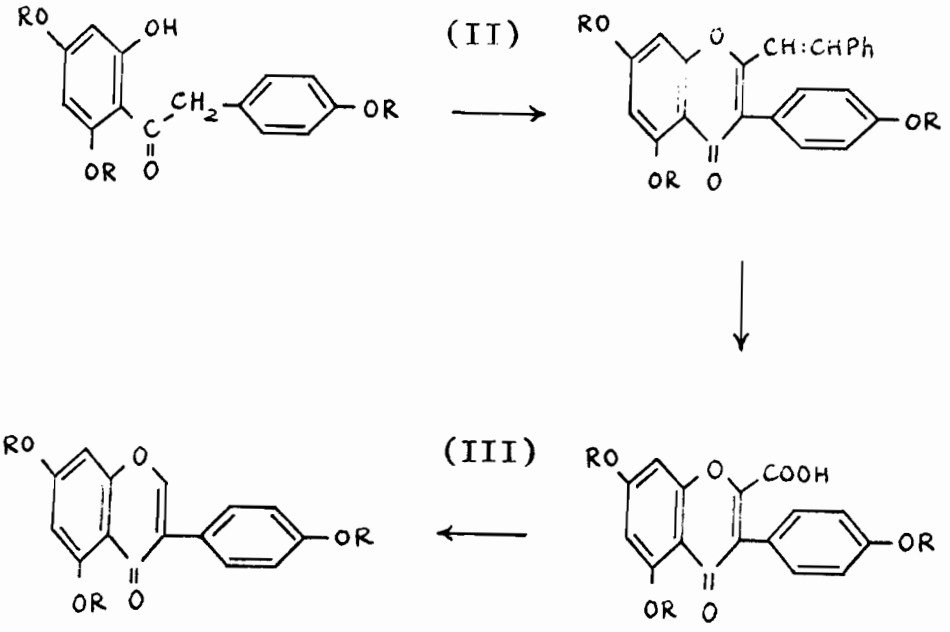


CHART III. Synthesis of Isoflavones: Method of Baker, Robinson and Simpson.

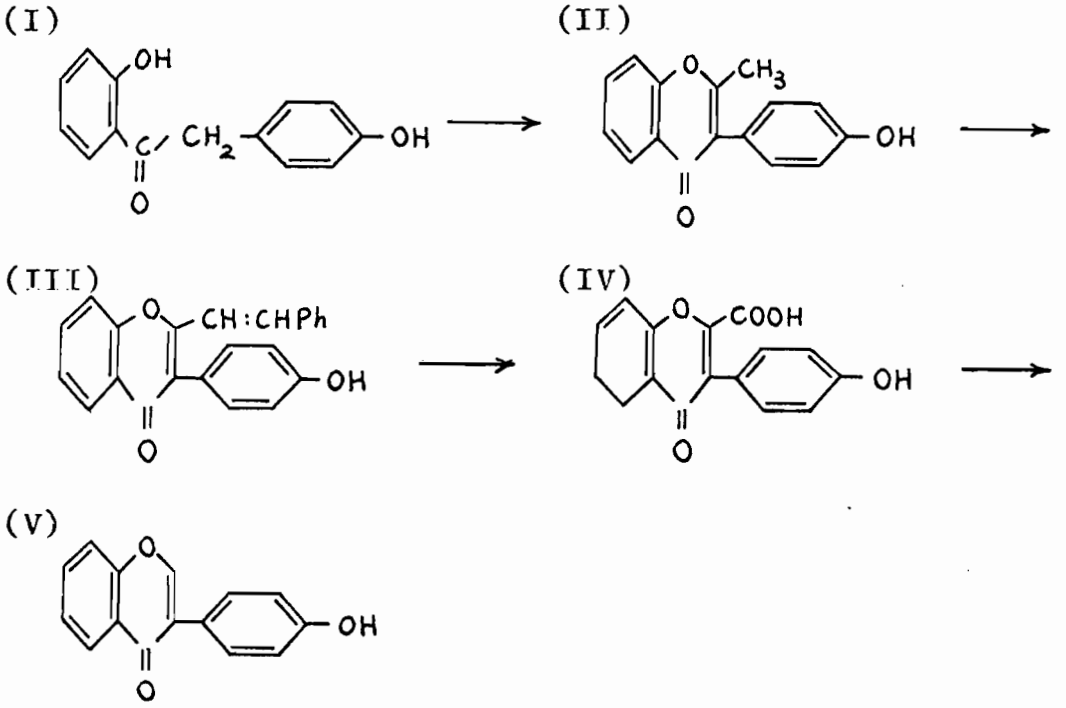


CHART IV. Synthesis of Isoflavones: Method of Späth and Lederer.

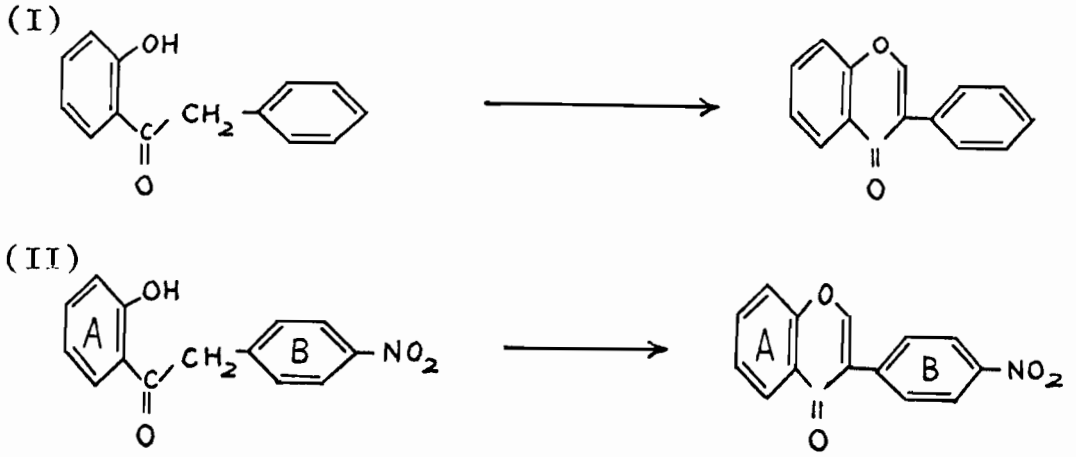
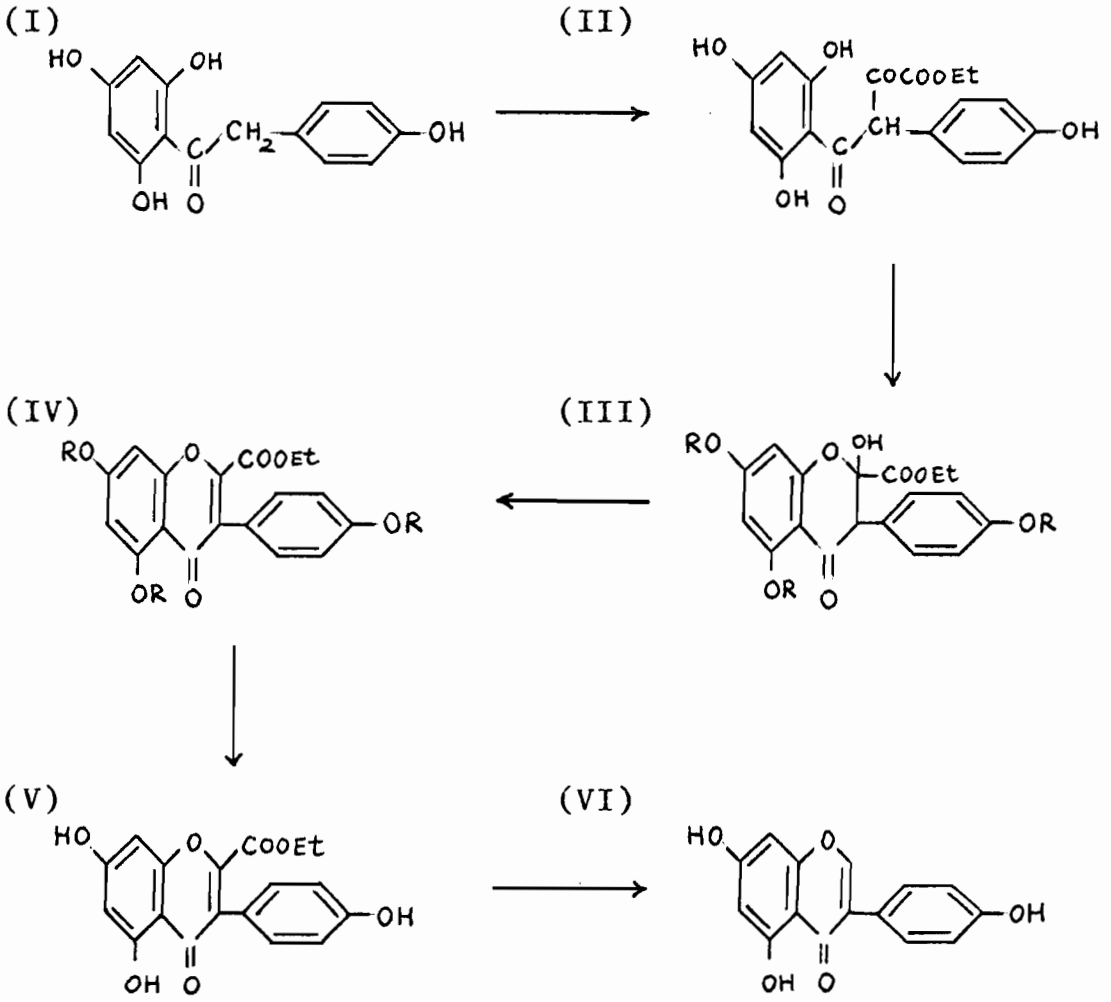


CHART V. Synthesis of Genistein: Method of Baker and Ollis et al..



R = -COCOOEt

CHART VI. Synthesis of Isoflavones: Method of Farkas *et al.*.

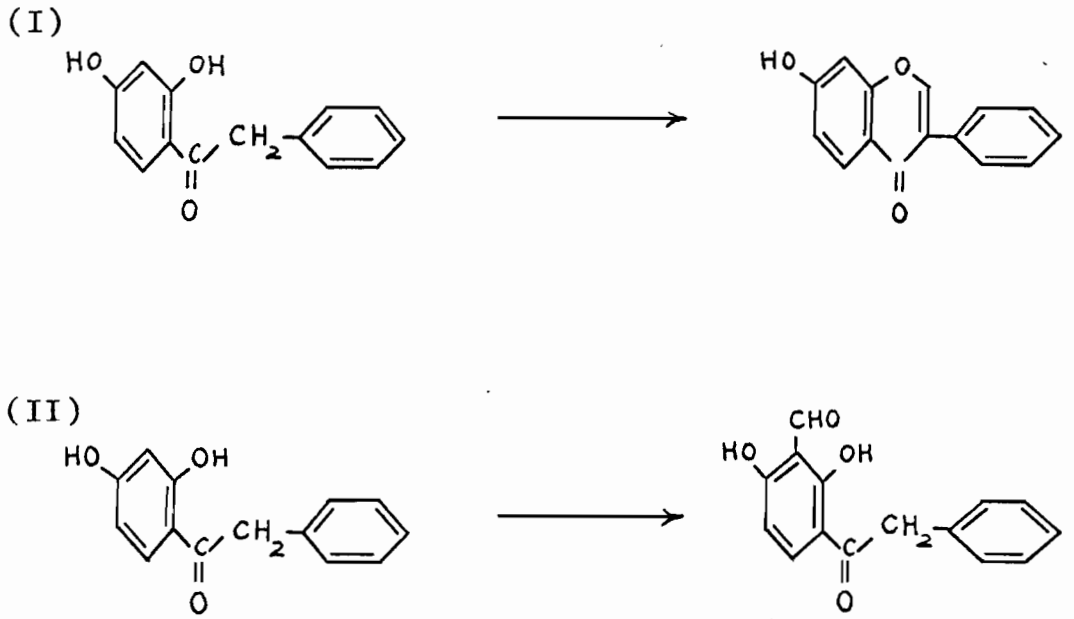


CHART VII. Preparation of p-Methoxybenzyl cyanide.

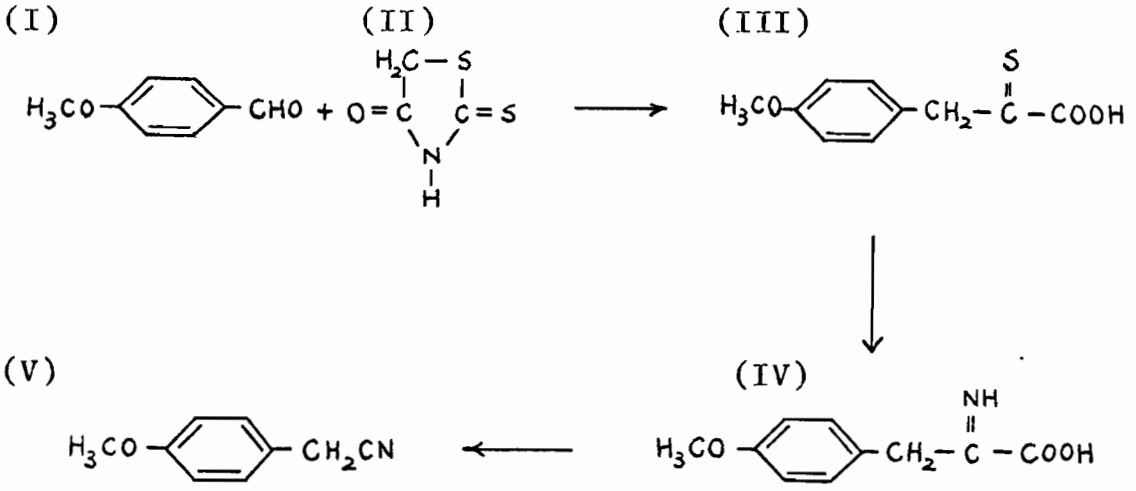
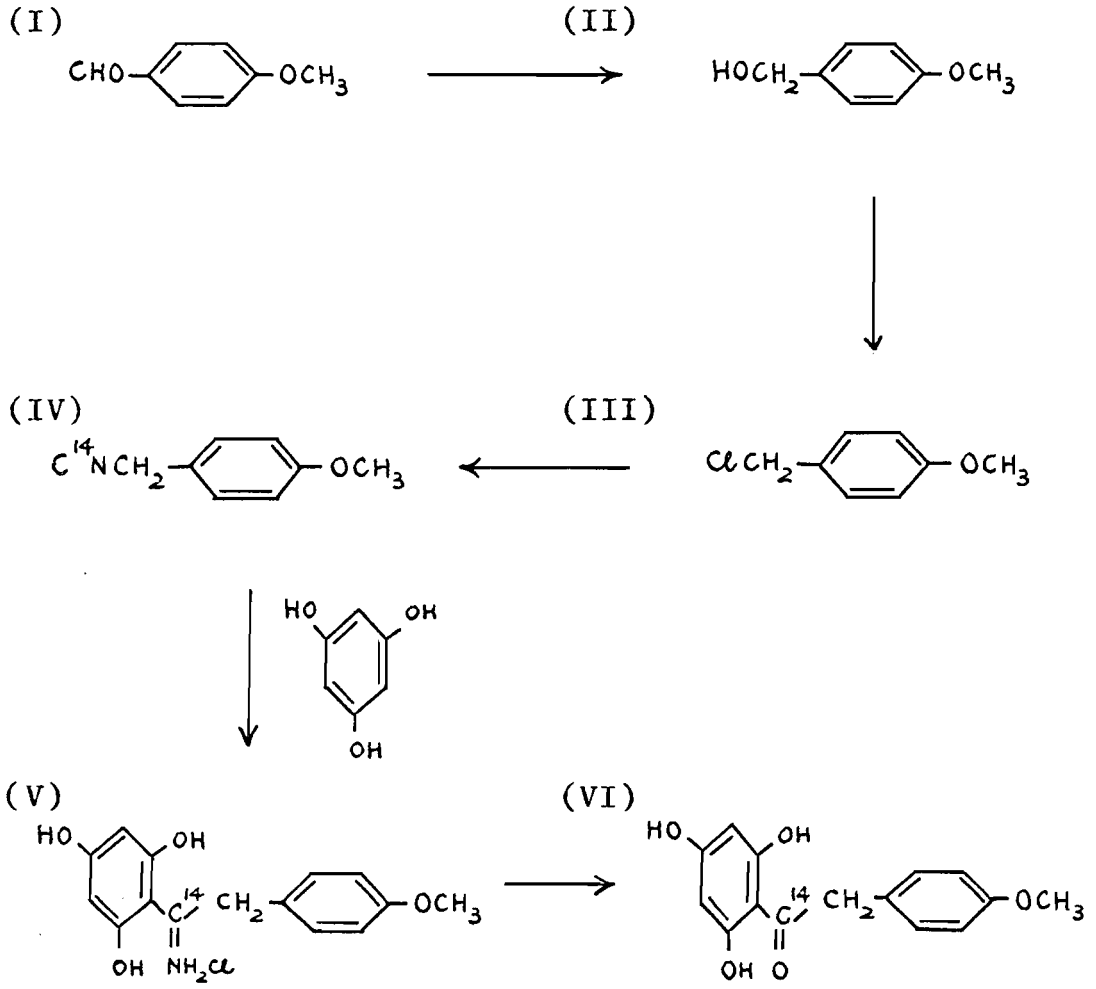


CHART VIII. Preparation of C^{14}-labelled Deoxybenzoin.



II. MATERIALS AND METHODS

II.1. General Materials and Apparatus

II.1. a. Reference compounds:

Biochanin A was presented by Dr. H. Grisebach, Chemical Laboratory, University of Freiburg-im-Breisgau, Germany.

Genistein was donated by Dr. Wilfred Lawson, Courtauld Institute of Biochemistry, London, England.

Daidzein was kindly supplied by Dr. E. M. Bickoff, Western Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Albany, California, U. S. A.

Formononetin was generously given by Dr. H. Grisebach and Dr. E. M. Bickoff.

II.1. b. Principal chemicals:

Anisaldehyde and ethyl oxalyl chloride were obtained from Eastman Organic Chemicals Distillation Products Industries, Rochester, New York, U. S. A.

Phloroglucinol, melting range 217°-219°C, and resorcinol, melting range 109.6°-110.7°C, were obtained from Fisher Scientific Company, Montreal, Canada.

Hydriodic acid, specific gravity 1.70, was also obtained from Fisher Scientific Company.

Potassium cyanide-C^{14} was obtained from New England Nuclear Corporation, 575 Albany Street, Boston, Massachusetts, U. S. A.

II.1. c. Organic solvents:

Methanol, acetone, and diethyl ether were obtained from Anachemia Chemicals Ltd., Montreal, Canada.

Benzene, cyclohexane and pyridine were "Analar" analytical reagents and were products of the British Drug Houses, Ltd., Toronto, Canada.

Ethyl acetate was obtained from Mallinckrodt Chemical Works and was distilled before use. Spectranalyzed grade methanol, a Fisher Certified reagent, was used without further treatment.

Preparation of dry acetone: Acetone was dried over about one-quarter its volume of granular calcium chloride for at least twenty-four hours. The dried acetone was then filtered and distilled before use (Rorig et al., 1956).

Preparation of peroxide-free dry diethyl ether: Diethyl ether was freed from peroxides according to the

procedure of Bauld (1956). A litre of diethyl ether was shaken with 3 x 100 ml of 0.8M ferrous sulfate in 0.4N sulfuric acid, washed with 2 x 50 ml water and finally distilled. It was then dried over potassium hydroxide pellets for one week and then over sodium metal. Finally, it was filtered and redistilled just before use.

Preparation of dry pyridine: Pyridine was dried over potassium hydroxide pellets for at least two weeks. Its anhydrous state was ensured by subsequent addition of sodium metal.

Preparation of absolute ethanol: Ninety-five percent ethanol was obtained from Consolidated Alcohols Ltd., Toronto, Canada. It was refluxed for twelve hours with 5 percent zinc dust as reducing agent and 5 percent sodium hydroxide pellets as dehydrating agent. The filtrate was then twice distilled.

II.1. d. Colour reagents:

Diazotized sulphanilic acid (DSA) reagent was prepared immediately before use according to the directions of Block and Bolling (1951). Two-gram of sulphanilic acid was mixed with 20 ml of concentrated hydrochloric acid and 200 ml of water. Two volumes of 0.5% sodium nitrite were added just before use. This reagent was found sensitive for use with

paper chromatograms. It gave less intense background coloration than other diazonium salts and the colours of the spots were stable for long periods without appreciable change.

A reagent prepared by dissolving 1 gm p-nitrobenzene-diazonium fluoroborate in 100 ml 50% glacial acetic acid was found to be more sensitive than the reagent of Block and Bolling for use with thin-layer chromatograms and had the further advantage of giving a lesser degree of background coloration.

II.1. e. Refractometer, densitometer and melting-point apparatus:

An Abbé refractometer was used to measure the refractive indices of some of the intermediate products. The refractometer was one made by Hilger & Watts, Ltd., London, England.

The DSA-stained chromatostrips were scanned with a manual densitometer (Model 52C), obtained from Photovolt Corporation, New York City, N. Y. No filter was used and the slit width was 1 millimeter.

The melting points of the various intermediate compounds and the different final products were determined by a Kofler micro-melting point apparatus obtained from Arthur

H. Thomas Company, Philadelphia, Pennsylvania, U. S. A.

II.1. f. Vacuum distillation unit:

The boiling points of some of the intermediate products were inconveniently high for direct distillation. Consequently, they were distilled under reduced pressure in an all-glass distillation assembly including a suitable Claisen flask. The pressure was checked on a manometer connected to the assembly and the Duo-Seal vacuum pump obtained from W. M. Welch Manufacturing Company, Chicago, Illinois, U. S. A., was protected by a trap refrigerated by liquid air.

II.2. General Methods

II.2. a. Thin-layer chromatography (Truter 1963):

Thin-layer chromatography (TLC) was used as one of the principal means for the identification of the synthesized products, because of the following five considerations:

(1) Scale

The synthesized products were radioactive and thus expensive. Consequently, only a minute amount of the samples could be made available for the identification work. The sample that can be conveniently handled on a single chromatoplate may be as small as 0.5 microgram, and hence TLC was the technique of choice for identification purposes.

(2) Resolution

The resolution obtainable in TLC often surpasses that attainable on paper. A mixture of as many as ten compounds can be separated into its components within a distance of 5 cm, a degree of resolution that is difficult of attainment with any other chromatographic process.

(3) Simplicity

The apparatus is not unduly expensive, the technique is simple and the results are readily reproducible.

(4) Sensitivity

By using an appropriate chromogenic reagent or a suitable combination of chromogenic reagents, it is possible to detect less than 0.1 microgram of many substances. In addition, the chemical inertness of the silica gel layers ordinarily used permits the use of a much wider range of chromogenic reagents than can be used in conjunction with paper chromatograms.

(5) Time

The whole process of TLC can be carried out within 30 minutes. Other chromatographic processes require hours, and occasionally days, for the same problem.

Preparation of the layers (Randerath, 1963):

The thin-layer chromatography apparatus used was that marketed by Shandon Scientific Company, Ltd., London, England.

Glass plates for TLC must be carefully cleaned and be completely free from grease. The plates were thoroughly scrubbed with Alconox detergent (Canadian Laboratory Supplies, Montreal) and left for ten minutes in chromic-sulfuric acid. They were then brushed under running tap water, rinsed clean with distilled water and dried on a metal drying rack in a drying cabinet.

Five 20 x 20 cm cleaned glass plates of uniform thickness were then placed on an aligning tray. The ends of the row of plates were completed with two 5 x 20 cm end-plates. The aligning tray was inflated to prevent the plates from sliding. A spreader with 250 micrometer slit-width was placed in position on the left-hand end-plate. Thirty gram of neutral Silica Gel G Merck was evenly mixed with 60 ml distilled water by vigorous shaking for one minute in a stoppered 250 ml conical flask. The mixture was then poured into the spreader. As soon as all the slurry was transferred, the spreader was held with both hands and drawn quickly but steadily across the plates to the further end-plate without applying much pressure.

The coated plates were left in position for about twenty minutes until their surface had become completely mat. They were then placed in a drying cabinet at 110°C for activation. The activity of the layer was determined by the heating time and temperature. The longer the heating time and the higher the heating temperature, the greater was the adsorption activity of the layer. For Silica Gel G, one hour's heating in 110°C oven was adopted. At the end of this time, a small strip was scraped off from the four sides of each plate with a spatula to even the edges and to facilitate handling. The plates were then stored, with the coated side up, over calcium chloride in a desiccator.

Preparation of the chromatograms:

Reference materials and synthesized materials were dissolved in spectranalyzed grade methanol and suitable portions of the solutions were spotted on to the plate by means of micro-pipettes and at a distance of 4 cm from the lowermost edge of the plate. A transparent plastic template was used to enable the substances to be applied in a straight line. The starting points, which were placed at intervals of 1.5 cm were marked off with a sharp, hard pencil. The outside starting points were at least 1.5 cm from the edges of the layer. A dividing line, 12 cm away from the starting points was drawn along the edge of the template with a

spatula at right angles to the direction of development and the resulting dust was blown away.

Ascending TLC was carried out at room temperature in standard rectangular chromatographic tanks 22 x 10 x 22 cm. Two glass rods were placed one along each shorter wall of the tank to support the plate in a horizontal position. Three sides of the tank were covered internally with a sheet of Whatman No. 1 filter paper so that it would dip into the solvent and facilitate the saturation of the tank. The appropriate solvent system (240 ml) was poured into the tank and equilibration was allowed to proceed for twelve hours. At the end of this time, the lid of the tank was partially removed and the charged plate was slipped in with the silica gel below the origin immersed in the solvent. This step was carried out as quickly as possible so as to disturb the saturation equilibrium of the tank as little as possible. As soon as the solvent front had reached the finishing line, the plate was removed from the tank. The chromatogram was then dried at room temperature. It was finally stained with p-nitrobenzenediazonium fluoroborate in 50% glacial acetic acid and recorded for reference.

II.2. b. Paper chromatography:

Paper chromatography was used to confirm the identities of the products synthesized and to check their radiochemical purities. Two solvent systems were used successfully. The benzene-acetic acid-water (125/72/3 by volume) and the chloroform-formamide (1/1 by volume) were proved to give a single narrow band for each sample in question and to afford a wide range of R_f values for different synthesized products.

Benzene-acetic acid-water solvent system:

This single phase benzene-acetic acid-water (125/72/3 by volume) solvent system was originally devised by Smith (1960) as an improvement on the organic phase of benzene-acetic acid-water (2/2/1 by volume), which has been commonly used for the chromatography of phenolic acids. In 1962, Wong and Taylor (1962) applied the monophasic benzene solvent system to the separation of ether-soluble polyphenols (Wong 1962) and certain hydrolysates of plant extracts. Excellent separations were obtained for flavones, isoflavones, etc. when two-dimensional chromatograms were run with this system as the first solvent and 2N ammonia (Wong and Taylor 1962) or aqueous acetic acid as the second solvent.

The chromatographic apparatus consisted of a rectangular glass chamber, 12 inches square and 24 inches high and a ground-glass cover with a small hole right in the middle

through which solvents might be added into the chamber. Inside the chamber, there was a glass solvent trough held horizontally in the upper part of the chamber by glass supports, which in turn were carried by stainless steel brackets screwed to opposite sides of the chamber.

Chromatography was carried out on Whatman No. 1 filter paper (6.5 x 57.0 cm). The paper was thoroughly extracted in a Soxhlet apparatus with a mixture of benzene and methanol (1/1, V/V) for 72 h. In order to run a radioactive sample, a non-radioactive sample and a reference sample on a single chromatogram with a view to eliminating the effects of variations in running conditions, such as the room temperature, the chamber saturation, etc., the paper was cut longitudinally into three strips in such a manner that the paper was left intact for 8.5 cm from one end. One strip was three times as wide as the other two. A line of application was drawn 10 cm from the intact end. A solution of the radioactive sample in spectranalyzed methanol (about 100 micrograms of material) was applied to the wide strip while the non-radioactive sample and the sample's reference were applied separately one on each of the two narrow strips. As a whole, the application line was an interrupted streak about 0.5 cm wide.

The chromatographic chamber was filled to a depth of

about 2 cm with benzene-acetic acid-water (125/72/3 by volume) and the charged three-armed paper strip was placed in position with the intact end lying on the glass trough. One hour was allowed for the preliminary equilibration. At the end of this time, the solvent was introduced into the glass trough through the central small hole by means of a funnel. The small opening was immediately closed again with a cork so as to disturb the chamber equilibrium as little as possible.

The monophasic benzene-acetic acid-water system was found to be very sensitive to temperature effects. However, when the temperature was kept within the range $20 \pm 2^{o}C$, the R_f values for the compounds studied were reproducible to within $\pm$ 0.02 and the solvent took 3.5-4 h to descend a length of 40 cm.

Chloroform-formamide solvent system:

The application of this system to the separation of steroid estrogens has been described in detail by Layne and Marrian (1958). The chromatographic apparatus was the same as that in the descending monophasic benzene-acetic acid-water solvent system.

Strips of Whatman No.42 paper (6.5 x 57.0 cm) were extracted in a Soxhlet apparatus with a mixture of methanol and chloroform (1/1, V/V) for 3 days. After drying, the

paper strips were impregnated with the stationary phase by immersing in a (2/1, V/V) methanol-formamide mixture. Each strip was then carefully blotted with filter paper between two sheets of plate glass and dried in a horizontal position within a drying cabinet at 37°C for 45 minutes.

Charged three-armed chromatographic strips were prepared in the same way as described above. Saturation of the chromatographic chamber was accomplished by filling the bottom of the chamber to a depth of 2 cm with the mobile phase (chloroform). The whole apparatus was equilibrated for at least 1 h at 20±2°C before the chromatograms were developed with chloroform. Under these conditions, development of the chromatograms usually required 4 h, by which time the solvent front had descended about 40 cm.

After the chromatograms were developed, whether in the monophasic benzene-acetic acid-water solvent system or in the chloroform-formamide solvent system, their arms were separated. A pilot strip, 1 cm wide was cut longitudinally from the wide arm for preliminary location of the radioactive sample. The bands of the radioactive sample on the pilot strip, the non-radioactive sample and the reference compound on the narrow strips were located by ultra-violet light and/or by use of diazotized sulphanilic acid as a colour reagent. The distribution of radioactivity on the chromatogram

of the radioactive sample was then determined by the technique described in the next section.

II.2. c. Measurement of radioactivity:

The distribution of radioactivity on paper chromatograms was determined by liquid scintillation spectrometry (Cayen, 1964). The scintillation fluid was prepared by dissolving 3 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in 1 liter of scintillation grade toluene. Both the PPO and POPOP were obtained from Packard Instrument Company, Inc., La Grange, Illinois, U. S. A., while the toluene was a scintillation grade product of Nuclear Enterprises, Winnipeg, Manitoba, Canada.

After a pilot strip, 1 cm wide, had been cut longitudinally from the wide arm of the three-armed chromatogram for staining, the remaining portion was divided into 0.5 cm bands along the length of the chromatostrip. Each band was placed in a standard 20-ml screw-top glass vial which was numbered beforehand. Spectranalyzed methanol (0.2 ml) was subsequently added to each vial and the contents were shaken for 3 minutes. Both the methanol and filter paper band were then flooded with 10 ml scintillation fluid. The shaking was continued for another 2 minutes.

The radioactivity of the contents of each loaded vial

was counted in a Packard Tri-Carb liquid scintillation spectrometer, Series 314E. The measured radioactivity (counts per minute) was then plotted against the distance along the chromatostrip. If only one single symmetrical high peak was found in the graph, and if the position of the peak corresponded to the positions of the stained bands in the pilot strip as well as in the two narrow strips, then the radioactivity purity of the sample in question was confirmed. Here it must be remembered that the radioactivity thus registered for each loaded vial was a relative quantity, and did not provide an absolute measurement of the radioactivity present.

III. PROCEDURES AND RESULTS

III.1. Syntheses of C^{14}-labelled Biochanin A, Formononetin, Genistein and Daidzein:

III.1. a. Preparation of p-methoxybenzyl alcohol (Davidson and Weiss, 1943).

The apparatus comprised a 500 ml three-necked, ground-glass-joint, round-bottomed flask fitted with a mercury-sealed mechanical stirrer, a reflux condenser, a dropping funnel, and a thermometer which reached almost to the bottom of the flask.

Eighty-four g (1.5 moles) of U.S.P. grade potassium hydroxide pellets (85% potassium hydroxide) and 130 ml of acetone-free absolute methanol were placed in the flask. Stirring was then begun. The bulk of the alkali dissolved in a few minutes, with the evolution of heat. The presence of traces of the undissolved alkali indicated the methanol had been saturated with potassium hydroxide. A mixture of 68 g (0.5 moles) of p-methoxybenzyl aldehyde (anis-aldehyde), 50 ml (0.65 moles) of 40% formaldehyde solution (formalin) and 50 ml of acetone-free absolute methanol was added through the dropping funnel at such a rate that the internal temperature was kept within the range 65-75^{o}C.

The addition required about 10 minutes. This internal temperature was maintained manually for three hours by an electric heating system. At the end of this time, the reflux condenser was replaced by a downward condenser and the temperature was increased to distil over the methanol, until the internal temperature reached 101°C. One hundred and fifty ml of cold water was then added to the warm residue and the mixture was left overnight.

Next morning, the resulting two layers were separated. The aqueous layer was then extracted with three successive 40 ml portions of benzene. The combined oil and extracts were washed with six 10 ml portions of water. The combined washings were then extracted with 15 ml of benzene, the benzene layer being added to the washed extract. The benzene solution was subsequently cleared by shaking it with 10 g anhydrous sodium sulphate and was distilled. After removal of the benzene, the p-methoxybenzyl alcohol was purified by distillation with diminished pressure.

Results and discussion: The product was a colourless oily liquid with a specific gravity of the order of 1.15. Its boiling point was 146-148°C at 20 mm Hg. On cooling, it formed snow-white, crystalline plates which melted at 19-21°C.

This preparation was first studied by Davidson and Bogert (1935) in 1935. They described the action of alkali on mixtures of aromatic aldehydes and formaldehyde as resulting in the practically complete conversion of the aromatic aldehydes to the corresponding alcohols. The conversion was a "Crossed Cannizzaro Reaction". Since then, it has been regarded as one of the most convenient methods for preparing certain aromatic alcohols, such as anisyl, piperonyl, and veratryl alcohols, of which the corresponding aldehydes are readily available.

Besides the above described "Crossed Cannizzaro Reaction", p-methoxybenzyl alcohol may be prepared by reducing anisaldehyde with lithium aluminium hydride in absolute ether or with sodium boron hydride in methanol (Nilsson et al., 1961a). Yields of 60% and 30% respectively, have been reported for the foregoing methods. In the present study, 58 g of p-methoxybenzyl alcohol was obtained. This corresponds to a yield of the order of 84%, which is more satisfactory than the yields reported for the reduction methods.

III.1. B. Preparation of p-methoxybenzyl cyanide-C^{14} (Rorig *et al.*, 1956).

In a 125 ml Erlenmeyer flask were placed 5.5 g of p-methoxybenzyl alcohol and 10 ml of concentrated hydrochloric acid. After stirring vigorously with an electro-magnetic stirrer for 15 minutes, the contents of the flask were transferred to a 125 ml separatory funnel. The lower layer (p-methoxybenzyl chloride) was separated, dried over 1 g of granular calcium chloride for about 30 minutes, and finally filtered to remove the drying agent.

A mixture of 4.3 g (0.028 moles) of the p-methoxybenzyl chloride, 1.5 g of sodium iodide, 2.0 g (0.037 moles) of potassium cyanide containing a small amount of C^{14}-marked potassium cyanide (1 millicurie) and 50 ml of dry acetone was introduced into a 250 ml ground-glass-joint round-bottomed flask fitted with a reflux condenser. The heterogeneous mixture was then refluxed with vigorous stirring by means of an electro-magnetic stirrer for 18 h.

At the end of this time, the reaction mixture was cooled to room temperature and finally filtered with suction. The solid on the filter was washed with 25 ml of dry acetone and discarded with due care. The combined filtrates were distilled to remove the acetone. At this stage, white

crystals appeared and at the same time the boiling solution began to bump. In order to reduce the bumping to a minimum, the distillation was carried out in a flash-evaporator (from Buchler Instruments, Fortlee, New Jersey, U. S. A.) under reduced pressure and at a temperature of 50°C. The residual oil was subsequently taken up in 40 ml of benzene and washed with three 20 ml portions of hot water (about 60°C). The benzene solution was then dried over anhydrous sodium sulphate for 15 minutes. The dried solution was transferred into a 150 ml round-bottomed flask and the solvent was distilled at 42-44°C with a pressure of 18 cm Hg. The residual p-methoxybenzyl cyanide-C^{14} was purified by distillation under greatly reduced pressure.

Results and discussion: Both the p-methoxybenzyl chloride and the p-methoxybenzyl cyanide-C^{14} were colourless liquids. The latter had a boiling point of 118-120°C at 2 mm Hg and a refractive index n_D^{25} 1.5285-1.5291. It was found to turn into a brown yellowish colour if stored for more than a few weeks.

p-methoxybenzyl cyanide has been prepared by different methods by various authors e.g., Baker and Robinson (1926; 1929), Kondo and Oshima (1931), Julian and Sturgis (1935), Shriner and Stephenson (1942) and Shriner and Hull (1945a; 1945b). In 1954, Yoder et al. (1954) described a route for

the preparation of p-methoxybenzyl cyanide, mainly based on the one suggested by Julian and Sturgis. Their starting materials were anisaldehyde and rhodanine. The procedure involved the anisaldehyde-rhodanine condensation, the alkaline cleavage of the condensation product, and the oximation, dehydration and decarboxylation of p-methoxyphenyl thiopyruvic acid to p-methoxybenzyl cyanide. Though this route appeared to be very promising as to yields and availability of the starting materials, yet it was not applicable if isotopic carbon was to be introduced by means of $KC^{14}N$.

In the present study, the preparation of p-methoxybenzyl cyanide-C^{14} was repeated twice, each time with 3 millicurie C^{14}-marked potassium cyanide. The average yield was 68%. It was found that the yield depended greatly on the purity of the alcohol used and on the anhydrous state of the heterogeneous mixture. The latter point was found to be particularly important. If it was not strictly observed not only were the yields low, but the product was brown in colour as well. This brown colouration indicated the presence of impurities and it could not be removed even by successive distillations.

III.1. c. Preparation of 2,4,6-trihydroxyphenyl p-methoxybenzyl ketone-C^{14} and 2,4-dihydroxyphenyl p-methoxybenzyl ketone-C^{14} (Yoder *et al.*, 1954).

A solution of 2.7 g (0.018 moles) p-methoxybenzyl cyanide-C^{14} and 2.4 g (0.018 moles) anhydrous phloroglucinol in 20 ml of dry diethyl ether in a 125 ml Erlenmeyer flask was cooled to $0^{o}C$ in an ice bath and a slow stream of dry hydrogen chloride was passed into the solution.

Dry hydrogen chloride gas was prepared by treating concentrated hydrochloric acid with concentrated sulphuric acid. A 500 ml pyrex Florence flask was fitted with a two-hole rubber stopper, equipped with a short-stem dropping funnel and a glass delivery tube. To the stem of the dropping funnel, there was attached a capillary tube of no greater than 1 mm internal diameter, and of such a length that, when the apparatus was assembled, its lower end reached within 1 to 2 mm of the bottom of the flask. Two hundred ml concentrated sulphuric acid was run into the flask. The stopper was put in place and concentrated hydrochloric acid was added very slowly through the dropping funnel. A stream of hydrogen chloride gas thus generated was dried by passing through a couple of flasks containing concentrated sulphuric acid.

After passing the gas into the solution for about

20 minutes, ketimine hydrochloride was seen to separate on the bottom of the 125 ml Erlenmeyer flask. The flask was then stoppered and set in a deep-freeze refrigerator for 12 h. The mixture was again saturated with dry hydrogen chloride gas and kept in the refrigerator for another 24 h.

The ether in the mixture was finally decanted and the crust washed with 5 ml ice-cold ether. The insoluble ketimine hydrochloride and the gummy precipitate were refluxed for 1 h with 110 ml of 2% (W/V) hydrochloric acid. Upon cooling the solution, the crude ketone precipitated as white flocculant mass, which was filtered. The product was purified by recrystallization from 50% methanol with the aid of Norit A as a decolorizing agent.

For the preparation of 2,4-dihydroxyphenyl p-methoxybenzyl ketone-C^{14}, resorcinol was used instead of phloroglucinol in the above procedure. Due to the relative inertness of resorcinol as compared with phloroglucinol, the time required to saturate the solution of cyanide, resorcinol and ether with dry hydrogen chloride gas was prolonged to at least 30 minutes before any white crystalline specks of ketimine hydrochloride appeared.

Results and discussion: Both the 2,4,6-trihydroxyphenyl p-methoxybenzyl ketone-C^{14} and its analogue,

2,4-dihydroxyphenyl p-methoxybenzyl ketone-C^{14}, were white crystals. Their melting points were very sharp and were at 194°C and 154°C, respectively. The yield of the former ketone was 37% while that of the latter was only 22%.

That phenols might be condensed with derivatives of aryl-acetic acids has been known since Hoesch (1915) reported this reaction in 1915. Baker and Robinson (1926; 1928) modified the "Hoesch Condensation Reaction" by eliminating the use of anhydrous zinc chloride. The reaction conditions were further modified by Yoder et al. (1954) in the preparation of various deoxybenzoins for the syntheses of estrogenic isoflavones. The procedure described here was practically the same as that suggested by Yoder et al. The commercially available phloroglucinol was dihydrated, and accordingly it had to be dehydrated before use by drying in an oven of 110°C for 30 minutes. Absolutely anhydrous conditions were completely essential for high and reproducible yields in this particular reaction.

III.1. d. Syntheses of 2-carboxy-5,7-dihydroxy-4'-methoxy isoflavone-4-C^{14} and 2-carboxy-7-hydroxy-4'-methoxy isoflavone-4-C^{14} (Bickoff, 1963).

Five hundred and fifty mg of the labelled 2,4,6-trihydroxyphenyl p-methoxybenzyl ketone-C^{14} was dissolved

in 2.8 ml of dry pyridine in a 125 ml Erlenmeyer flask, and 0.82 ml of ethyl oxalyl chloride was added drop-wise, the temperature being maintained below 25°C by means of an ice-bath. After the addition was complete, the temperature was increased to 65°C for 20 minutes. The flask was then brought to room temperature and 1 ml of diethyl ether was added to redissolve some crystalline needles which appeared at this stage. After mixing, the solution was poured onto a mixture of 15 ml ice-water and 5 ml concentrated hydrochloric acid. The acidic mixture was then vigorously stirred. A sticky precipitate formed. The entire mixture was subsequently extracted with five separate 10 ml portions of diethyl ether. The ethereal solution was dried over anhydrous sodium sulphate, evaporated to about 2 ml and stored in a refrigerator overnight.

The next morning, the oily material was diluted with 10 ml of water and concentrated sodium hydroxide (50% W/V) was slowly added until all the solids were in solution. The pH was then about 12. The solution was held at room temperature for 3 h. By the end of this time, the oxalyl ester was completely saponified. (It is necessary that this saponification be complete). The entire mixture was then acidified with 50% V/V hydrochloric acid, while being stirred and cooled in an ice bath. Addition of acid to a pH of 2 liberated a

water-insoluble yellow precipitate which was collected on a filter, washed with a small amount of ice cold water, and dried in a vacuum desiccator.

With the described procedure, 0.01 mole of 2,4-dihydroxyphenyl p-methoxybenzyl ketone-C^{14} required 0.02 mole of ethyl oxalyl chloride to produce almost colourless 2-carboxy-7-hydroxy-4'-methoxy isoflavone-4-C^{14}.

Results and discussion: The 2-carboxy-5,7-dihydroxy-4'-methoxy isoflavone-4-C^{14}, after recrystallized from 50% methanol, gave a melting point of 270-272°C, while the 2-carboxy-7-hydroxy-4'-methoxy isoflavone-4-C^{14} melted at 260-262°C.

As indicated in the section entitled "Historical Review", ring closure of various deoxybenzoins to form the inner pyrone ring of isoflavones was the critical step in the synthesis of isoflavones and was the subject of numerous investigations. Baker and Robinson (1928) synthesized genistein in 1928 by cinnamoylization of the exposed 6-hydroxy group of methylated deoxybenzoin, and then by dehydration and inner ring closure, they obtained the 2-styryl isoflavone. Shriner and Hull (1945a; 1945b) in 1945 shortened the synthesis by formylating the unmethylated, unprotected hydroxybenzoin with sodium powder and ethyl formate in the synthesis of biochanin A.

These methods were complicated and gave low and erratic yields. It was in 1952 that Baker and Ollis *et al.* (1952a; 1952b; 1953) first introduced the use of ethyl oxalyl chloride in a pyridine solution of the deoxybenzoin followed by mild saponification as described above. Two years later, Yoder *et al.* (1954) adopted this method in their syntheses of isoflavones with a yield of 80%. Recently Nilsson *et al.* (1961a) changed the procedure slightly in their synthesis of biochanin A-4-C^{14}. However, the yields obtained by this procedure in the course of the present study were undesirably low. Subsequently, as an outcome of a personal communication from Bickoff (1963), the writer abandoned Nilsson's modification and shifted back to the procedure used by Yoder *et al.*, but with a slight change in the reaction temperature. The average yield was of the order of 83%, which is somewhat better than that reported by Yoder *et al.* themselves and surpasses Nilsson's reported yield of 69%.

III.1. e. Syntheses of 5,7-dihydroxy-4'-methoxy isoflavone-4-C^{14} (biochanin A-4-C^{14}) and 7-hydroxy-4'-methoxy isoflavone-4-C^{14} (formononetin-4-C^{14}) (Nilsson, 1961).

The dried 2-carboxy-5,7-dihydroxy-4'-methoxy isoflavone-4-C^{14}, 0.2 g, was tamped into the bottom of a 10 ml pyrex

test-tube which was plunged into a Fisher-wax bath heated 5°C above the melting point of the acid, i.e., 275°C. After evolution of carbon dioxide had ceased, which took less than 5 minutes, the tube was removed and air-cooled. The glassy melt was triturated into solution in a minimum amount of boiling methanol, and was then discoloured with Norit A. After concentrating the hot methanol solution and adding an equal volume of distilled water, the isoflavone biochanin A-4-C^{14} crystallized out as pale yellow needles.

The analogue, formononetin-4-C^{14} as obtained from 2-carboxy-7-hydroxy-4'-methoxy isoflavone-4-C^{14} by the above procedure, formed very faint yellowish plates from 50% methanol.

Results and discussion: Both biochanin A-4-C^{14} and formononetin-4-C^{14}, after two recrystallizations from 50% methanol, were found to give very sharp melting points, 209-210°C and 252-253°C, respectively. They were then mixed with their corresponding reference compounds and the mixed melting points were found to be unchanged.

Decarboxylation through pyrolysis in the syntheses of isoflavones was first used as early as 1928 by Baker and Robinson (1928). This step has been found most satisfactory. A yield as high as 98% has been reported by Baker et al. (1952a). In the present syntheses of C^{14}-tagged material,

however, yields were lower, almost certainly because of the relatively small amounts of the starting materials. For biochanin A-4-C^{14}, the yield was 75% and for formononetin-4-C^{14}, the yield was only 52%.

III.1. f. Syntheses of 4',5,7-trihydroxy isoflavone-4-C^{14} (genistein-4-C^{14}) and 4',7-dihydroxy isoflavone-4-C^{14} (daidzein-4-C^{14}) (Shriner and Hull, 1945b).

To 0.05 g of labelled biochanin A-4-C^{14} contained in a 75 ml pyrex test-tube was added 1 ml of hydriodic acid of specific gravity 1.7. Another smaller pyrex test-tube was inserted into the former one and was used as a cold-finger condenser. The mixture was refluxed for 4 h. At the end of this time, the excess acid was neutralized with 30% (W/V) potassium hydroxide solution and the mixture was then made slightly acidic by adding glacial acetic acid dropwise. Upon cooling, white needles of genistein-4-C^{14} separated out from the solution.

The analogous isoflavone, daidzein-4-C^{14}, was obtained by the same procedure but starting with formononetin-4-C^{14} instead of biochanin A-4-C^{14}. The daidzein-4-C^{14} crystallized out in microscopic needles.

Results and discussion: The labelled genistein-4-C^{14},

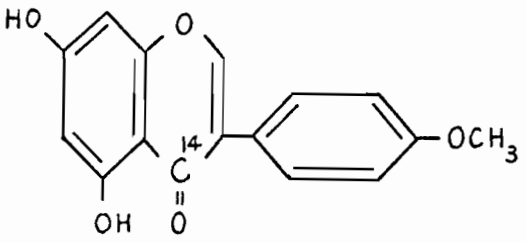
after one crystallization from 50% methanol, melted at 295-297°C. The melting point for daidzein-4-C^{14} was even higher; it decomposed at 316-319°C.

As early as 1910, Finnemore applied the classical reagent, hydriodic acid, to the demethylation of prunetin to the trihydric phenol, prunetol. The procedure described here is practically the same as that used by Shriner and Hull (1945a; 1945b). This method proved very serviceable, though the yield was not particularly high. In this study, it was 42%, which was the same as that obtained by Shriner and Hull when they were demethylating biochanin A to genistein.

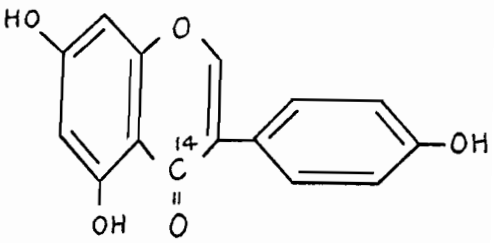
III.2. Syntheses of Non-labelled Biochanin A, Formononetin, Genistein and Daidzein:

The non-labelled naturally-occurring isoflavones, biochanin A, formononetin, genistein and daidzein were all synthesized practically in the same way as described in III.1., with the exception that no C^{14}-labelled potassium cyanide was employed in the preparation of the p-methoxybenzyl cyanide.

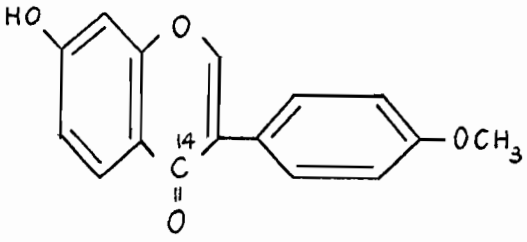
Fig. 1. Structural formulae of the four synthesized C^{14}-labelled isoflavones.



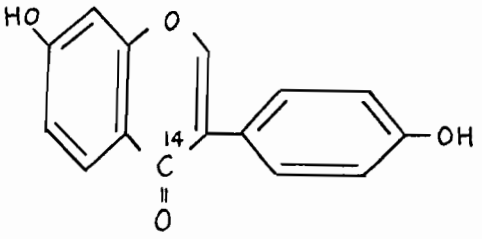
Biochanin A-4-C^{14}



Genistein-4-C^{14}

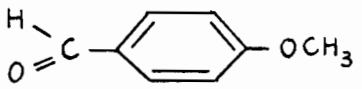


Formononetin-4-C^{14}



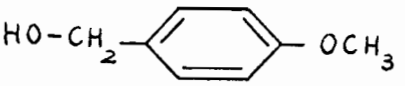
Daidzein-4-C^{14}

CHART IX. A General Flow Sheet For the Synthesis of C^{14}-labelled Isoflavones.



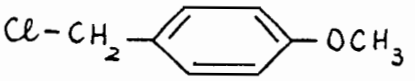
p-methoxybenzyl aldehyde

↓ HCHO + KOH



p-methoxybenzyl alcohol

↓ HCl



p-methoxybenzyl chloride

↓ $KC^{14}N$

Chart IX (cont'd):

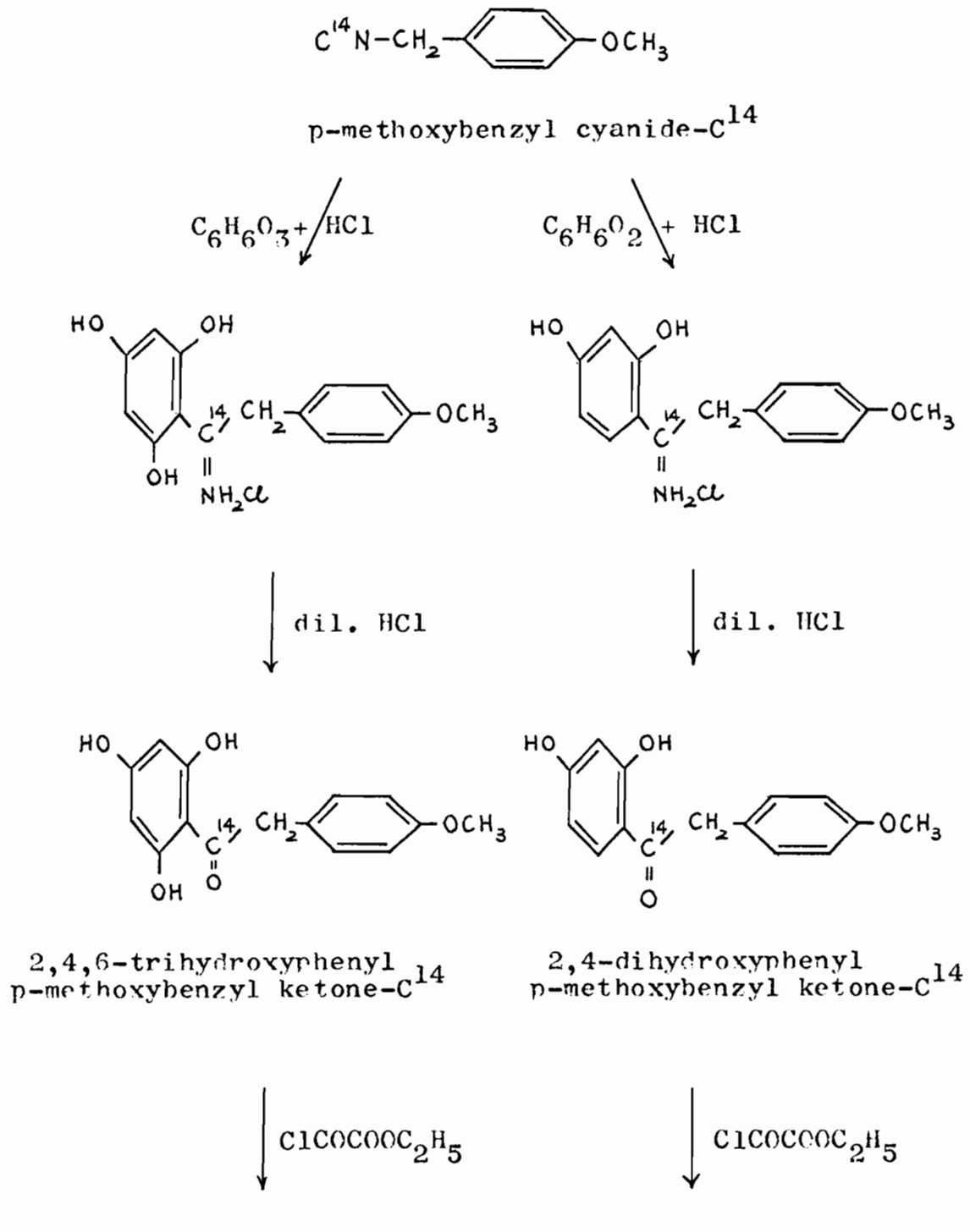


Chart IX (cont'd):

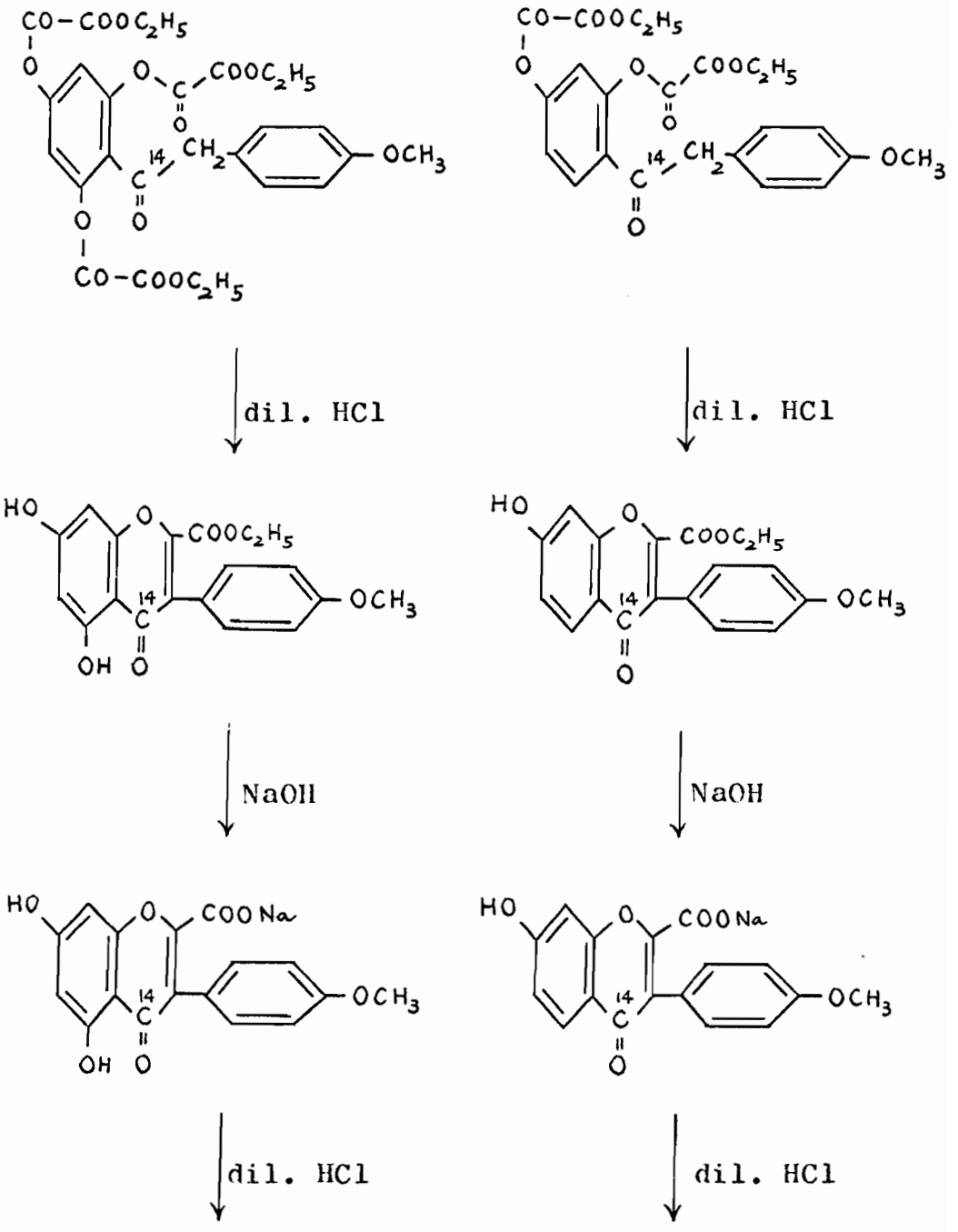
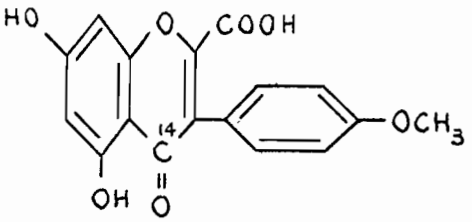
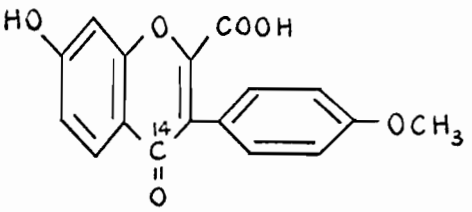


Chart IX (cont'd):



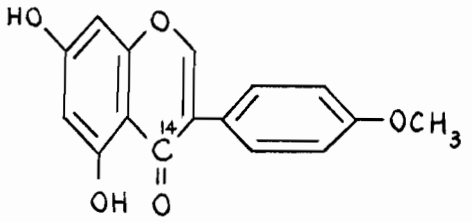
2-carboxy-5,7-dihydroxy-4'-methoxy isoflavone-4-C^{14}



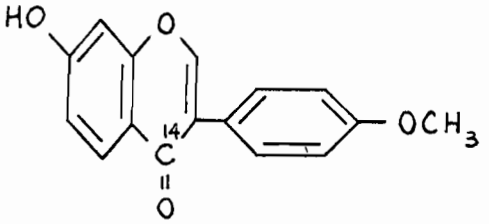
2-carboxy-7-hydroxy-4'-methoxy isoflavone-4-C^{14}

Heat

Heat



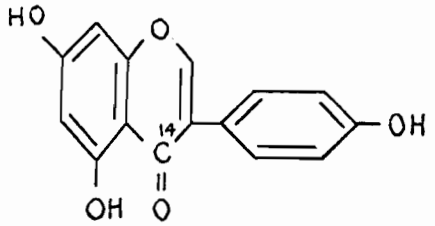
Biochanin A-4-C^{14}



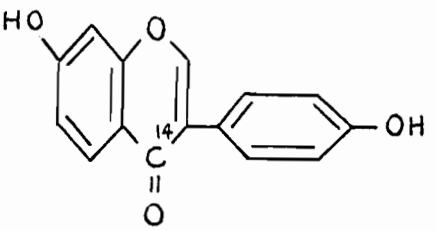
Formononetin-4-C^{14}

HI

HI



Genistein-4-C^{14}



Daidzein-4-C^{14}

Table I

The Yield Percentages

for the Various Steps in the Syntheses of Isoflavones-4-C^{14}

Principal Reactant (M.W.)	Weight of Reactant	Principal Product	Theoretical Yield	Actual Yield	Yield Percentage
p-Methoxybenzyl aldehyde (136)	68.0 g	p-Methoxybenzyl alcohol	69.0 g	58.0 g	84%
p-Methoxybenzyl alcohol (138)	5.5 g	p-Methoxybenzyl chloride	6.2 g	5.4 g	87%
p-Methoxybenzyl chloride (156.5) + potassium cyanide-C^{14}	4.3 g	p-Methoxybenzyl cyanide-C^{14}	4.04 g	2.74 g	68%
p-Methoxybenzyl cyanide-C^{14} (147) + Phloroglucinol	2.7 g	2,4,6-Trihydroxy-phenyl p-methoxy-benzyl ketone-C^{14}	5.03 g	1.86 g	37%

Table I (cont'd)

Principal Reactant (M.W.)	Weight of Reactant	Principal Product	Theoretical Yield	Actual Yield	Yield Percentage
p-Methoxybenzyl cyanide-C^{14} (147) + Resorcinol	2.7 g	2,4-Dihydroxyphenyl p-methoxybenzyl ketone-C^{14}	4.74 g	1.04 g	22%
2,4,6-Trihydroxyphenyl p-methoxybenzyl ketone-C^{14} (274)	550 mg	2-Carboxy-5,7-dihydroxy-4'-methoxy isoflavone-4-C^{14}	658 mg	545 mg	83%
2,4-Dihydroxyphenyl p-methoxybenzyl ketone-C^{14} (258)	550 mg	2-Carboxy-7-hydroxy-4'-methoxy isoflavone-4-C^{14}	666 mg	545 mg	82%
2-Carboxy-5,7-dihydroxy-4'-methoxy isoflavone-4-C^{14} (328)	200 mg	Biochanin A-4-C^{14}	173 mg	130 mg	75%

Table I (cont'd)

Principal Reactant (M.W.)	Weight of Reactant	Principal Product	Theoretical Yield	Actual Yield	Yield Percentage
2-Carboxy-7-hydroxy-4'-methoxy isoflavone-4-C^{14} (312)	200 mg	Formononetin-4-C^{14}	172 mg	90 mg	52%
Biochanin A-4-C^{14} (284)	50 mg	Genistein-4-C^{14}	47.5 mg	20 mg	42%
Formononetin-4-C^{14} (268)	50 mg	Daidzein-4-C^{14}	47.5 mg	20 mg	42%

III.3. Identifications of the Synthesized C^{14}-labelled and Non-labelled Biochanin A, Formononetin, Genistein and Daidzein:

III.3. a. Identification of the synthesized C^{14}-labelled and non-labelled isoflavones by their melting points and their mixed melting points:

The melting points of the intermediate compounds and the final products in the syntheses of isoflavones were firstly compared with those recorded in the literature. Each final product was then mixed with its corresponding reference compound and the mixed melting point was determined.

Results and discussion: The various melting points of the intermediate compounds (Table II) were either the same or nearly the same as those recorded in the literature. The melting points of the various final products (Table II) were subsequently observed and they were in reasonable agreement with those recorded in the literature. In addition, it was also found that the mixed melting point of each sample was the same as its own melting point. This provided strong evidence that the synthesized presumptive isoflavones and their corresponding reference compounds were identical.

Table II

Comparison of the Melting Points of the Intermediate Compounds and the Final Products of the Syntheses of Isoflavones with those Reported in the Literature

Compound	Observed Melting Point	Reported Melting Point
p-Methoxybenzyl alcohol	19-21°C	19-21°C (Hodgman et al., 1961)
2,4,6-Trihydroxyphenyl p-methoxybenzyl ketone-C^{14}	194°C	192-194°C (Nilsson et al., 1961)
2,4-Dihydroxyphenyl p-methoxybenzyl ketone-C^{14}	154°C	154°C (Yoder et al., 1954)
2-Carboxy-5,7-dihydroxy-4'-methoxy isoflavone-4-C^{14}	270-272°C	270-276°C (Nilsson et al., 1961)
2-Carboxy-7-hydroxy-4'-methoxy isoflavone-4-C^{14}	260-262°C	263°C (Yoder et al., 1954)
Biochanin A-4-C^{14}	209-210°C	207-210°C (Nilsson et al., 1961)
Formononetin-4-C^{14}	252-253°C	255°C (Yoder et al., 1954)
Genistein-4-C^{14}	295-297°C	295°C (Yoder et al., 1954)
Daidzein-4-C^{14}	316-319°C	315-320°C (Mayer, 1943)

III.3. b. Identification of the synthesized C^{14}-labelled and non-labelled isoflavones by thin-layer chromatography:

Both the C^{14}-labelled and the non-labelled isoflavones were dissolved in methanol (spectranalyzed grade) at a concentration of 1 mg per ml. A 20 x 20 cm cleaned glass plate of uniform thickness was coated with Silica Gel G Merck as described in II.2. a. By means of a suitable micropipette, solutions of 6 microliters of the C^{14}-labelled isoflavones, the non-labelled isoflavones and their corresponding references were delivered in spots at a distance of 4 cm from the bottom of the plate. The spots were made as compact as possible and in no case were their diameters greater than 0.5 cm. A "finishing" line was drawn with a pointed hard pencil through the mat 16 cm away from the bottom of the plate to limit the distance of development.

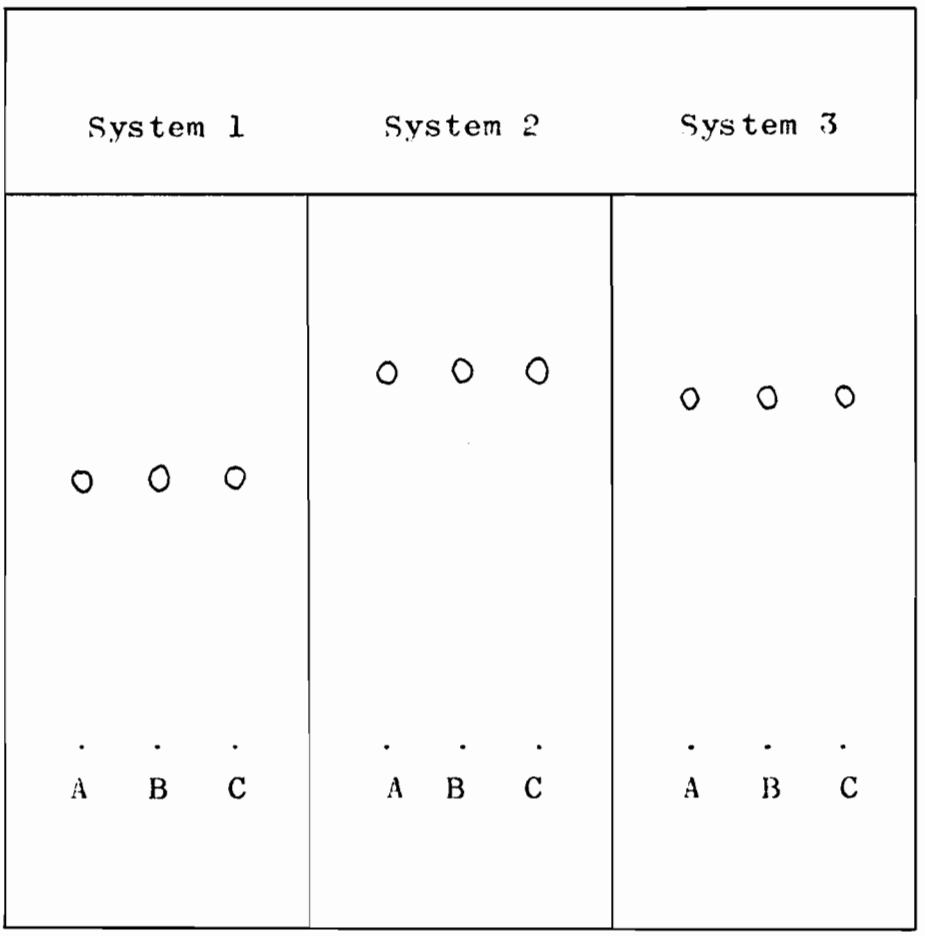
In order to economize in use of Silica Gel and to save the time for plate preparation, three different solvent systems were run on a single plate. This was done by dividing the mat into three equal columns with two straight lines drawn perpendicularly to the finishing line. Solutions of the C^{14}-labelled sample, the non-labelled sample and the corresponding reference compound were separately delivered onto one column of the mat as three equidistant isolated

spots. The reference spot was in between the spots of the synthesized products. The charged plate was subsequently developed in solvent system No.1. As soon as the solvent front had reached the finishing line (it took about 30 min.), the plate was removed from the rectangular chamber, and left to dry at room temperature. When the chromatogram was completely dry, the portion of the mat 1 cm below the application spots of the developed column of the plate was carefully scraped off, without touching any other parts of the plate. Solutions of samples and corresponding reference were spotted onto the next column and the chromatogram was developed in solvent system No.2. Care was taken at this point to ensure that the second solvent system was prevented from running over the already developed column of the mat. When the development was completed, the portion of the mat 1 cm below the application spots was removed in the same manner as before. Finally, the whole procedure was repeated once again, using the remaining column of the divided plate and employing solvent system No.3. The three solvent systems used were cyclohexane-ethyl acetate (50/50, V/V), cyclohexane-ethyl acetate-absolute ethanol (45/45/10, V/V/V) and benzene-glacial acetic acid-water (110/87/3, V/V/V). They were numbered as solvent systems No. 1, 2 and 3, respectively. After all the three columns of the plate had been developed, the spots were made visible with p-nitrobenzenediazonium

fluoroborate in 50% glacial acetic acid as a colour reagent, or located by means of fluorescence in ultra-violet light.

Results and discussion: The stained chromatograms were drawn and are shown in the accompanying Figures 2 - 5. Each of the C^{14}-labelled as well as the non-labelled samples under study appeared as a compact, discrete and non-tailing spot. This suggested that each sample had only one component as far as the resolving power of the different solvent systems employed was concerned. In addition, the C^{14}-labelled sample, the non-labelled sample and their corresponding reference of each of the four synthesized isoflavones all lined up in a straight line in each of the three solvent systems tested. This provided further evidence that the synthesized products and their corresponding references were identical. Their R_f values in the three different solvent systems are presented in Table III.

Fig. 2. Chromatogram of the non-labelled (A), the reference (B) and the labelled (C) biochanin A in three different solvent systems.

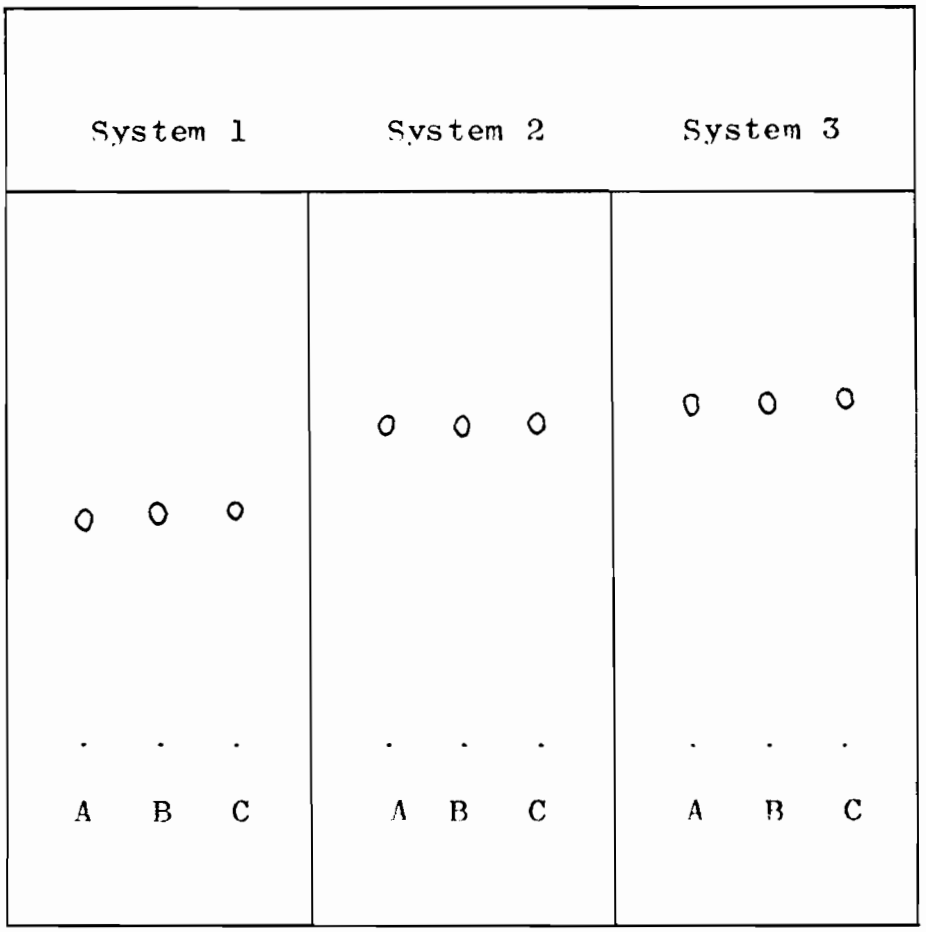


System 1: cyclohexane-ethyl acetate (50/50, V/V).

System 2: cyclohexane-ethyl acetate-absolute ethanol (45/45/10, V/V/V).

System 3: Benzene-glacial acetic acid-water (110/87/3, V/V/V).

Fig. 3. Chromatogram of the non-labelled (A), the reference (B) and the labelled (C) formononetin in three different solvent systems.

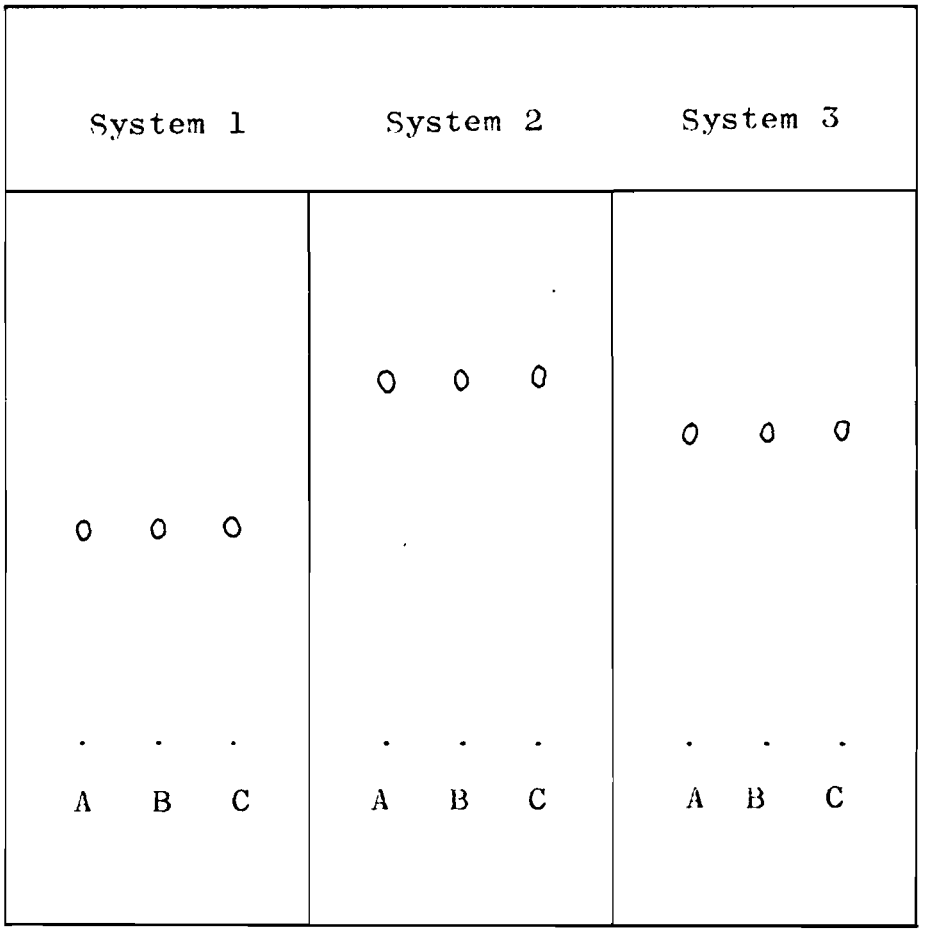


System 1: cyclohexane-ethyl acetate (50/50, V/V).

System 2: cyclohexane-ethyl acetate-absolute ethanol (45/45/10, V/V/V).

System 3: Benzene-glacial acetic acid-water (110/87/3, V/V/V).

Fig. 4. Chromatogram of the non-labelled (A), the reference (B) and the labelled (C) genistein in three different solvent systems.

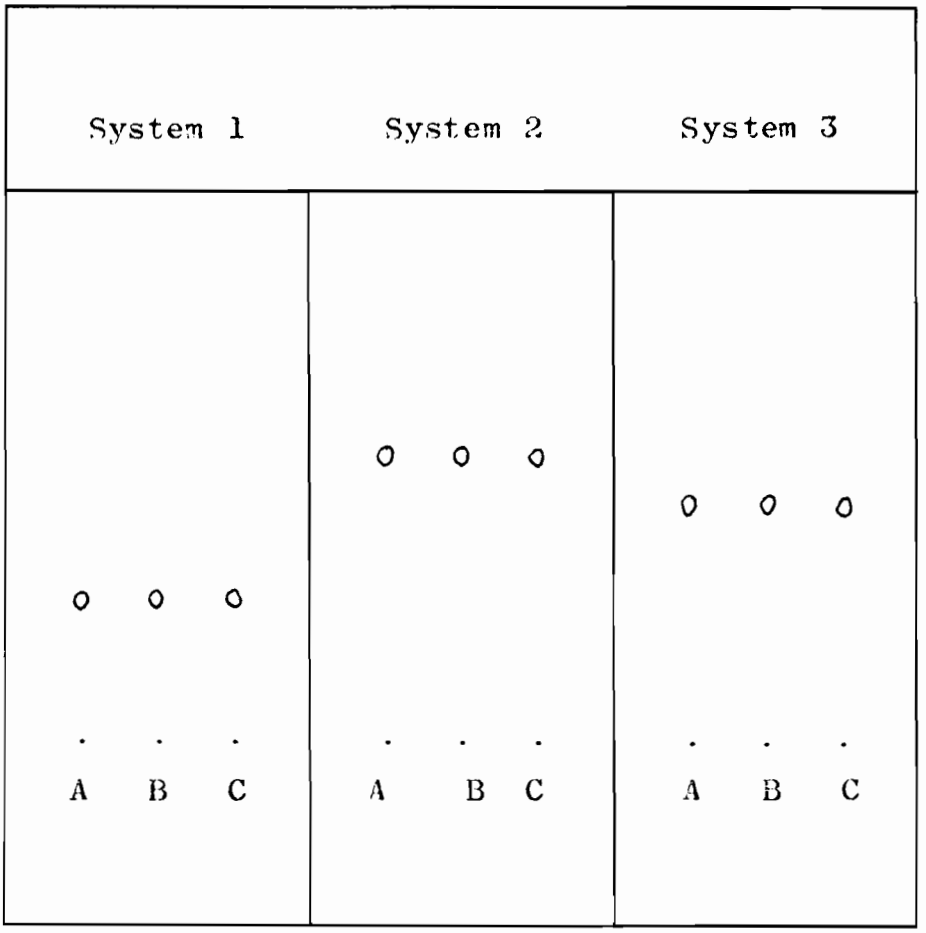


System 1: cyclohexane-ethyl acetate (50/50, V/V).

System 2: cyclohexane-ethyl acetate-absolute ethanol (45/45/10, V/V/V).

System 3: Benzene-glacial acetic acid-water (110/87/3, V/V/V).

Fig. 5. Chromatogram of the non-labelled (A), the reference (B) and the labelled (C) daidzein in three different solvent systems.



System 1: cyclohexane-ethyl acetate (50/50, V/V).

System 2: cyclohexane-ethyl acetate-absolute ethanol (45/45/10, V/V/V).

System 3: Benzene-glacial acetic acid-water (110/87/3, V/V/V).

Table III

R_f values

of the Synthesized Isoflavones and their Colour Reactions with p-Nitrobenzenediazonium Fluoroborate in 50% glacial Acetic Acid

Compound	R_f value System 1	System 2	System 3	Colour with NBDF
Biochanin A	0.48	0.68	0.63	pinkish
Formononetin	0.42	0.57	0.62	-
Genistein	0.38	0.65	0.56	brown
Daidzein	0.26	0.52	0.43	yellow

III.3. c. Determination of the specific activities of the synthesized C^{14}-labelled isoflavones:

The synthesized C^{14}-labelled material was dissolved in spectranalyzed grade methanol and made up to 5 ml or 10 ml. A suitable aliquot of this solution was then diluted with methanol so as to give a solution containing an amount of the order of 1 mg per ml. For each sample, five 10 microliters of the diluted solution were measured with a microsyringe (Hamilton Company Incorporated P.O. Box 307, Whittier, California, U. S. A.) and transferred into five

standard screw-top glass scintillation vials. Ten ml of scintillation fluid was subsequently pipetted into each vial and mixed gently. Each sample was then counted five times for one minute in a Packard Tri-Carb liquid scintillation spectrometer, Series 314E. Blanks were also prepared with 10 microliters spectranalyzed methanol replacing the sample solution.

Results and discussion: The mean of the five trials in each vial and the mean of five vials for each sample were calculated. The total mean was then expressed in counts per minute. Corrections for the blank and the spectrometer efficiency were made. The specific activity of each of the four synthesized C^{14}-labelled isoflavones was determined and the results are tabulated below (Table IV).

Table IV

Specific Activities of the Synthesized C^{14}-labelled Isoflavones

Compound	Specific Activity
Biochanin A-4-C^{14}	364 microcurie/gm
Formononetin-4-C^{14}	225 microcurie/gm
Genistein-4-C^{14}	69.5 microcurie/gm
Daidzein-4-C^{14}	214 microcurie/gm

In the preparation of genistein-4-C^{14}, one microcurie of isotopic carbon in the form of potassium cyanide (3.8 mg) was employed to react with p-methoxybenzyl chloride, while in the syntheses of formononetin-4-C^{14}, daidzein-4-C^{14} and biochanin A-4-C^{14}, triple the foregoing amount of isotopic carbon was introduced into the reaction mixture. It was, therefore, logical to expect that the specific activities of formononetin-4-C^{14} and daidzein-4-C^{14} should be roughly three times greater than that of genistein-4-C^{14}. The difference in the specific activities of the synthesized biochanin A-4-C^{14} and formononetin-4-C^{14}, however, called for an explanation. It was probably due to the fact that the weight of the C^{14}-marked potassium cyanide used was different, although the radioactivity was the same. In the course of the radioactive biochanin A synthesis, 3 x 7.3 mg of C^{14}-marked potassium cyanide was introduced whereas only 3 x 3.8 mg of potassium cyanide with isotopic carbon was used for the synthesis of formononetin-4-C^{14}. Accordingly, the specific activity of the radioactive potassium cyanide employed in the synthesis of formononetin-4-C^{14} was higher than that of the radioactive potassium cyanide used in the preparation of biochanin A-4-C^{14}. Thus if an equal amount of the radioactive cyanide was lost during synthesis, there would be a greater drop in the final specific activity of formononetin-4-C^{14} than in that of biochanin A-4-C^{14}.

In view of this, the specific activity of biochanin A-4-C^{14} would be expected to be somewhat higher than that of formononetin-4-C^{14}, as realized experimentally in this study.

III.3. d. Identification of the synthesized C^{14}-labelled and non-labelled isoflavones by paper chromatography:

A three-armed paper strip was prepared as described in II.2. b. An interrupted line of application was drawn across the "arms" of the paper strip and was 10 cm from the intact end. A length of 40 cm was measured and a finishing line was drawn to define the distance of development. Twenty micrograms of one of the four synthesized non-labelled samples in methanol was applied to the outside narrow arm, while the same amount of its reference compound was transferred to the middle narrow arm. Both arms were about 1 cm in width. An amount of 80 to 100 micrograms of the synthesized corresponding C^{14}-labelled isoflavone was evenly distributed along the line of application on the wide arm of the paper strip which was about 3 cm in width. These three arms were partially separated by cutting away narrow strips between each of them. In order to evaporate the methanol solution as quickly as possible and consequently to prevent the solution from spreading wide apart, a draught was maintained during application.

For each sample, two paper chromatograms were developed,

one in each of the two solvent systems: the monophasic benzene-acetic acid-water (125/72/3, V/V/V) and the chloroform-formamide (1/1, V/V). In both cases, descending technique was employed. The detail for the developing procedure has been described in II.2. b.

As soon as the solvent front had reached the finishing line, the paper strip was removed from the rectangular chromatographic chamber. It was then dried in room temperature. The arms were separated by cutting the intact portion of the paper strip. A pilot strip, about 1 cm wide was then cut longitudinally along the wide arm of the chromatogram. The two narrow arms and the pilot strip of the wide arm were all stained with diazotized sulphanilic acid, for the location of the sample under test. The DSA-stained strips of each sample were then scanned with a densitometer.

Results and discussion: Biochanin A, genistein and daidzein showed up as distinctive bands along the chromatostrip after staining. Formononetin, on the other hand, did not react with the colour reagent. However, it gave a bluish fluorescence under ultra-violet light. The densitometer readings of each of the two narrow strips and the pilot strip from the same chromatogram were plotted individually against their respective lengths of the chromatostrip. The resulting graphs are presented in Figures 6 - 11. The

relevant R_f values and colour reactions with diazotized sulphanilic acid are tabulated below (Table V).

Table V

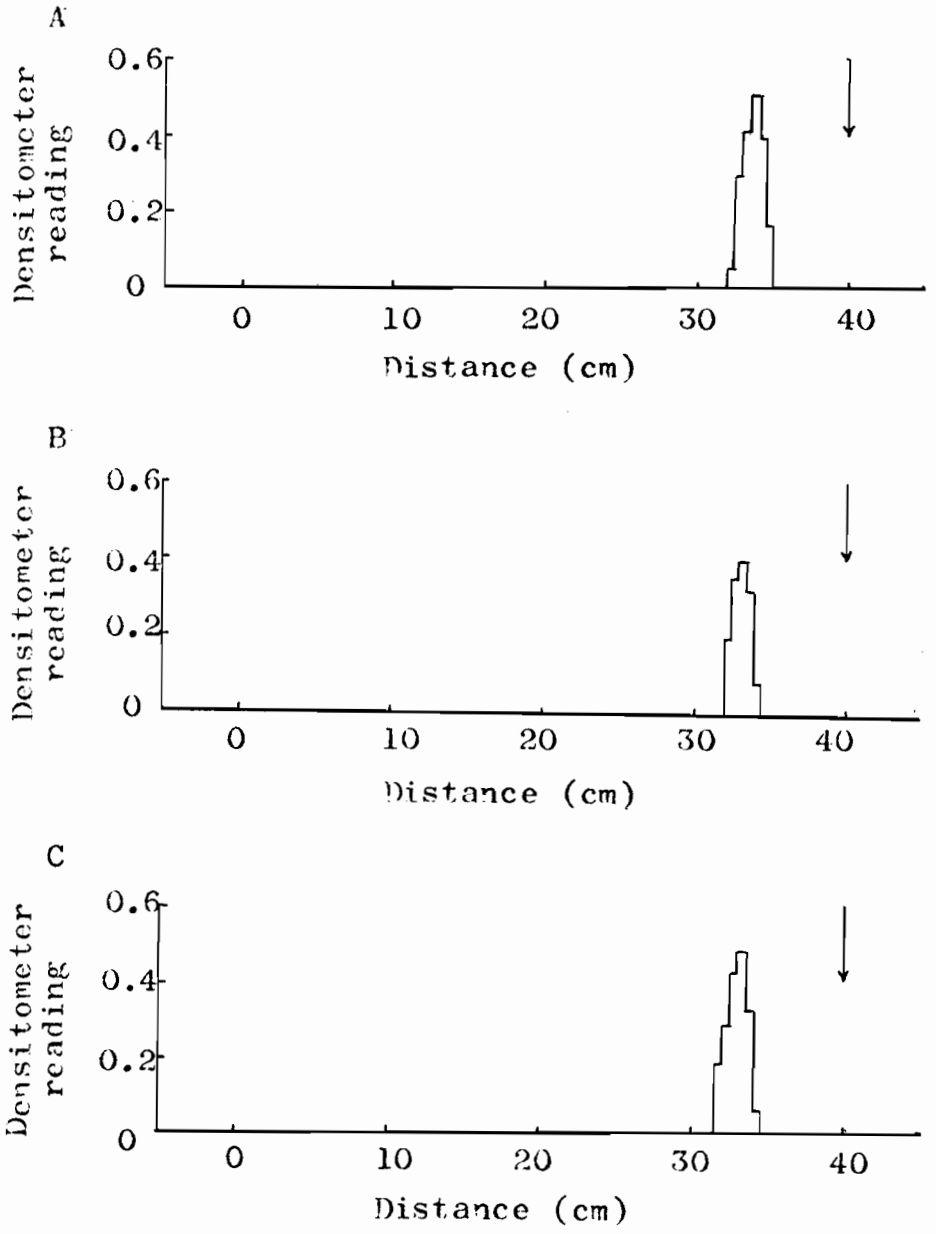
R_f values of the Synthesized Isoflavones and their Colour Reactions with Diazotized Sulphanilic Acid

Compound	R_f values		Colour with DSA
	System 1	System 2	
Biochanin A	0.84	0.36	yellow
Formononetin	0.78	0.24	-
Genistein	0.49	0.025	brown
Daidzein	0.37	0.0125	pink

Solvent system 1: Benzene-acetic acid-water (125/72/3, V/V/V)

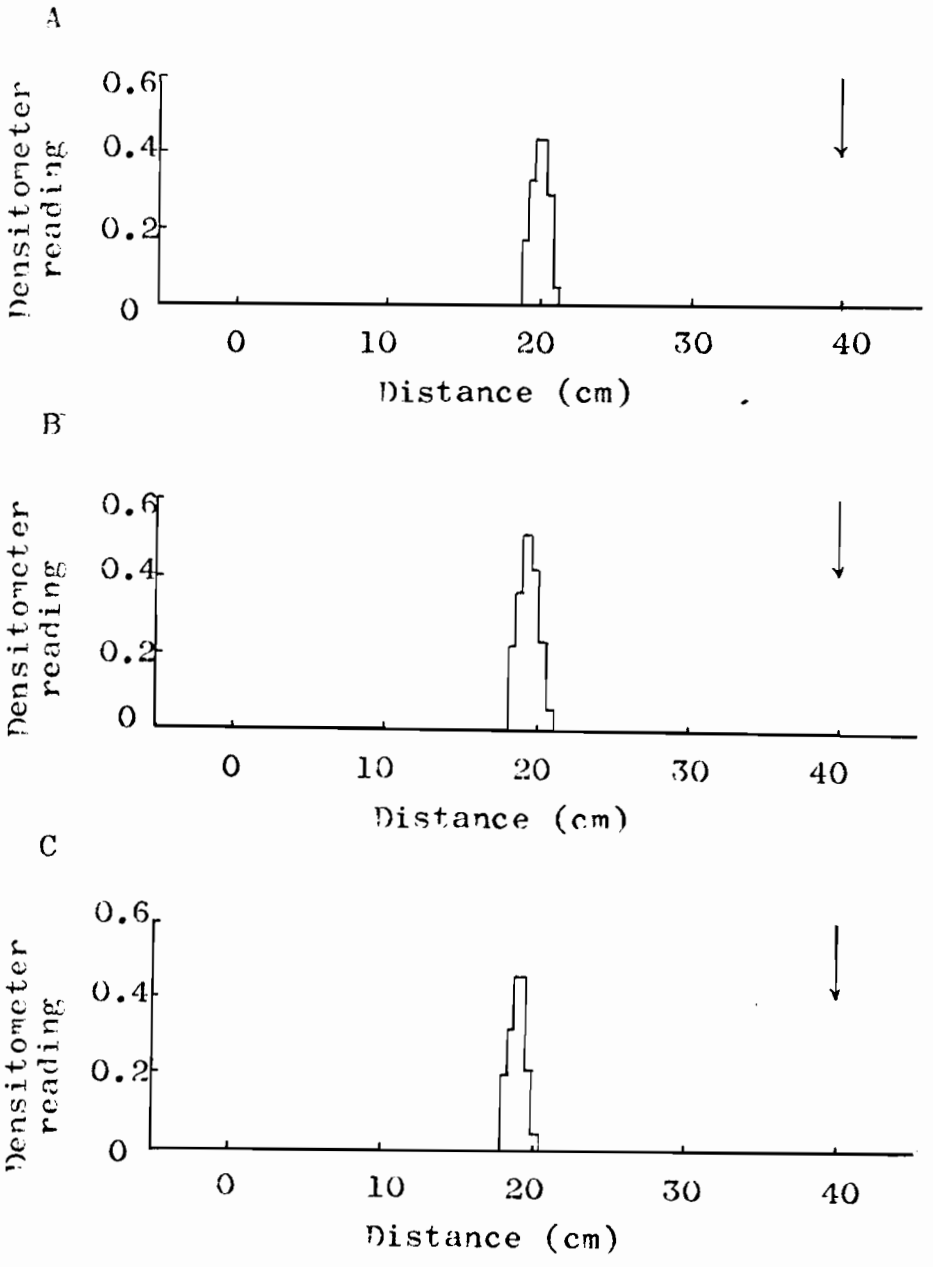
Solvent system 2: Chloroform-formamide (1/1, V/V)

Figure 6. Distribution of staining (DSA) on paper chromatogram of the non-labelled (A), the reference (B) and the labelled (C) biochanin A run in the benzene-acetic acid-water solvent system.



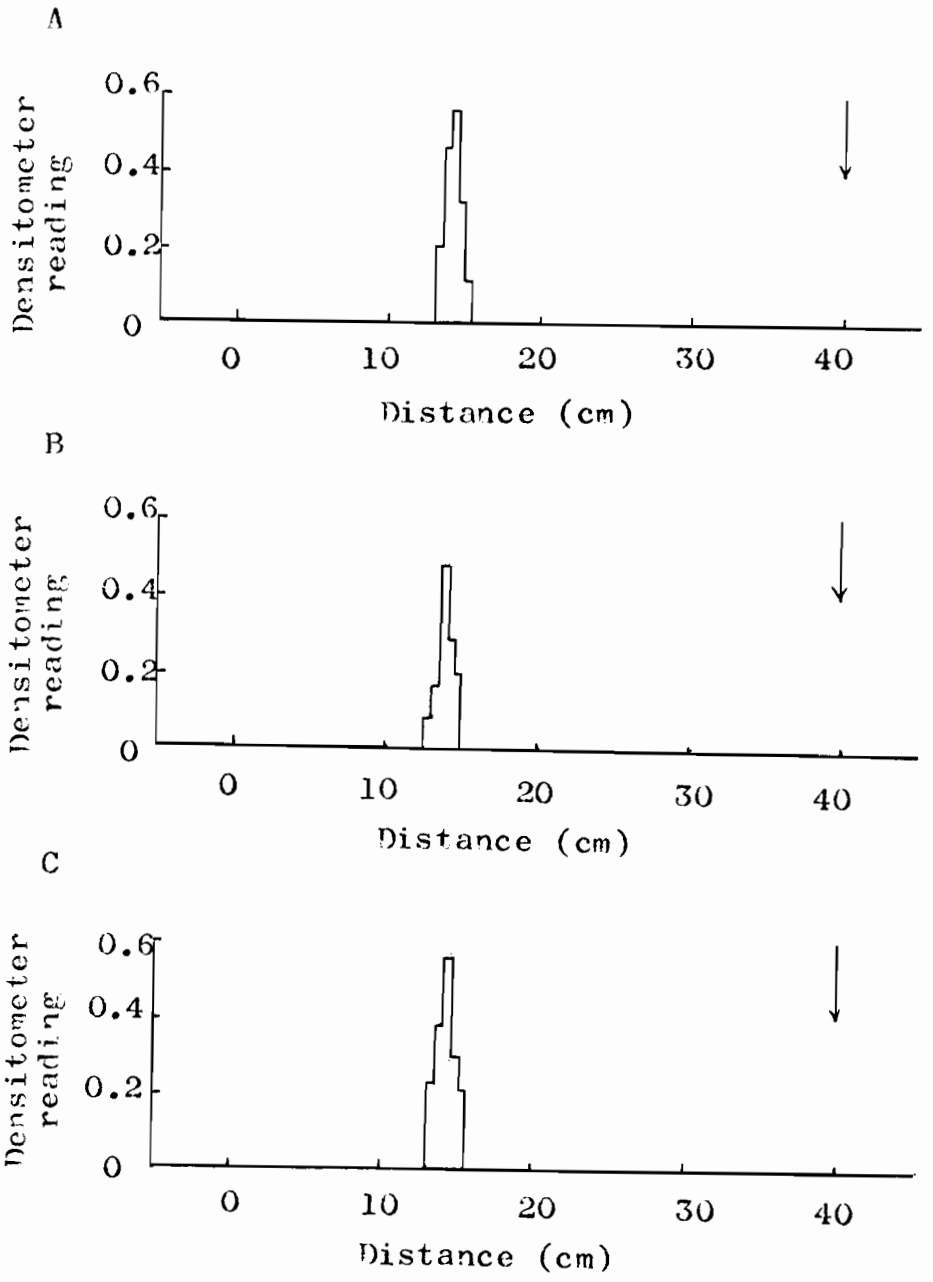
Vertical arrows indicate the position of the solvent front

Figure 7. Distribution of staining (DSA) on paper chromatogram of the non-labelled (A), the reference (B) and the labelled (C) genistein run in the benzene-acetic acid-water solvent system.



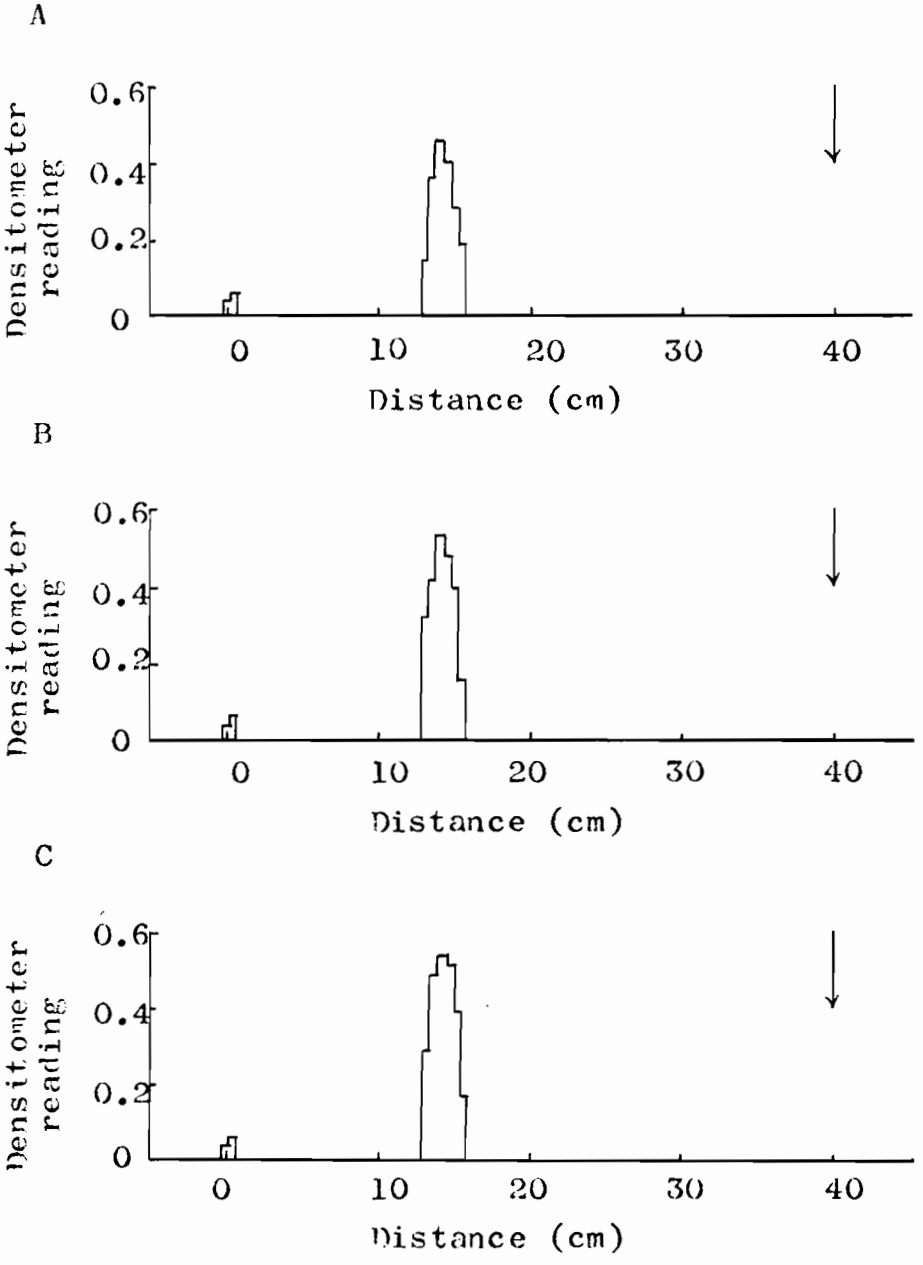
Vertical arrows indicate the position of the solvent front

Figure 8. Distribution of staining (DSA) on paper chromatogram of the non-labelled (A), the reference (B) and the labelled (C) daidzein run in the benzene-acetic acid-water solvent system.



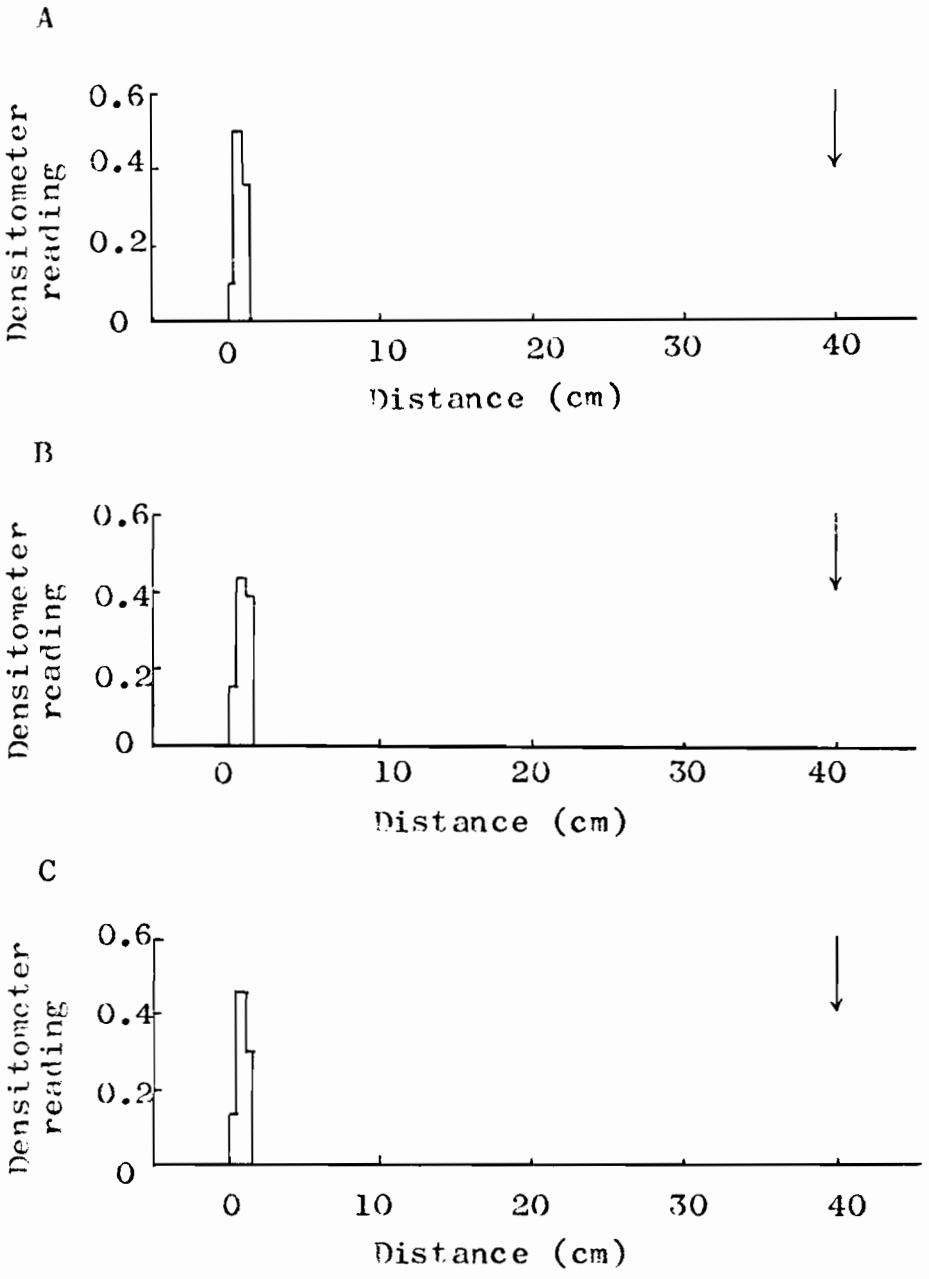
Vertical arrows indicate the position of the solvent front

Figure 9. Distribution of staining (DSA) on paper chromatogram of the non-labelled (A), the reference (B) and the labelled (C) biochanin A run in the chloroform-formamide solvent system.



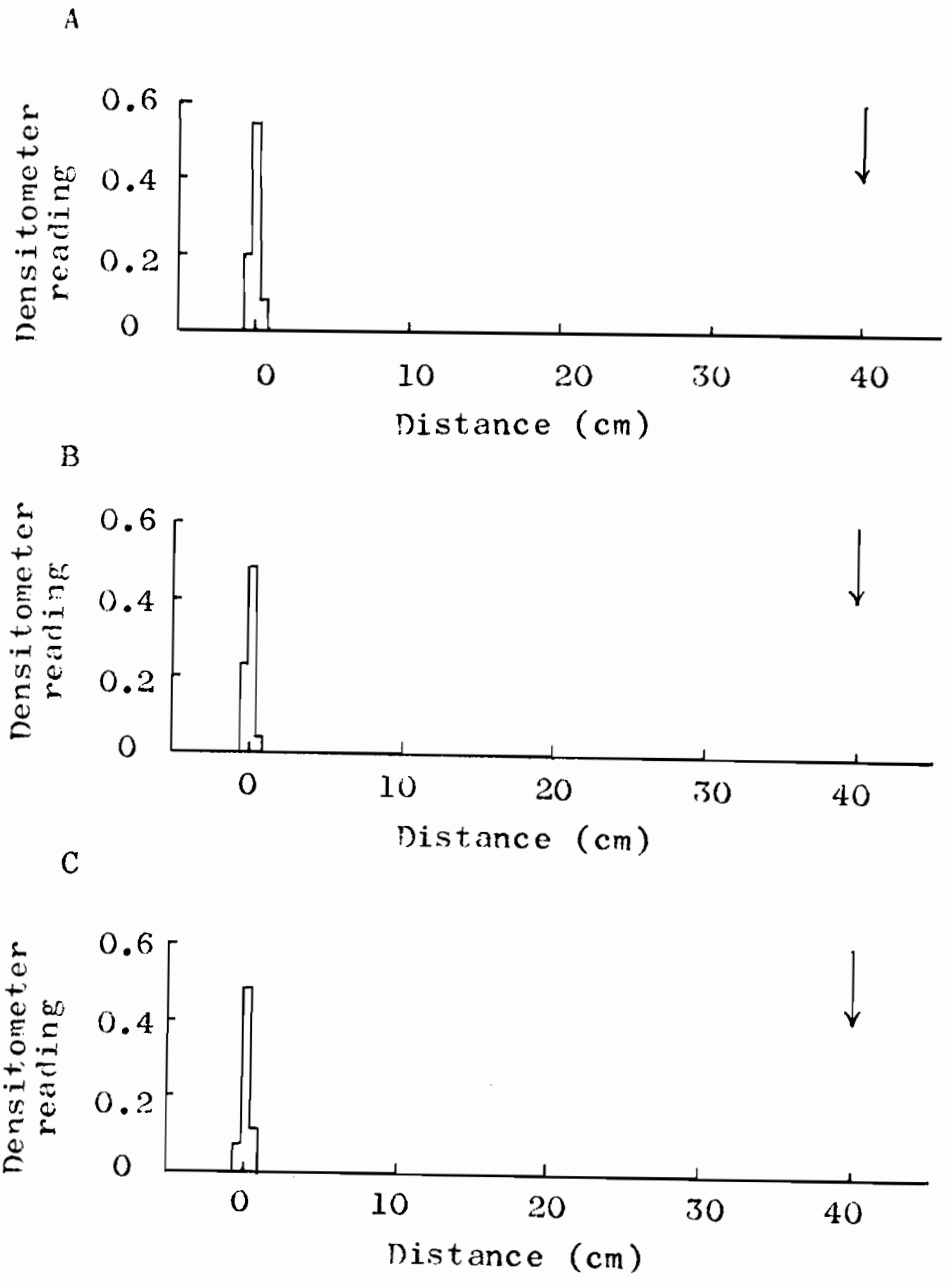
Vertical arrows indicate the position of the solvent front

Figure 10. Distribution of staining (DSA) on paper chromatogram of the non-labelled (A), the reference (B) and the labelled (C) genistein run in the chloroform-formamide solvent system.



Vertical arrows indicate the position of the solvent front

Figure 11. Distribution of staining (DSA) on paper chromatogram of the non-labelled (A), the reference (B) and the labelled (C) daidzein run in the chloroform-formamide solvent system.



Vertical arrows indicate the position of the solvent front

Since there was only one single symmetrical high peak in every strip of the DSA-sensitive samples studied, each of these compounds could be said to comprise only one component as far as the resolving power of the solvent systems and the staining power of the colour reagent were concerned. Furthermore, since the locations of the peaks of the non-labelled as well as the C^{14}-labelled isoflavones corresponded to the locations of the peaks of their respective reference compounds, their R_f values were the same. This fact provided further evidence that the synthesized isoflavones were identical to their respective reference compounds. In the case of formononetin, which was DSA-negative, its band was located by means of its fluorescence in ultra-violet light.

III.3. e. Determination of the radioactive purity of the synthesized C^{14}-labelled isoflavones:

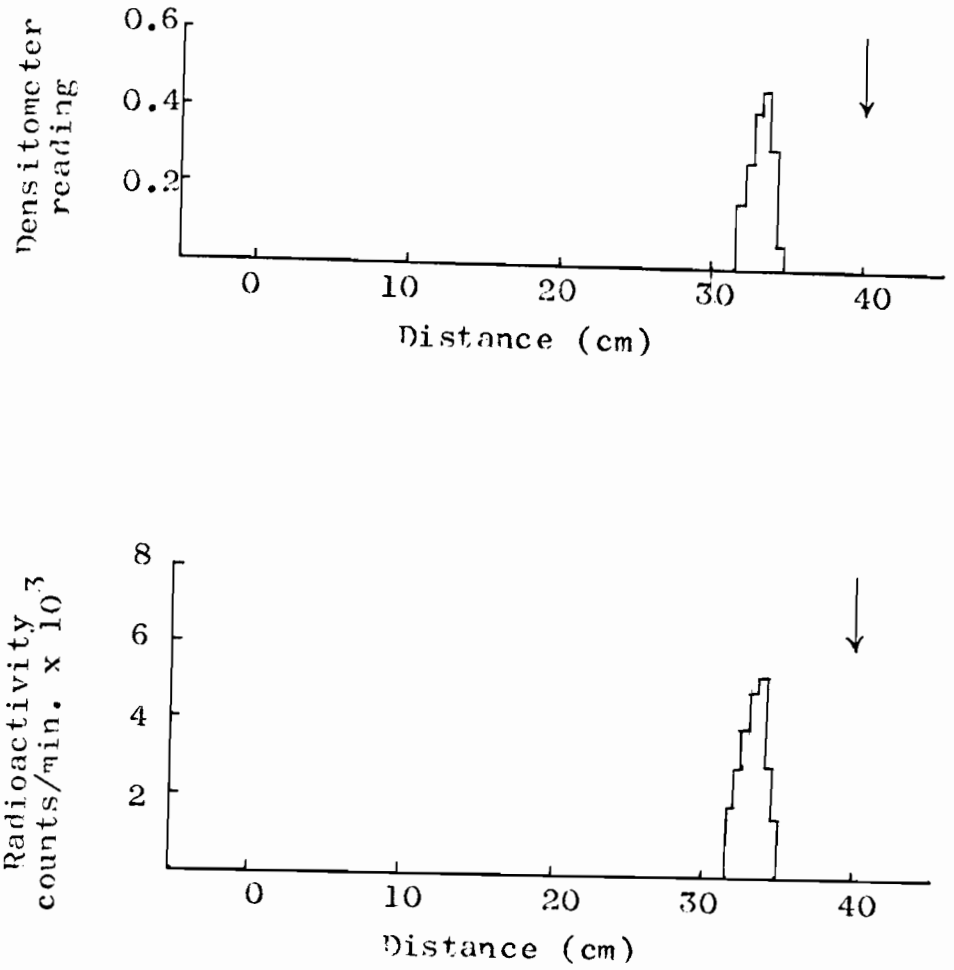
After the pilot strip had been cut away, the remaining portion of the wide arm was divided into ½ cm bands along the chromatostrip as described in II.2. c. Four ½ cm bands just in front of the application line and another four immediately beyond the solvent front were also cut. The chromatostrip proper, being 40 cm in length, was divided accordingly into 80 equal bands. For each chromatostrip, eighty-eight ½ cm bands were consequently prepared for the determination of

the radioactivity distribution, and thus the radioactive purity of the synthesized C^{14}-labelled isoflavones could be perceived. Each band was placed into a separate standard screw-top glass scintillation vial which was numbered beforehand. Spectranalyzed methanol and scintillation fluid were added to each vial in the same manner as described in II.2. c. The radioactivity of the contents of each loaded vial was determined by counting in a Packard Tri-Carb liquid scintillation spectrometer. The registered number of counts per minute was then plotted against the length of the paper chromatostrip.

Results and discussion: A radioactivity distribution graph of each of the four synthesized C^{14}-labelled isoflavones is shown in the accompanying figures 12 - 19. Each graph displayed a single symmetrical high peak for each of the samples studied. These observations provided evidence that the synthesized compounds were radiochemically pure. Moreover, the radioactive peak of each C^{14}-labelled sample corresponded in location to the staining peak of the pilot strip. The stained band of the latter, in turn corresponded to that of the reference compound. Therefore it was reasonable to conclude that all the radioactivity of the isoflavone solutions was from the C^{14}-labelled isoflavones themselves, and not from any other compounds. The above information,

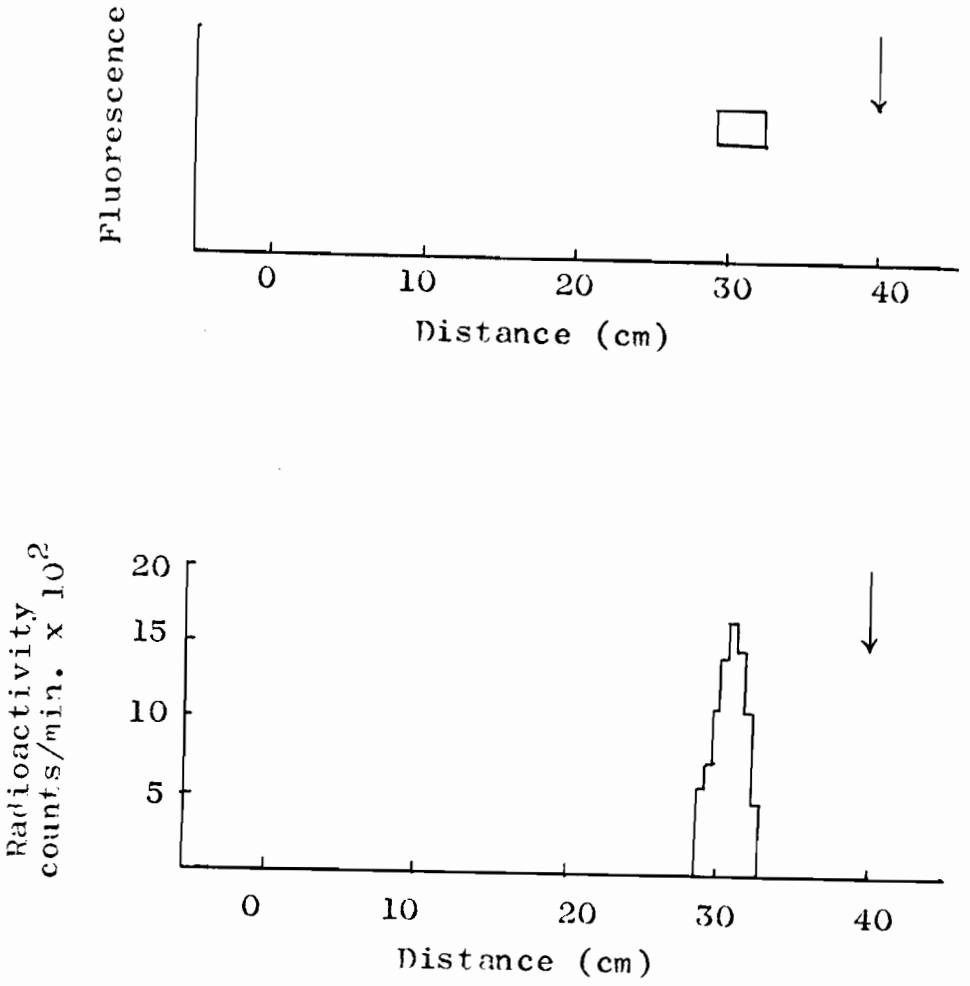
together with the evidence set out in the previous parts of this section, permitted the conclusion that all four synthesized C^{14}-labelled compounds were radiochemically pure isoflavones.

Figure 12. Distribution of staining (DSA) and radioactivity on paper chromatogram of the synthesized labelled biochanin A-4-C^{14} run in the benzene-acetic acid-water solvent system.



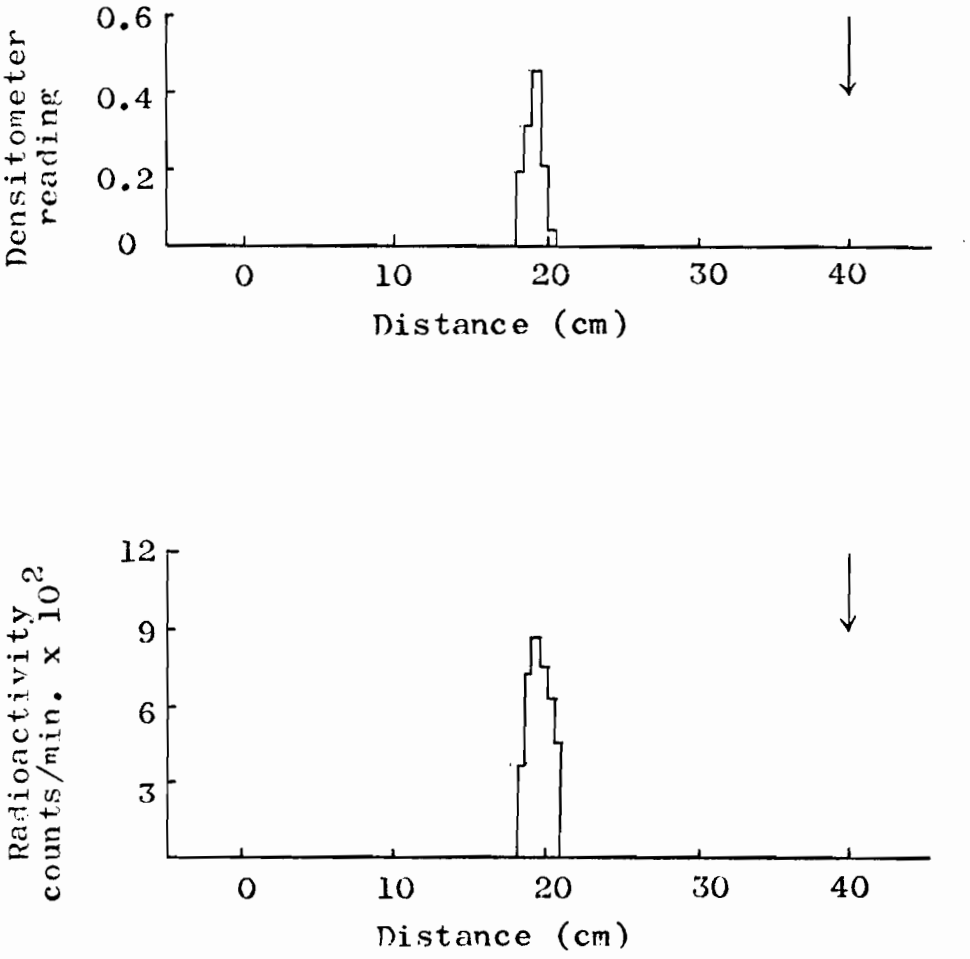
Vertical arrows indicate the position of the solvent front

Figure 13. Distribution of fluorescence and radioactivity on paper chromatogram of the synthesized labelled formononetin-4-C^{14} run in the benzene-acetic acid-water solvent system.



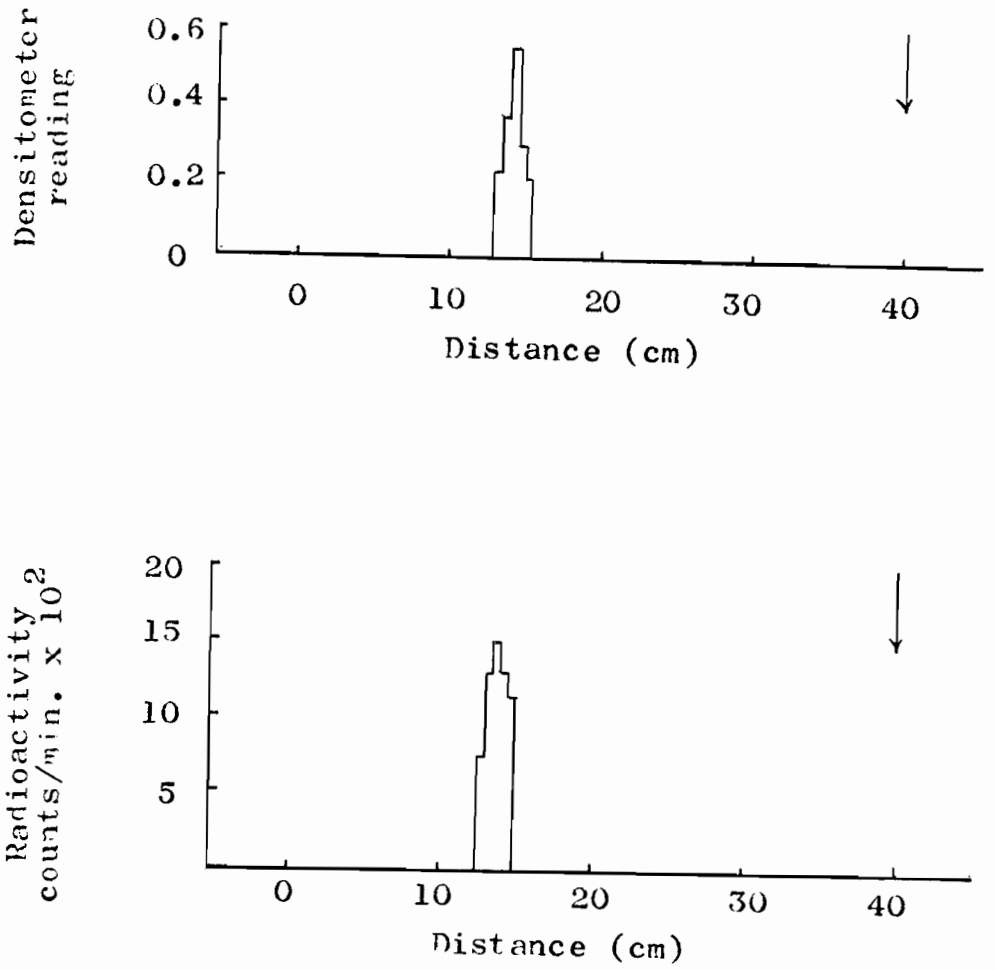
Vertical arrows indicate the position of the solvent front

Figure 14. Distribution of staining (DSA) and radioactivity on paper chromatogram of the synthesized labelled genistein-4-C^{14} run in the benzene-acetic acid-water solvent system.



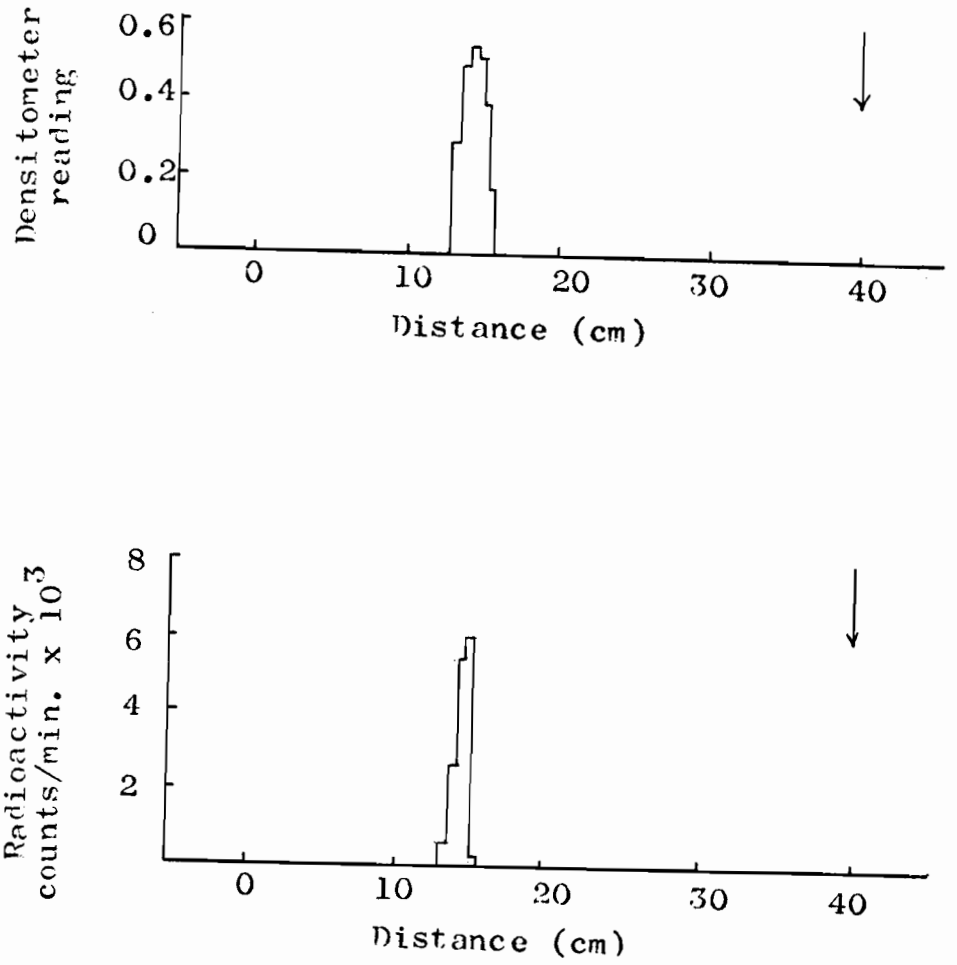
Vertical arrows indicate the position of the solvent front

Figure 15. Distribution of staining (DSA) and radioactivity on paper chromatogram of the synthesized labelled daidzein-4-C^{14} run in the benzene-acetic acid-water solvent system.



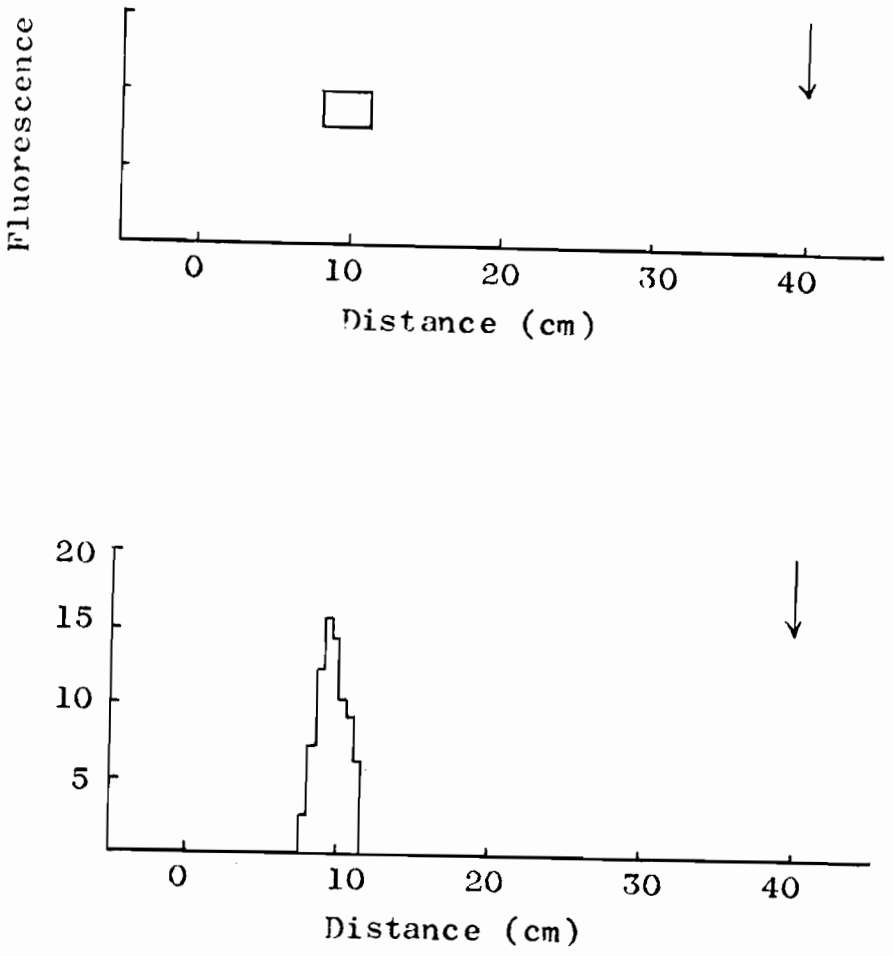
Vertical arrows indicate the position of the solvent front

Figure 16. Distribution of staining (DSA) and radioactivity on paper chromatogram of the synthesized labelled biochanin A-4-C^{14} run in the chloroform-formamide solvent system.



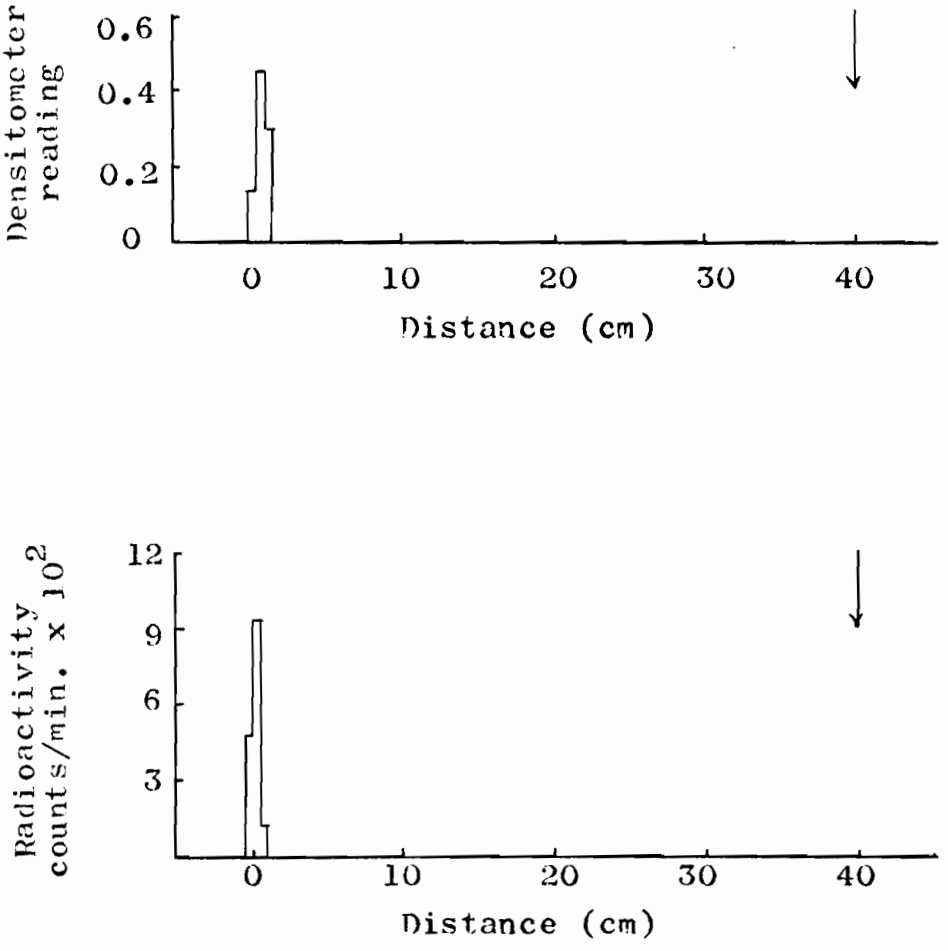
Vertical arrows indicate the position of the solvent front

Figure 17. Distribution of fluorescence and radioactivity on paper chromatogram of the synthesized labelled formononetin-4-C^{14} run in the chloroform-formamide solvent system.



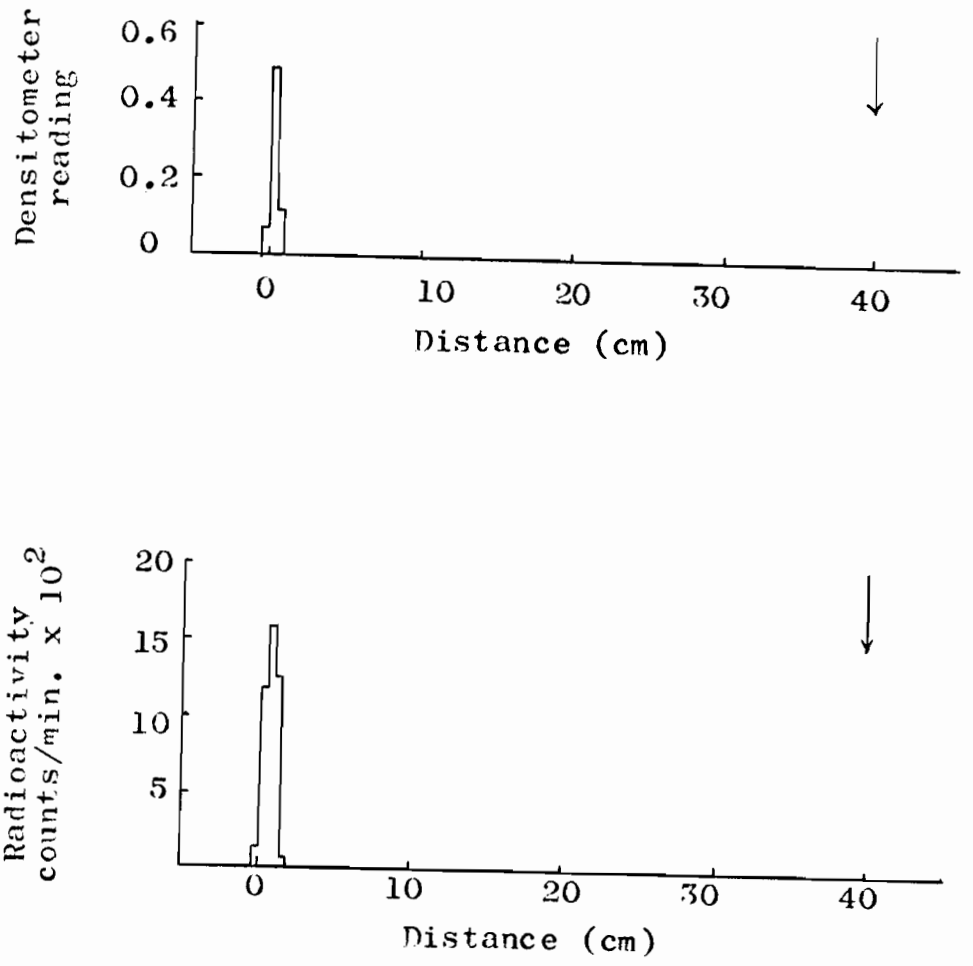
Vertical arrows indicate the position of the solvent front

Figure 18. Distribution of staining (DSA) and radioactivity on paper chromatogram of the synthesized labelled genistein-4-C^{14} run in the chloroform-formamide solvent system.



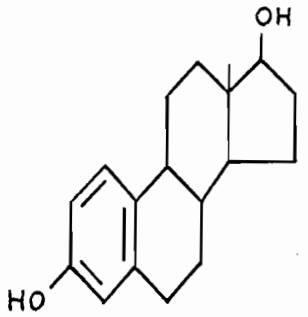
Vertical arrows indicate the position of the solvent front

Figure 19. Distribution of staining (DSA) and radioactivity on paper chromatogram of the synthesized labelled daidzein-4-C^{14} run in the chloroform-formamide solvent system.

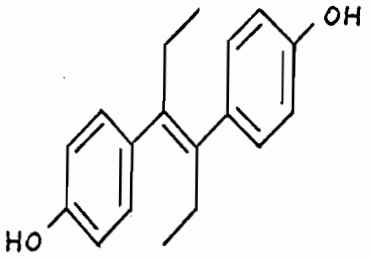


Vertical arrows indicate the position of the solvent front

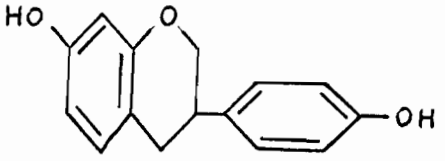
Figure 20. Structural formulae of some of the important compounds discussed.



Oestradiol



Diethylstilbestrol



Equol

SUMMARY

Detailed procedures have been presented for the syntheses of the naturally-occurring isoflavones, biochanin A, formononetin, genistein and daidzein. The various steps in the syntheses were based on the methods adopted by Yoder *et al.* (1954) and Nilsson *et al.* (1961a). For all the isoflavones synthesized, the starting chemical was anisaldehyde, p-methoxybenzyl aldehyde. The isotope used was C^{14} in the form of potassium cyanide, which was introduced into the cyano group of p-methoxybenzyl cyanide, one of the intermediate products. The labelled carbon was finally sited in the C-4 position of the resulting isoflavones.

Commercially available anisaldehyde was first converted to p-methoxybenzyl alcohol by the crossed Cannizzaro reaction. The alcohol was then transformed into p-methoxybenzyl chloride by direct reaction with concentrated hydrochloric acid. The resulting p-methoxybenzyl chloride was refluxed with the C^{14}-labelled potassium cyanide in dried acetone to yield the radioactive p-methoxybenzyl cyanide. Through the Hoesch condensation of the p-methoxybenzyl cyanide with phloroglucinol or resorcinol, crystalline 2,4,6-trihydroxy- and 2,4-dihydroxy- phenyl-p-methoxybenzyl ketones were formed, respectively. The inner pyrone ring formation of the isoflavones was then achieved by the Baker-Ollis cyclization

reaction in the presence of ethyl oxalyl chloride. The carbethoxy isoflavones thus obtained were hydrolyzed to the corresponding carboxy acids. Decarboxylation of the carboxy acids by pyrolysis yielded the isoflavones desired. Biochanin A-4-C^{14} and formononetin-4-C^{14} were thus synthesized.

Genistein-4-C^{14} was prepared by demethylation of biochanin-A-4-C^{14} with hydriodic acid (specific gravity 1.7), while daidzein-4-C^{14} was obtained similarly through the demethylation of formononetin-4-C^{14}. For the syntheses of the non-labelled isoflavones, the same routes were followed, except no radioactive potassium cyanide was introduced into the chloride-cyanide refluxing mixture.

All eight synthesized products, four C^{14}-labelled and four non-labelled, were characterized by the comparison of their melting points with that given in the literature. Mixed melting points were also determined to confirm the identity of each synthesized product with its reference compound.

Thin-layer chromatographic technique which was believed to have adequate resolving power was employed to check the chemical purity of the products, while paper chromatographic technique was used with the purposes of checking the chemical purity as well as the radiochemical purity of the four synthesized isoflavones.

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