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OCCURRENCE OF METABOLICALLY-ACTIVE BOUND FORMS OF CINNAMIC ACID AND ITS PHENOLIC DERIVATIVES IN ACETONE POWDERS OF WHEAT AND BARLEY PLANTS*

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Abstract—Acetone powders of wheat and barley shoots contain bound forms of cinnamic, *p*-coumaric, caffeic and ferulic acids. The free acids can be released by an enzymatic autolysis as well as by the action of acids and alkali. The bound forms can be extracted by cold buffer and a preliminary fractionation by ammonium sulphate precipitation and gel filtration on Sephedax shows some association with the protein fractions. Photosynthesis in $^{14}CO_2$ (2 hr) or feeding of ^{14}C-labelled phenylalanine prior to preparation of the acetone powder caused a rapid labelling of the bound cinnamic acids thus showing them to be metabolically active. Analysis of the degree of labelling supports the sequence:

cinnamic acid→*p*-coumaric acid→caffeic acid→ferulic acid.

Differential autolysis showed that several bound forms of cinnamic and ferulic acid were present. It is suggested the actual pool of phenylpropanoid intermediates involved in the biosynthesis of lignin and related compounds is to be found in these acetone-insoluble cinnamic acid derivatives.

INTRODUCTION

IT HAS been well established that esters, and to a lesser extent glycosides, of the hydroxylated cinnamic acids, *p*-coumaric, caffeic, ferulic, and sinapic acids, are widely distributed in vascular plants.[1,2] In contrast cinnamic acid itself, which is the parent molecule, seems to have a very restricted distribution. It has been recorded to occur as the free acid or its methyl ester in essential oils and resins of a few plant species.[3,4] In a previous paper of this series[5] we have shown that several unidentified esters of hydroxylated cinnamic acids occur in ethanolic extracts of wheat. In addition, much larger quantities of the acids were found to occur in ethanol-insoluble forms from which they could be liberated by mild alkaline hydrolysis.

Numerous tracer and enzyme studies[6] have implicated the phenolic cinnamic acids as key intermediates in the biosynthesis of lignin and flavonoid compounds. Further work from this laboratory[7] has indicated that the actual intermediates in lignin biosynthesis in wheat are

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[1] E. C. BATE-SMITH, In *Wood Extractives* (Edited by W. E. HOLLIS, p. 136. Academic Press, New York (1962).

[2] R. E. KREMERS, *Tappi* **40**, 262 (1957).

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[4] W. VON MILLER, *Ann. Chem.* **188**, 184 (1877).

[5] SAID Z. EL-BASYOUNI and G. H. N. TOWERS, *Can. J. Biochem.* **42**, 203 (1964).

[6] A. C. NEISH, In *Formation of Wood in Forest Trees* (Edited by M. ZIMMERMANN), p. 219. Academic Press, New York (1964).

[7] SAID Z. EL-BASYOUNI, A. C. NEISH and G. H. N. TOWERS, *Phytochem.* **3**, 627 (1964).

the forms of hydroxycinnamic acids which are insoluble in ethanol. It was also evident that compartmentation of the hydroxycinnamic acids occurs in both the ethanol-soluble and the ethanol-insoluble pools and that only a small fraction of the latter pool is actually involved in the biosynthetic process. The present report provides further confirmation for the earlier results and describes the isolation of cinnamic acid itself, from a metabolically-active bound form.

RESULTS AND DISCUSSION

In previous work hot 80% ethanol was used to extract soluble phenolic compounds and the insoluble residue was investigated for phenolic acids released on acid or alkaline hydrolysis.[7] In the present investigation the plant material was extracted with cold acetone in order to maintain enzymatic activity in the residue.

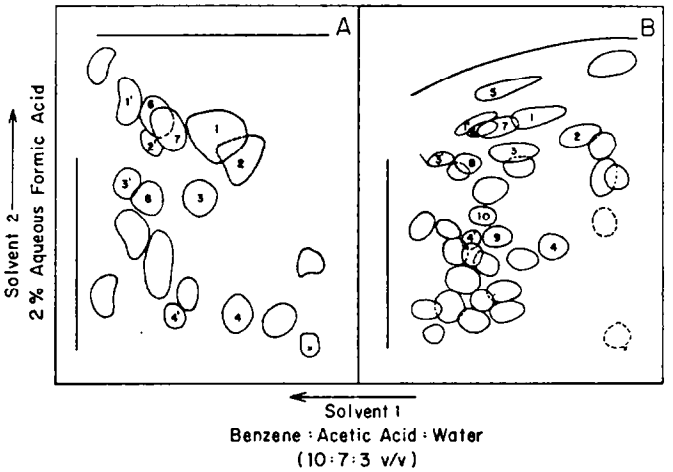

FIG. 1. PAPER CHROMATOGRAMS OF ETHER-SOLUBLE ACIDS OBTAINED BY ALKALINE HYDROLYSIS OF AN ACETONE EXTRACT OF WHEAT SEEDLING SHOOTS (A) AND BY AUTOLYSIS OF THE ACETONE POWDER (B).
1,1′=Ferulic acid; 2,2′=sinapic acid; 3,3′=*p*-coumaric acid; 4,4′=caffeic acid; 5=cinnamic acid; 6=vanillic acid; 7=syringic acid; 8=*p*-hydroxybenzoic acid; 9=protocatechuic acid; 10=gentisic acid. The remaining spots were not identified.

Cold acetone extraction removes a variety of phenolic substances (Fig. 1A) and is similar to hot ethanol in this respect. The insoluble residue from cold acetone extraction was found to undergo autolysis, during incubation at room temperature in a buffer at pH 7·4, thereby releasing cinnamic acid and its hydroxy derivatives as well as a variety of other phenolic substances (Fig. 1B). This autolysis is probably due to enzymes since acetone powders inactivated by heating the incubation mixture in a boiling water bath for 10 min do not exhibit this phenomenon. Small amounts of *p*-coumaric and ferulic acids are released on heating but do not increase on further incubation of the heat-treated material at room temperature. Apparently these plant materials contain a wide variety of acetone-insoluble phenolic compounds that are susceptible to hydrolysis by enzymes present in the plant.

Cinnamic acid has not been previously identified from these sources. The spot ascribed to cinnamic acid (No. 5, Fig. 1B) was identified by its u.v. absorption spectrum and by co-chromatography and co-crystallization of authentic cinnamic acid mixed with a ^{14}C-labelled sample isolated from plants following photosynthesis in $^{14}CO_2$.

It was observed that buffer extraction of acetone powders at 0° removed phenolic substances soluble in both water and ether (Table 1). These low-temperature buffer extracts do not contain free cinnamic, *p*-coumaric, caffeic, ferulic or sinapic acids. However these acids are released on acid or alkaline treatment of the buffer extract, presumably by hydrolysis of the unknowns listed in Table 1, as well as from some minor components also present in the low-temperature buffer extracts. These unknowns are absent or present in very small

TABLE 1. CHROMATOGRAPHIC DATA OF MAJOR ETHER-SOLUBLE UNKNOWNS EXTRACTED FROM WHEAT ACETONE POWDER BY AN AQUEOUS SOLUTION AT LOW TEMPERATURE*

Compound	R_f on paper†		Behaviour in u.v. light‡	Colour with fast bordeaux salt B.D.§
	Solvent 1	Solvent 2		
A	0·83	0·70	absorbs	purple–brown
B	0·66	0·76	absorbs	violet–brown
C	0·24	0·82	none	blue–grey
D	0·00	0·80	absorbs	violet–brown

* Acetone powder, pre-chilled to −20° was extracted with 15 parts of 0·1 M phosphate buffer at 0° and pH 7·4 for 2 hr. Precautions were taken to keep the temperature from rising above 0°.

† Solvent 1 is upper layer of benzene–acetic acid–water (10:7:3, v/v). Solvent 2 is 2% aqueous formic acid.

‡ Short wavelength, mainly 253 nm.

§ A 1% aqueous solution oversprayed with 5% Na_2CO_3.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN ACIDS RELEASED BY AUTOLYSIS OF ^{14}C-LABELLED ACETONE POWDERS OF WHEAT SHOOTS

Compound administered to plants*	Total ^{14}C in the ether extract (counts/min)†	Distribution of ^{14}C (counts/min)‡			
		Cinnamic acid	*p*-Coumaric acid	Caffeic acid	Ferulic acid
$^{14}CO_2$	21,800	4120	3160	2460	2220
	100%	18·9%	14·5%	11·3%	10·2%
L-(U-^{14}C) Phenylalanine	36,200	7630	4550	4280	4100
	100%	21%	12·6%	11·8%	11·3%

* Intact seedlings were exposed to $^{14}CO_2$ in light for 2 hr. L-(U-^{14}C) Phenylalanine was fed to shoots for 90 min.[7]

† 39 per cent of the ^{14}C was in an unknown compound in autolysates from plants activated by $^{14}CO_2$ and 41 per cent from plants activated by L-(U-^{14}C) phenylalanine.

‡ Radioactivity in sinapic acid was not determined.

amounts in buffer extracts made at 24 ± 2° although then the free cinnamic acids are readily detectable. These results can be explained by assuming that these unidentified phenolic substances undergo enzymatic hydrolysis at 24°, but not at 0°, releasing free cinnamic acids.

Radiotracer experiments have shown that the cinnamic acid derivatives found in acetone powders are metabolically active. Wheat shoots were treated with $^{14}CO_2$ in light or with L-[U-^{14}C]-phenylalanine and then converted to acetone powders which were subjected to autolysis at room temperature (Table 2). Previous work has shown that bound phenolic

cinnamic acid derivatives are readily labelled by these precursors.[7] The results in Table 2 confirm this and show, in addition, that bound cinnamic acid itself is also labelled. The amounts of ^{14}C incorporated into each fraction support the hypothesis that:

cinnamic acid→*p*-coumaric acid→caffeic acid→ferulic acid

Here the sequence refers to the acetone-insoluble bound forms of these acids.

In another experiment barley shoots were labelled by photosynthesis in $^{14}CO_2$, and converted to an acetone powder which was allowed to autolyse. In this experiment a distinction was made between the acids released early in autolysis and those obtained after more prolonged treatment. The results (Table 3) show that several bound forms of these acids are present which are hydrolysed at different rates. For example the metabolically-active (i.e. readily labelled) bound forms of cinnamic acid are hydrolysed more slowly than the bound forms which are less readily labelled, whereas the reverse is true for ferulic acid esters. This

TABLE 3. AUTOLYTIC RELEASE OF ^{14}C-LABELLED CINNAMIC, *p*-COUMARIC AND FERULIC ACIDS ON INCUBATION OF BARLEY-SHOOT ACETONE POWDERS*

	Cinnamic acid	*p*-Coumaric acid	Ferulic acid
First incubation (2 hr)			
μg Acid released per g acetone powder	67·0	13·2	12·3
Total ^{14}C (disintegrations/min)	3620	2460	1060
Specific activity (μc/mmole)	3·6	13·8	7·5
Second incubation (8 hr)			
μg Acid released per g acetone powder	14·8	12·0	20·0
Total ^{14}C (disintegrations/min)	3200	2080	1020
Specific activity (μc/mmole)	16·7	12·8	4·5

* The acetone powders were prepared from barley seedlings which had assimilated $^{14}CO_2$ photosynthetically, for 1 hr.[7] The first incubation was carried out in 0·1 M phosphate buffer (pH 7·3) at 24±2° for 2 hr, the soluble compounds were removed by filtration and the insoluble residue re-incubated in fresh buffer under the same conditions for 8 hr.

can be seen by comparing the specific activities of these acids obtained at the two different times of autolysis (Table 3). Apparently these plants contain a mixture of acetone-insoluble esters of cinnamic acids which vary in their metabolic activity.

It would be of great value to determine the nature of these acetone-insoluble esters. One possibility is that they are acylated enzymes, so experiments were designed to see if it could be shown that complexes with proteins were involved. Some support was obtained for this view but more work is needed to obtain a conclusive result. It was found however that appreciable amounts of bound cinnamic acid derivatives, extracted from wheat shoot acetone powders at 0° could be precipitated with ammonium sulphate at 30–70 per cent saturation although most of the activity remained in solution (Table 4). This experiment was done with ^{14}C-labelled acetone powders, to ensure that metabolically-active forms were being followed. Other experiments employed gel filtration on Sephadex columns (Fig. 2). It appears that some compounds in the molecular size range of proteins are present but most of the bound cinnamic acids are in compounds which may be somewhat below this range in molecular size.

TABLE 4. FRACTIONATION OF BOUND CINNAMIC ACID DERIVATIVES OF WHEAT SHOOT-ACETONE POWDERS BY AMMONIUM SULPHATE PRECIPITATION*

Fraction	^{14}C in ether-soluble acids (counts/min)
0–30% $(NH_4)_2SO_4$	
precipitate	200
dialyzate	nil
30–70% $(NH_4)_2SO_4$	
precipitate	5680
dialyzate†	2020
Supernatant, 70% $(NH_4)_2SO_4$	26,600‡

* Wheat shoots were activated by photosynthetic assimilation of $^{14}CO_2$ for 1 hr.[7] Acetone powders prepared from these were extracted with 0·1 M phosphate buffer, at 0° and pH 7·3, and subjected to ammonium sulphate fractionation. The ^{14}C was determined in the ether-soluble acids released by acid hydrolysis.[5]

† Dialyzed for 12 hr against 0·02 M phosphate buffer pH 7·4 at 4°.

‡ 5200 counts/min in Cinnamic acid, 4300 counts/min in *p*-coumaric acid, and 2900 counts/min in ferulic acid.

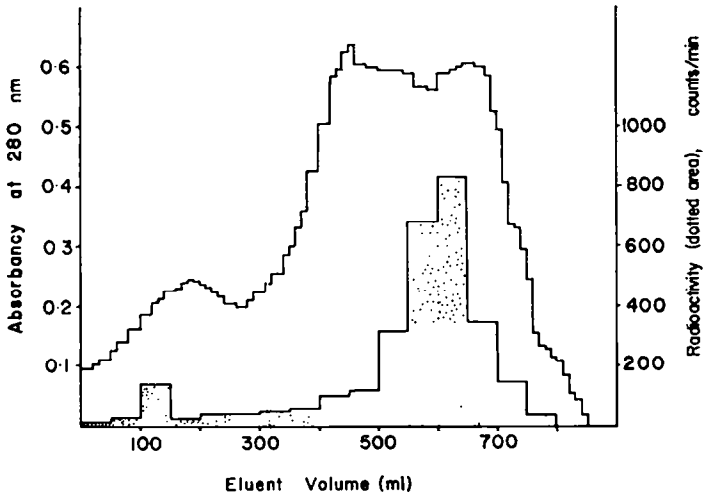

FIG. 2. FRACTIONATION OF BOUND CINNAMIC ACID ON A SEPHADEX COLUMN.

Wheat acetone powder (2 g) was extracted by 50 ml of 0·1 M phosphate buffer at 0°. The extract was treated with $(NH_4)_2SO_4$ and the fraction precipitated at 50–100% saturation was taken up in 10 ml of buffer and put on a 5 × 50 cm column of Sephadex G-200, which was then developed at 4°. The radioactivity was determined on cinnamic acid released by acid hydrolysis.[5] Ammonium ions started to appear at about 750 ml.

Brown and Neish[8] first observed the incorporation of ^{14}C-labelled cinnamic acid into the lignin of wheat plants and since then it has been established that cinnamic acid can be converted, not only to lignin, but to related compounds such as coumarins and flavonoids by a number of plant species (see reviews by Neish[6, 9] and by Brown[10]). However cinnamic acid was never shown to be a *natural intermediate* in these experiments since it was not shown to occur naturally in the plant material being used. In the present paper it is shown to be present in wheat and barley plants in metabolically-active forms. Preliminary results of a survey which will be published later,[11] have also shown the occurrence of bound cinnamic acid in carrot shoots and leaves of spinach and pine.

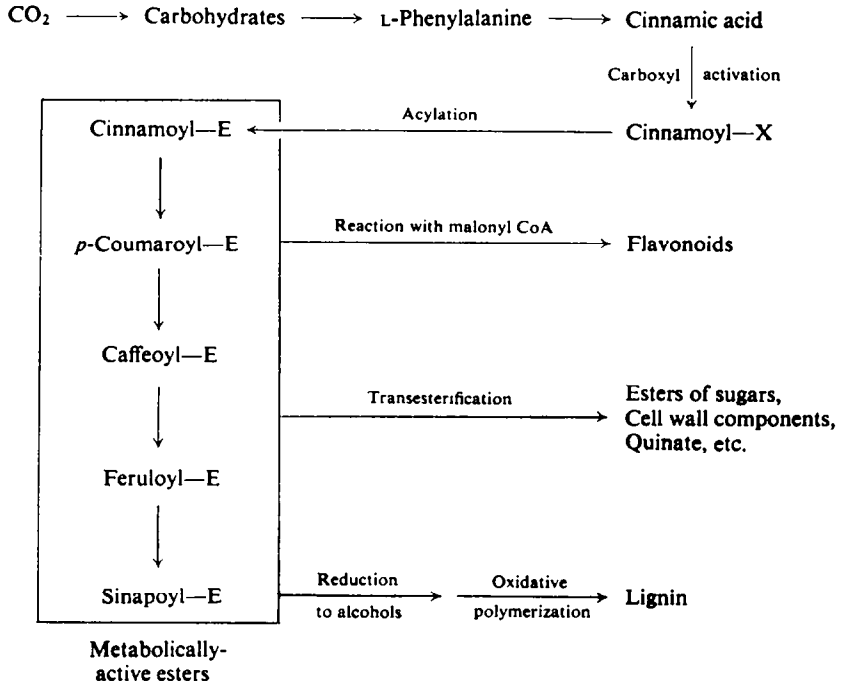

FIG. 3. HYPOTHETICAL OUTLINE OF THE ORIGIN OF SOME COMMON PHENYLPROPANOID CONSTITUENTS OF PLANTS.

As mentioned above these acetone-insoluble compounds of cinnamic acid probably act as precursorts of its phenolic derivatives such as *p*-coumaric acid and ferulic acid which are known to occur widely in vascular plants. Previously it has been suggested that the sequence:

cinnamic acid→*p*-coumaric acid→caffeic acid→ferulic acid

involves the free acids[12] or the quinate esters.[13] Some support for the involvement of free acids was obtained by Nair and Vining[14] who observed hydroxylation of cinnamate to *p*-

[8] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* **33**, 948 (1955).
[9] A. C. NEISH, In *Biochemistry of Phenolic Compounds* (Edited by J. B. HARBORNE), p. 295. Academic Press, New York (1964).
[10] S. A. BROWN, In *Biochemistry of Phenolic Compounds* (Edited by J. B. HARBORNE), p. 361. Academic Press, New York (1964).
[11] SAID Z. EL-BASYOUNI, Unpublished results.
[12] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 537 (1959).
[13] C. C. LEVY and M. ZUCKER, *J. Biol. Chem.* **235**, 2418 (1960).
[14] P. M. NAIR and L. C. VINING, *Phytochem.* **4**, 161 (1965).

coumarate by an enzyme preparation from spinach. However, the results of the present investigation and a previous paper[7] show the biosynthetic sequence involves esters insoluble in acetone or hot 80% ethanol. This eliminates free acids or quinate esters as important intermediates.

A hypothetical outline of the origin of some common phenylpropanoid constituents of plants is shown in Fig. 3.

Cinnamic acid, formed by L-phenylalanine ammonia-lyase,[15] is assumed to undergo carboxyl-activation to give "cinnamoyl-X", which might be the CoA ester. This could give rise to a series of esters but it is believed the metabolically-important reaction is formation of "cinnamoyl-E" which represents the active acetone-insoluble esters described above. Hydroxylation and methylation reactions could then give a pool of ring-substituted cinnamoyl-E derivatives, from which intermediates may be drawn for the biosynthesis of flavonoids, lignins, phenylpropanoid esters and possibly coumarins and phenolic glycosides. It is possible these cinnamoyl-E derivatives represent acylated enzymes in some instances. It will be necessary to learn more about the nature of these derivatives and the mechanism of their formation before a more detailed explanation can be given.

EXPERIMENTAL

Plant Material

Wheat (*Triticum aestivum* L. emend Thell. spp. *vulgare* var. Kharkov 22 M.C.) and barley (*Hordeum vulgare* var. Montcalm) seeds were germinated and grown as described previously.[5] Seedlings were harvested 10–15 days after germination.

Radioactive Compounds

L-(U-^{14}C)Phenylalanine and $Na_2{}^{14}CO_3$ were purchased from Merck, Sharp and Dohme, Montreal. Methods of administration of labelled phenylalanine and $^{14}CO_2$ have been described.[7]

Preparation of Acetone Powders

Fresh plant material (shoots, roots or leaves) was harvested, cut into pieces with suitable length (1–2 in) and homogenized in acetone at $-20°$ in a chilled Waring Blendor for two successive 45-sec intervals. The homogenate was filtered under suction on a Whatman No. 1 filter disc supported on a Buchner funnel. The residue was washed with cold acetone until the final washings were colourless. Air was sucked through for 5 min and the resultant mixture of powder and fibers was spread on a filter paper in a fume hood and air-dried until the odour of acetone was no longer detectable (10–15 min). It was finally dried over $CaCl_2$ in a vacuum desiccator at room temperature for 1 hr, transferred to a clean screw-cap jar and kept in a freezer until used. There was no noticeable difference in the results between freshly prepared powders and powders stored for six months.

Autolysis of the Acetone Powder

Acetone powder was mixed with fifteen times its weight of 0·1 M sodium phosphate buffer, pH 7·3–7·4 in a 125 ml Erlenmeyer flask. The mixture was incubated for 2 hr at $24 \pm 2°$ in a temperature-regulated water bath.

[15] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).

Analyses for Phenolic Acids

The incubation medium, from the above step, was filtered, the filtrate acidified with conc. HCl to pH 4 and continuously extracted with ether for 20 hr. Methods for chromatographic separation and quantitative determination of phenolic acids have been described previously.[5]

The u.v. absorption spectrum of cinnamic acid was scanned on a Bausch and Lomb Spectronic 505 recording spectrophotometer. Quantitative spectrophotometric determinations of phenolic acids were obtained with a Beckman DU spectrophotometer. Measurements of radioactivity were carried out with a Packard Tri-Carb series 314A Liquid Scintillation Spectrometer.

Identification of Cinnamic Acid

Cinnamic acid was detected by paper chromatography (Fig. 1B) and found to have the same R_f as an authentic sample in the benzene–acetic acid–water and the formic acid solvents. The cinnamic acid spot did not give any colour reactions with fast Bordeaux salt B.D., diazotized *p*-nitro-aniline or diazotized sulfanilic acid. It showed absorption of u.v. light (253 nm) on the chromatogram and a sample eluted from the paper and purified by rechromatography on washed paper gave a u.v. absorption spectrum identical with an authentic specimen. Cinnamic acid labelled with ^{14}C was isolated by paper chromatography from an acetone powder of wheat prepared from shoots following photosynthesis in $^{14}CO_2$. It was mixed with authentic cinnamic acid and the radioactivity was found to co-chromatograph with the cinnamic acid in both solvents. Furthermore the specific activity was constant (4520, 4430 and 4450 disintegrations/min 10 mg respectively) during three successive crystallizations from water.

Ammonium Sulfate Fractionation

A known aliquot of wheat acetone powder was extracted with twenty-five times its weight of 0·1 M sodium phosphate buffer, pH 7·3–7·4 at 0° for 1 hr with occasional stirring. The buffer extract was squeezed through a double layer of cheese cloth and centrifuged at $18 \times 10^3 g$ for 20 min in Servall RC-2 refrigerated centrifuge to remove cell debris. To the supernatant, stirred slowly at 0°, was added solid ammonium sulfate to bring the concentration up to 30 per cent saturation in 5 min. The solution was left for 30 min, centrifuged at $10 \times 10^3 g$ for 20 min, decanted and the precipitate collected by centrifugation. The precipitation step was carried out on the supernatant after it had been brought to 70 per cent saturation and the precipitate, collected by centrifugation and suspended in one-tenth of original volume of fresh buffer. It was dialyzed against two changes of 0·01 M phosphate buffer, pH 7·4, for 12 and 24 hr respectively. To each of the two precipitates and the supernatant remaining after 70 per cent saturation was added enough conc. HCl to make a 2 N solution. The solutions were heated under reflux on a steam bath for 45 min and analysed for phenolic acids.[5]

Gel Filtration

Sephadex G-25 and G-200 were purchased from Pharmacia Chemicals, Sweden. Fine particles were removed by suspending the powder in 10 volumes of distilled water for 30 min and decanting several times. It was then allowed to swell in 5 volumes of 0·5 M-phosphate buffer, pH 7·4, for 24 and 72 hr respectively for G-25 and G-200, and washed several times with distilled water.

The columns were packed with the gel suspended in 0·02 M phosphate buffer and equilibrated for 24–48 hr before being used. Samples of 10 ml volume were placed on each column and eluted with the same buffer. Ten-ml fractions were collected by means of a fraction collector. The absorbancy of the fractions at 280 mμ was measured with a Beckman DU spectrophotometer. Successive fractions were combined, hydrolysed and analysed for their phenolic acid content and radioactivity.