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FURTHER STUDIES ON THE ROLE OF CHALCONE AND FLAVANONE IN BIOSYNTHESIS OF FLAVONOIDS

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Abstract—Competitive feeding experiments using 4,2′,4′-trihydroxychalcone[-carbonyl-^{14}C] and (−)-7,4′-dihydroxyflavanone-[T] have been carried out with seeds of subterranean clover (*Trifolium subterraneum* L.) and garbonzo bean (*Cicer arietinum* L.) and with cell-free extracts of garbanzo seedlings. T/^{14}C ratios of the radioactive products 3,7,4′-trihydroxyflavanone (garbanzol), 7,4′-dihydroxyflavonol, 7,4′-dihydroxyflavone and 7-hydroxy-4′-methoxyisoflavone (formononetin) were determined at various times after feeding. Results obtained support the previous conclusion that chalcone is a more immediate precursor than flavanone for the biosynthesis of these flavonoids.

INTRODUCTION

IN PREVIOUS work,[1] results from parallel competitive feeding experiments in which either (*a*) isoliquiritigenin-^{14}C (4,2′,4′-trihydroxychalcone) (I) diluted with an equal amount of (–)-liquiritigenin (7,4′-dihydroxyflavanone) (II) or (*b*) liquiritigenin-^{14}C diluted similarly with isoliquiritigenin were fed as precursors were interpreted as indicating that chalcone is the more immediate precursor than flavanone for the biosynthesis of flavanonols, flavones and isoflavones. We have now carried out further studies on this problem, using garbanzo (*Cicer arietinum*) and clover (*Trifolium subterraneum*) seedlings as in the previous work, but with simultaneous feeding of isoliquiritigenin-[^{14}C] and (–)-liquiritigenin-[T]. T/^{14}C ratios of the flavonoid compounds were determined at various times after feeding. Garbanzol (3,7,4′-trihydroxyflavanone) (III) formation was studied in cell-free extracts of garbanzo seedlings and in the intact seedling, and the biosynthesis of 7,4′-dihydroxyflavone and formononetin (7-hydroxy-4′-methoxyisoflavone) was studied in intact seedlings of both garbanzo and clover. Results presented here support the previous conclusion that the chalcone isoliquiritigenin is a more immediate precursor than the flavanone liquiritigenin for the biosynthesis of these compounds.[1]

RESULTS

The radioactive precursor in all the following experiments consisted of an approximately equal molar mixture of isoliquiritigenin-[carbonyl-^{14}C] (0·467 mc/mmole) and (–)-liquiritigenin‡ [3′,5′-T] (2·11 mc/mmole) prepared from the corresponding chalcone by means of the chalcone-flavanone isomerase.[2]

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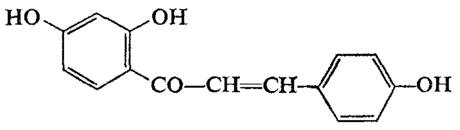
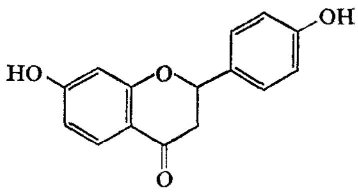
‡ For brevity and emphasis, the two compounds isoliquiritigenin (I) and (–)-liquiritigenin (II) will be referred to simply as chalcone and flavanone respectively in the remainder of this paper.

[1] E. WONG, *Phytochem.* 7, 1715 (1968).

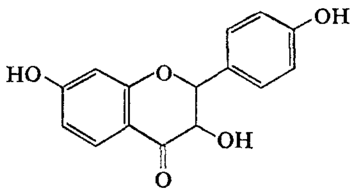
[2] E. MOUSTAFA and E. WONG, *Phytochem.* 6, 625 (1967).



(I)
Isoliquiritigenin



(II)
Liquiritigenin



(III)
Garbanzol

Biosynthesis of Garbanzol in Cell-free Extracts of Garbanzo Seedlings

The mixture of chalcone-[^{14}C] and flavanone-[T] was incubated with a cell-free extract of garbanzo seedlings for 7 min and for 20 min. After separation and purification by paper chromatography of the flavonoid products, the T/^{14}C ratios were determined by liquid scintillation counting. In this work, Y_1 and Y_2, the other products of incubation previously identified as 6,4′-dihydroxy-2-(α-hydroxybenzyl)-coumaranone,[3] were also isolated and studied. The results are shown in Table 1.

Table 1. Ratio of T and ^{14}C activities (cpm) and precursor composition* of flavonoid compounds from cell-free extracts of garbanzo bean incubated with chalcone-^{14}C and flavanone-T†‡

	Flavanone	Chalcone	Garbanzol	Y_1+Y_2
After 7 *min*				
T/^{14}C	4·73	1·81	1·24	1·28
ch/fl	0·63	1·78	2·60	2·54
After 20 *min*				
T/^{14}C	4·77	2·56	1·63	1·80
ch/fl	0·63	1·26	1·97	1·79

* ch/fl, molar ratio of original chalcone-^{14}C and flavanone-T incorporated (fl/ch = (T/^{14}C/3·22†).

† Ratio of specific activities of precursors (cpm§), flavanone-T/chalcone-^{14}C = 3·22.

‡ Precursor mixture fed, T/^{14}C = 2·90, ch/fl = 1·11.

§ The relative efficiency of counting in the ^{14}C and T-channels, ^{14}C/T was found to be 1·41, independent of quenching in the efficiency range encountered.

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[3] E. Wong, *Phytochem.* **6**, 1227 (1967).

Biosynthesis of Garbanzol, 7,4′-Dihydroxyflavonol, 7,4′-Dihydroxyflavone and Formononetin in Intact Garbanzo Seedlings

Dry garbanzo seeds were soaked in the precursor mixture and allowed to germinate, after which portions were extracted after 54 and 78 hr. In the previous work with clover seedlings[1] the flavonoids were recovered after initial acid hydrolysis of the extracts and therefore represent the total free and combined forms of the compounds present. In the present work, in both experiments with intact garbanzo and clover seedlings (next section), phenolic compounds were isolated both before (Fr A) and after (Fr B) acid hydrolysis and the two fractions were analysed separately. Compounds found in Fr B are assumed to be derived from glycosides. Garbanzol, 7,4′-dihydroxyflavonol, 7,4′-dihydroxyflavone and formononetin were isolated from these fractions, separated and purified by paper chromatography, and their radioactivity was determined. Results are summarized in Table 2.

TABLE 2. RATIO OF T AND ^{14}C ACTIVITIES (cpm) AND PRECURSOR COMPOSITION* OF FLAVONOID COMPOUNDS FROM GARBANZO SEEDLINGS FED CHALCONE-^{14}C AND FLAVANONE-T†‡

	After 54 hr				After 78 hr			
	Fraction A		Fraction B		Fraction A		Fraction B	
	T/^{14}C	ch/fl	T/^{14}C	ch/fl	T/^{14}C	ch/fl	T/^{14}C	ch/fl
Flavone	8·42	1/2·62	7·56	1/2·35	8·04	1/2·50	7·45	1/2·31
Chalcone	2·99	1·08	4·91	1/1·52	3·57	1/1·08	4·90	1/1·52
Garbanzol	2·95	1·09	3·30	1/1·03	2·85	1·13	3·34	1/1·04
7′4′-Dihydroxyflavonol	2·65	1·22	4·23	1/1·32	3·22	1·00	4·50	1/1·40
7′4′-Dihydroxyflavone	2·89	1·11	3·48	1/1·08	2·96	1·09	3·63	1/1·13
Formononetin	3·06	1·05	3·15	1·02	3·12	1·03	3·18	1·01

* ch/fl, molar ratio of original chalcone-^{14}C and flavanone-T incorporated (fl/ch = (T/^{14}C)/3·22†).

† Ratio of specific activities of precursors (cpm§), flavanone-T/chalcone-^{14}C = 3·22.

‡ Mixture fed, T/^{14}C = 3·38, ch/fl = 1/1·05, but after allowing for amounts not absorbed (see Experimental) actual ch/fl taken up ≑ 1/1·15.

§ See Table 1.

Biosynthesis of 7,4′-Dihydroxyflavone and Formanonetin in Clover Seedlings

The mixture of chalcone-[^{14}C] and flavanone-[T] was fed to dry clover seeds which were then allowed to germinate for 30, 54 and 78 hr (Experiment I) or 24 and 48 hr (Experiment II). Phenolic compounds were again separated into fractions A (before hydrolysis) and B (after hydrolysis) and purified by paper chromatography. 7,4′-Dihydroxyflavone and formononetin were isolated as described earlier[1] (daidzein, which occurs only in traces in the seedlings, was not studied further in this work). An additional minor radioactive spot was noted on two-dimensional chromatograms of all A fractions and was tentatively identified as a mixture of fisetin[4] (7,3′,4′-trihydroxyflavonol) and the corresponding chalcone butein. The concentration of these two compounds was too low to allow further separation and purification in the present work; they were therefore eluted together and the radioactivity of the spot was measured as a whole. Radioactivity data for the compounds studied are given in Table 3.

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[4] E. WONG and C. M. FRANCIS, *Phytochem.* 7, 2123 (1968).

TABLE 3. RATIO OF T AND ^{14}C ACTIVITIES (cpm) AND PRECURSOR COMPOSITION* OF FLAVONOID COMPOUNDS FROM CLOVER SEEDLINGS FED CHALCONE-^{14}C AND FLAVANONE-T†‡

	Flavanone		Chalcone		7,4′-Dihydroxy-flavone		Formononetin		Fisetin/butein§
	Fr A	Fr B	Fr A	Fr B	Fr A	Fr B	Fr A	Fr B	Fr A
Experiment I									
1. *After* 30 hr									
T/^{14}C	19·62	4·06	1·12	1·20	1·98	—	3·07	2·84	0·95
ch/fl	1/6·10	1/1·26	2·87	2·68	1·62	—	1·05	1·13	3·40
2. *After* 54 hr									
T/^{14}C	8·62	4·96	1·43	2·77	2·38	—	2·83	3·06	1·08
ch/fl	1/2·68	1/1·54	2·25	1·16	1·35	—	1·14	1·05	2·98
3. *After* 78 hr									
T/^{14}C	8·32	6·47	1·61	5·50	2·15	2·71	2·79	2·94	1·40
ch/fl	1/2·59	1/2·01	2·00	1/1·71	1·50	1·19	1·15	1·09	2·30
Experiment II									
1. *After* 24 hr									
T/^{14}C	9·10	—	1·01	—	2·52	—	2·49	—	0·74
ch/fl	1/2·83	—	3·19	—	1·28	—	1·29	—	4·30
2. *After* 48 hr									
T/^{14}C	7·60	5·05	1·10	3·94	2·18	2·90	2·12	2·45	0·80
ch/fl	1/2·36	1/1·57	2·85	1/1·25	1·48	1·11	1·52	1·31	4·00

* ch/fl, molar ratio of original chalcone-^{14}C and flavanone-T incorporated (fl/ch = (T/^{14}C/3·22†).

† Ratio of specific activities of precursors (cpm¶), flavanone-T/chalcone-^{14}C = 3·22.

‡ Mixture fed: Expt. I, T/^{14}C = 3·31; Expt. II, T/^{14}C = 3·20.

§ Data corrected for presumed 50 per cent loss of T.

¶ See Table 1.

DISCUSSION

In the cell-free experiment, the T/^{14}C and ch/fl values for garbanzol (Table 1), when compared with those of the chalcone and flavanone, indicate clearly that garbanzol was synthesized directly from the chalcone pool. The close relationship of garbanzol to chalcone is more clearly illustrated in Fig. 1. Not only does the ch/fl value for garbanzol decrease with time in the same way as that of the chalcone pool, but the fact that the garbanzol curve lies above that of the chalcone constitutes necessary evidence that garbanzol has come from the chalcone pool. A product of the chalcone pool at any given time comes from earlier pools of chalcone, the average ch/fl values of which must be higher than the value for the chalcone pool at that time. As support for these interpretations, Fig. 1 shows that Y_1 and Y_2 behave qualitatively in the same way as garbanzol in relation to chalcone. From *a priori* structural considerations and from results from studies with purified enzymes,[5] it is almost certain that Y_1 and Y_2 come more directly from chalcone than from flavanone.

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[5] J. M. WILSON and E. WONG, unpublished work.

In contrast to results from cell-free experiments, results from intact feeding experiments are in general less amenable to precise interpretation. Nevertheless, in the experiment with intact garbanzo seedlings the results seem remarkably clear cut. Comparison of the ch/fl values for the products in Fr A with the values for the chalcone and flavanone (Fig. 2) show that garbanzol, 7,4′-dihydroxyflavone and formononetin are closely related to the chalcone. These results therefore strongly suggest that all of these compounds are derived directly from chalcone. Furthermore, since all these isolated products have a precursor composition even richer in ^{14}C-chalcone than that of the chalcone pool, it is unlikely that they could have originated from any flavanone pool.

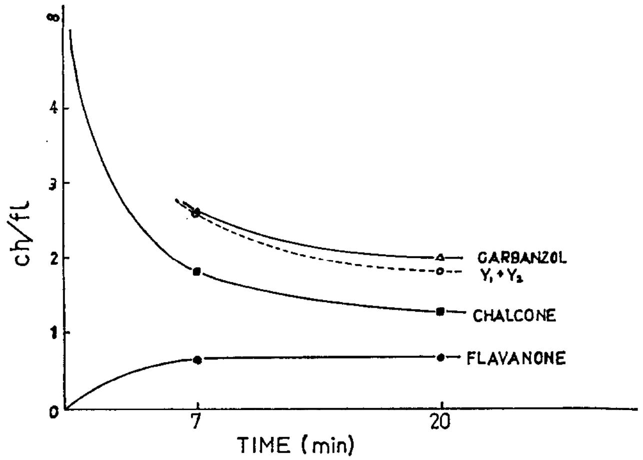

FIG. 1. COMPOSITION OF COMPOUNDS FROM CELL-FREE EXTRACTS OF GARBANZO INCUBATED WITH CHALCONE-^{14}C AND FLAVANONE-T.

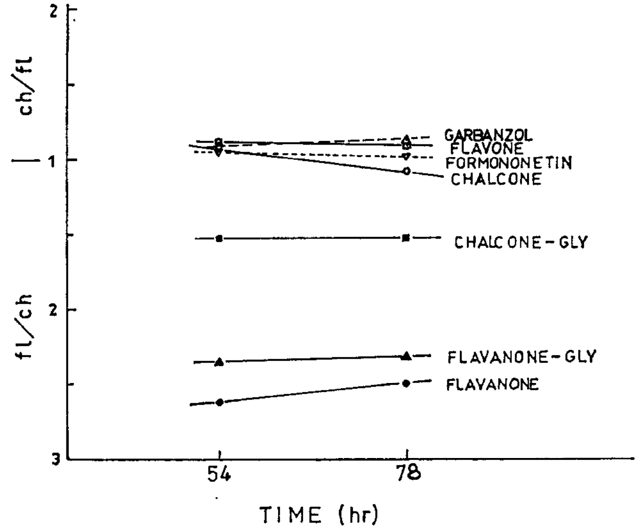

FIG. 2. COMPOSITION OF FLAVONOID COMPOUNDS FROM GARBANZO AFTER FEEDING CHALCONE-^{14}C AND FLAVANONE-T.

Results from the intact clover experiments (Table 3), on the other hand, could not be so readily interpreted. The ch/fl values for the flavone and formononetin do show higher incorporation of chalcone than of flavanone, suggesting that they are more probably derived from chalcone, but the time curves for these products (typical results from Experiment I, Fr A, are plotted in Fig. 3) could not be related to that of the chalcone in any obvious way. On the other hand it is even more difficult to try to relate them to the curve for the flavanone. Since a relatively large excess of chalcone and flavanone precursors were fed in this work, the problem may be due to the possibility that the apparent chalcone and flavanone pools

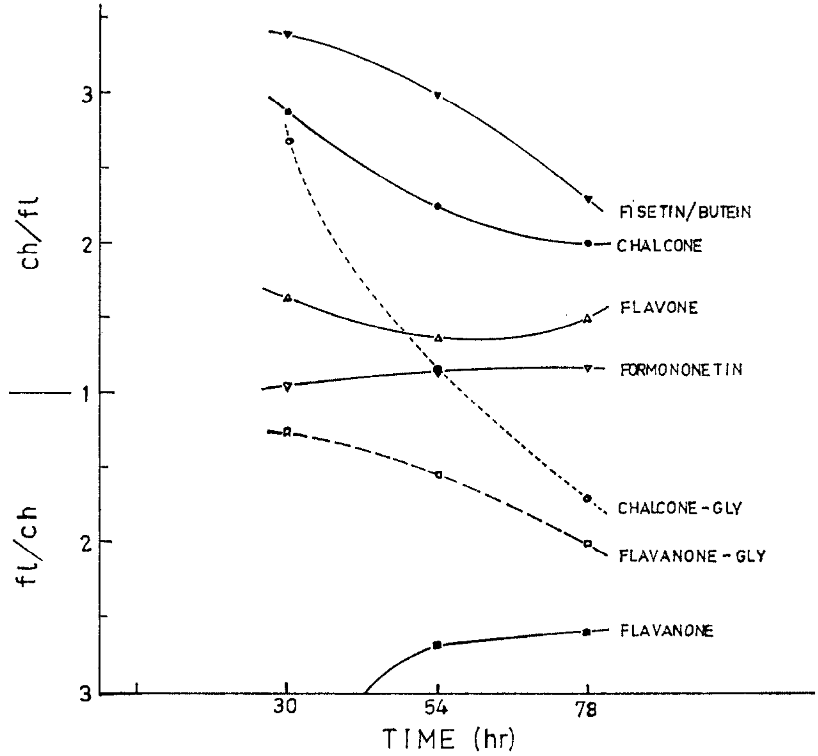

FIG. 3. COMPOSITION OF FLAVONOID COMPOUNDS FROM CLOVER AFTER FEEDING CHALCONE-^{14}C AND FLAVANONE-T.

measured do not represent the true metabolic pools of these compounds in the plant. One possible way for this situation to arise is that part of the chalcone and flavanone feed is only adsorbed on the surface of the seeds, which can be extracted but takes no part in the metabolic changes inside the plant. Some evidence for such adsorption having occurred on garbanzo seeds is described in the Experimental section.

A further possible complication is that even after adsorption of the chalcone and flavanone into the plant, more than one precursor pool may exist due to compartmentation and localization of sites of biosynthesis. That this was probably true for isoflavone biosynthesis in the clover experiments is revealed when specific activity data for parallel experiments given in the previous work[1] is analysed in more detail. Thus in experiments Ia and Ib (Table 1, Ref. 1) specific activity values (and hence dilution factors) for 7,4′-dihydroxyflavone indicate that this product was totally derived from 2 parts of the chalcone to 1 part of the flavanone fed (i.e. ch/fl = 2·0). By contrast, the high dilution of activity of formononetin in both experiments (*a*) and (*b*) indicates that a large portion of the formononetin isolated did not originate from the chalcone or flavanone fed (this portion may be termed the "extra" pool). It can be calculated from the specific activity data that the ratios of the amounts derived from the three sources are ch:fl:"extra" pool = 2·2:1:12·1. In other words nearly 80 per cent of the formononetin isolated in this experiment did not come from the pool of chalcone and flavanone fed. This "extra" pool was assumed earlier to be due to the presence of endogenous formononetin in the dry seed.[1] Results from the present work, however, show that very little formononetin exists in the seedling until after the first day. This "extra" pool of formononetin must therefore also have been synthesized during the experiment but apparently did so independently of the chalcone and flavanone fed.

Results presented in Table 2 and 3 also show that the bound forms of chalcone and flavanone (Fr B) have very different ch/fl values than the apparent free forms. By contrast, values for the free and bound forms of the products are in general similar. These results are consistent with the assumption that the biosynthetic sequence is product→product-glycoside.

Although results from the feeding experiments with intact clover seedlings cannot be unequivocally interpreted, results presented in this work as a whole can be taken as further support for the previous conclusion that flavanonols, flavones and isoflavones are more directly biosynthesized from chalcone than from flavanone. In the case of the flavanonol, the kinetic data from the cell-free experiment provides conclusive proof of this point.

EXPERIMENTAL

(−)-*Liquiritigenin* [3′,5′-T_2]

This flavanone was prepared from the corresponding chalcone-[3,5-T_2] by means of chalcone-flavanone isomerase.[1,2] The chalcone was prepared in the normal way by condensation of resacetophenone and *p*-hydroxybenzaldehyde-[3,5-T_2] (see below). Isoliquiritigenin-[carbonyl-^{14}C] was available from previous work.[1]

4-*Hydroxybenzaldehyde*-[3,5-T_2]

Tritium labelling of 4-hydroxybenzaldehyde was accomplished by exchange with tritiated water according to the method of Kirby and Ogunkoya.[6] A mixture of the aldehyde (2 mmole), THO (0·5 ml, 3·6 mc/mmole) and triethylamine (2 mmole) was heated in a sealed tube at 100° for 3 days. After removal of THO *in vacuo*, the product was used directly for condensation with resacetophenone (2 mmole). The yield of purified chalcone was about 25 per cent, with a specific activity of 2·11 mc/mmole. The position of labelling in the aromatic ring was shown in a parallel exchange experiment with D_2O; NMR spectral data showed that, as expected, exchange had taken place only at the positions *ortho* to the hydroxyl group. Conversion of the deuterated aldehyde to the chalcone also showed that no loss or exchange of labelling took place during the condensation step.

Counting of Radioactivity

Radioactivity was counted in a Beckman LS-100 liquid scintillation counter using two channels. The contribution of ^{14}C counts in the tritium channels was calculated from a calibration curve prepared with standard chalcone-^{14}C and flavanone-^{14}C samples, quenching being measured by the instrumental external standard. The relative efficiency of counting in the two channels, ^{14}C/T was found to be 1·41, independent of quenching in the efficiency range encountered (^{14}C, 48–62 per cent; T, 34–45 per cent). T/^{14}C ratios determined in this work are estimated to be accurate to 5–10 per cent.

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[6] G. Kirby and L. Ogunkoya, *J. Chem. Soc.* 6919 (1965).

Feeding and Incubation Experiments

These experiments were carried out essentially as previously described,[1] with the addition here that in each experiment aliquots were taken at various times and analysed separately. Furthermore, for the experiments with intact seedlings, phenolic compounds were extracted in this work both before and after hydroylsis. After washing with petroleum ether, the 70% alcoholic extract was evaporated, taken up in water and extracted with ether to give Fr A. The residue was then hydrolysed in 1% HCl (in 50% ethanol) and again subjected to ether extraction, giving Fr B. Relevant details in the feeding experiments are as follows:

Garbanzo, cell-free. 1·23 mg of chalcone-[^{14}C], 4·65 × 10^6 dpm, and 1·14 mg of flavanone-T, 19·47 × 10^6 dpm, were incubated at 37° with 20 ml of cell-free extract from 20 g of 3·5-day-old garbanzo seedlings.

Garbanzo, intact. 10 g of seeds were fed 2·48 mg of chalcone-^{14}C, 9·34 × 10^6 dmp, and 2·61 mg of flavanone-T, 44·70 × 10^6 dpm. The seeds were first soaked in the radioactive solution for 6 hr, then placed on a layer of plastic sponge to germinate. After 18 hr the seeds were washed for a few minutes in water. The water washing was found to contain the following amounts of radioactivity: ^{14}C, 1·14 × 10^6 dpm (12·1 per cent of total fed); T, 1, 34 × 10^6 dpm (3·0 per cent of total fed). These high activities very likely represent the amounts of chalcone and flavanone adsorbed on the surface of the seeds only. Total activity recovered in the extracts: ^{14}C, 43·8 per cent; T, 59·3 per cent.

Clover, experiment I. 3 g of seeds were fed 1·37 mg of chalcone-^{14}C and 1·41 mg of flavanone-T. After soaking, 2·5 per cent and 0·63 per cent respectively of the ^{14}C and T fed were found to have been left in the glass container. Total activities in the extracts: ^{14}C, 46·8 per cent; T, 52·4 per cent.

Clover, experiment II. 4 g of seeds, fed 1·68 mg each of chalcone-^{14}C and flavanone-T. Amount not taken up: ^{14}C, 9 per cent T, 6 per cent. Total recovered in extracts: ^{14}C, 47·3 per cent; T, 41·0 per cent.

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