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RATE OF INCORPORATION OF $^{14}CO_2$, 1-^{14}C-ACETATE AND 2-^{14}C-DL-MEVALONATE INTO FATTY ACIDS AND TRITERPENOIDS IN THE SHOOTS OF *CALENDULA OFFICINALIS* AT DIFFERENT LIGHT INTENSITIES

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Abstract—$^{14}CO_2$, 1-^{14}C-acetate and 2-^{14}C-DL-MVA were fed to shoots of *Calendula officinalis* at three light intensities (3000, 20,000 and 40,000 lx). Mevalonate is the most effective precursor of triterpenoids, and the acetate of fatty acids at both illuminations. The light intensity does not affect the rate and value of incorporation of 2-^{14}C-DL-MVA into triterpenoids and fatty acids. At higher illumination a distinct increase of incorporation into triterpenoids is observed for $^{14}CO_2$ and a decrease for 1-^{14}C-acetate. The biosynthesis of fatty acids from $^{14}CO_2$ and 1-^{14}C-acetate is lower at 40,000 lx than at 3000 lx. These facts are discussed in regard to the way in which the precursors are supplied and the permeability of the chloroplastic membrane.

INTRODUCTION

IN STUDIES on biosynthesis of triterpenoids 2-^{14}C mevalonate is most frequently used precursor.[1] Use is also made of $^{14}CO_2$ and ^{14}C-acetate[2,3] no work being done however on the comparison of the rate of incorporation of these precursors into triterpenoids in *Calendula officinalis*. In our previous investigations 1-^{14}C-acetate has been applied as precursor of triterpenoids.[4,5]

The aim of the present work was to compare the incorporations of three different precursors, namely: $^{14}CO_2$, 1-^{14}C-acetate and 2-^{14}C-DL-mevalonate into triterpenoid compounds and into fatty acids as well as to investigate the effect of light intensity on the dynamics of labelling of these compounds. The precursors used exhibit different affinity to triterpenoids, CO_2 being the most remote and mevalonate the closest. CO_2 is also the natural precursor of the compounds of the photosynthetic cycle and the rate of incorporation of CO_2 into these compounds is strictly correlated with the intensity of light. It seemed therefore of interest to investigate to what extent the intensity of the photosynthetic incorporation of $^{14}CO_2$ can influence the rate of labelling by this precursor the triterpenoids synthesized outside the chloroplasts. For comparative purposes we investigated also the influence of the light intensity on the labelling of triterpenoids by mevalonate and acetate supplied in aqueous solution.

RESULTS AND DISCUSSION

In our previous investigations, incorporation of 1-^{14}C-acetate into triterpenoids in the shoots of *Calendula* was studied at illumination of 3000 lx. Now, in order to find out the

[1] G. R. WALLER, *Progress in the Chemistry of Fats and other Lipids*, Vol. X, Part 2, Pergamon Press, Oxford (1969).
[2] T. W. GOODWIN, *Biochem. J.* **70**, 612 (1958).
[3] H. J. NICHOLAS, *J. Biol. Chem.* **237**, 1476 (1963).
[4] Z. KASPRZYK and Z. WOJCIECHOWSKI, *Phytochem.* **8**, 1921 (1969).
[5] Z. KASPRZYK, Z. WOJCIECHOWSKI and W. JANISZOWSKA, *Phytochem.* **9**, 561 (1970).

TABLE 1. RADIOACTIVITY IN FATTY ACIDS, NON-SAPONIFIED AND GLYCOSIDIC FRACTIONS AND TRITERPENOIDS IN SHOOTS OF *C. officinalis* AFTER ADMINISTRATION OF $^{14}CO_2$, 1-^{14}C ACETATE AND 2-^{14}C DL-MVA AT THREE LIGHT INTENSITIES

Radioactive precursor	3000 lx			20,000 lx			40,000 lx		
	Non-saponif. fraction	Fatty acids	Glycosidic fraction	Non-saponif. fraction	Fatty acids	Glycosidic fraction	Non-saponif. fraction	Fatty acids	Glycosidic fraction
$^{14}CO_2$	1·25	7·20	0·18	1·38	6·40	0·40	1·78	4·10	0·71
1-^{14}C acetate	3·20	17·50	3·80	3·90	14·00	2·00	4·50	9·20	1·00
2-^{14}C-MVA	14·00	3·90	4·80				13·80	3·80	5·00

	Sterols	Methyl-sterols	β-Amyrin	Oleanolic acid	Sterols	Methyl-sterols	β-Amyrin	Oleanolic acid	Sterols	Methyl-sterols	β-Amyrin	Oleanolic acid
$^{14}CO_2$	0·38	0·16	0·04	0·02	0·63	0·30	0·25	0·14	0·85	0·48	0·40	0·21
1-^{14}C acetate	0·88	0·60	0·24	0·20	0·90	0·60	0·16	0·13	0·90	0·62	0·12	0·08
2-^{14}C-MVA	7·00	0·95	0·96	1·45					6·85	0·90	1·02	1·54

The results are presented in percentage of radioactivity administered.

optimal conditions of labelling for $^{14}CO_2$, 1-^{14}C-acetate and 2-^{14}C-DL-mevalonate, the effect of illumination on incorporation of these precursors into triterpenoids and fatty acids was studied.

The shoots of *Calendula* were given with $^{14}CO_2$, 1-^{14}C-acetate and 2-^{14}C-DL-mevalonate and illuminated with light of intensity 3000, 20,000 or 40,000 lx. The plants were processed after 81 hr since it was known from the previous experiments with 1-^{14}C-acetate, that after this time high rate of incorporation of radioactivity into triterpenoids, i.e. sterols, β-amyrin and oleanolic acid, is observed. The level of radioactivity was determined in fatty acids, non-saponifying and glycosidic fractions as well as in components of these fractions such as sterols, methylsterols, triterpenic monohydroxy alcohols and oleanolic acid. TLC revealed, in agreement with earlier results,[4] that the fraction of monohydroxy alcohols is identical with β-amyrin.

The results presented in Table 1 indicate that at higher illumination a distinct increase of incorporation rate in fractions containing triterpenoids is observed only after administration of $^{14}CO_2$. In the case of 1-^{14}C-acetate radioactivity in non-saponified fraction increases slightly and in glycosidic fraction decreases almost four times at illumination of 40,000 lx in comparison with 3000. A distinct decrease was observed in the fraction of fatty acids at higher illumination after administration of $^{14}CO_2$ and 1-^{14}C-acetate. No basic changes were observed with respect to incorporation rate into triterpenoids and fatty acids after administration of 2-^{14}C-DL-mevalonate at various illuminations.

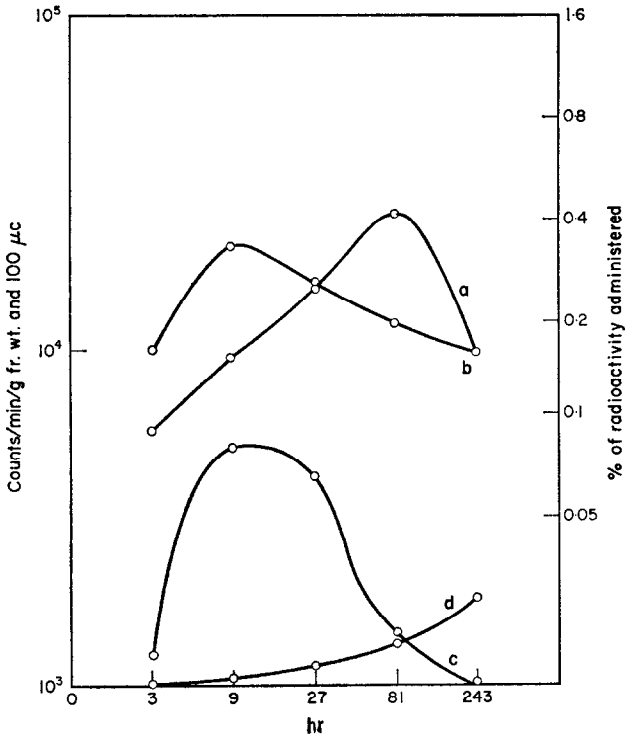

FIG. 1. THE DYNAMICS OF INCORPORATION OF $^{14}CO_2$ INTO: (a) STEROLS, (b) METHYLSTEROLS, (c) β-AMYRIN, (d) OLEANOLIC ACID IN THE SHOOTS OF *C. officinalis* AT ILLUMINATION OF 3000 lx.

The dynamics of incorporation of various precursors into shoots of *Calendula* was compared at illuminations of 3000 and 40,000 lx after administration of $^{14}CO_2$ and 1-^{14}C-acetate, and at 3000 lx in case of 2-^{14}C-DL-mevalonate. The plants were processed at time intervals from 3 to 243 hr after administration of the radioactive precursor. The results, expressed as percentage of radioactivity incorporated into nonsaponified, glycosidic and fatty acid fractions, are presented in Table 2. Figures 1–5 show the dynamics curves for

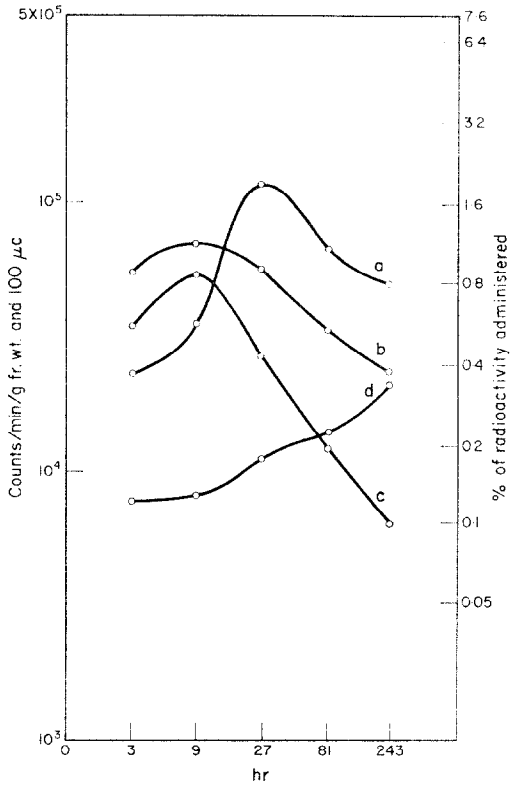

FIG. 2. THE DYNAMICS OF INCORPORATION OF $^{14}CO_2$ INTO: (a) STEROLS, (b) METHYLSTEROLS, (c) β-AMYRIN, (d) OLEANOLIC ACID IN THE SHOOTS OF *C. officinalis* AT ILLUMINATION OF 40,000 lx.

sterols, methylsterols, β-amyrin and oleanolic acid after administration of precursors investigated at 3000 lx for $^{14}CO_2$, 1-^{14}C-acetate and 2-^{14}C-DL-mevalonate and at 40,000 lx (for $^{14}CO_2$ and 1-^{14}C-acetate). The results are presented in counts/min calculated per g fr. wt. and 100 μc of the precursor as well as in percent of radioactivity assimilated by the plant.

The results in Table 2 indicate that at 3000 lx 2-^{14}C-DL-mevalonate is the most effective precursor of triterpenoids as compared with $^{14}CO_2$ and 1-^{14}C-acetate. 1-^{14}C-acetate is the precursor most intensively incorporated into fatty acids. It labels the non-saponifying fraction slightly better than $^{14}CO_2$ at both illuminations.

The effect of light on incorporation of radioactivity from 1-^{14}C-acetate into the non-saponified fraction is not significant. On the other hand the fraction of glycosides is labelled by 1-^{14}C-acetate several times less intensively at higher than at lower illumination.

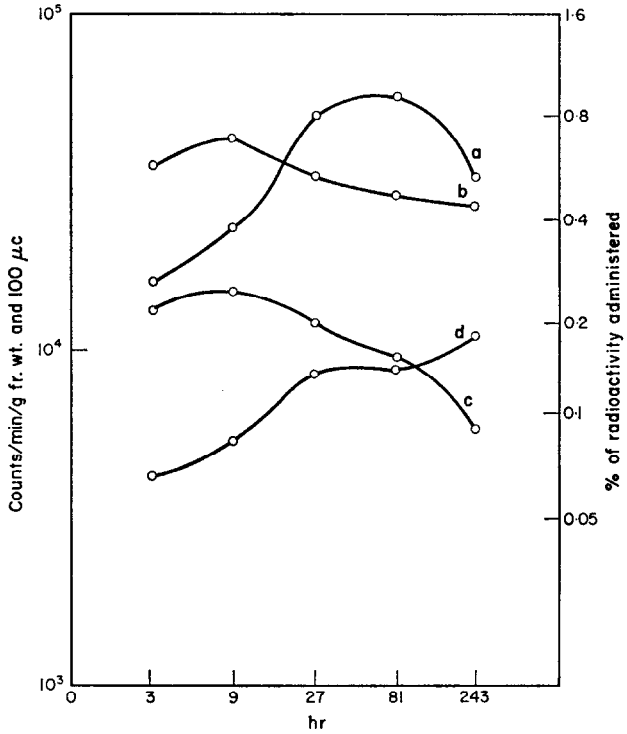

FIG. 3. THE DYNAMICS OF INCORPORATION OF 1-^{14}C-ACETATE INTO: (a) STEROLS, (b) METHYLSTEROLS, (c) β-AMYRIN, (d) OLEANOLIC ACID IN THE SHOOTS OF *C. officinalis* AT ILLUMINATION OF 3000 lx.

The dynamics of incorporation of radioactivity at various illuminations into the non-saponified fraction, containing mainly β-amyrin and sterols, shows an interesting relationship with the changes in the level of radioactivity in the fraction of fatty acids. It is seen from Table 2, that in the case of $^{14}CO_2$ and 1-^{14}C-acetate the maximal labelling of non-saponifying fraction at illumination of 3000 lx occurs after 81 hr, but at 40,000 lx the maxima reach higher values and occur already after 27 hr. In the fraction of fatty acids labelled with $^{14}CO_2$, the maximum is decreasing and shifts from 27 hr at illumination of 3000 lx to 81 hr at 40,000 lx. The value of maximal incorporation of 1-^{14}C-acetate and $^{14}CO_2$ into fatty acids fraction decreases at higher illumination. The maximum of incorporation of $^{14}CO_2$ into this fraction is shifted from 27 to 81 hr. This indicates that there is competition for the radioactive precursor between fatty acids and some compounds of the photosynthetic cycle.

The effect of light on labelling of individual triterpenoids is illustrated in Figs. 1–5. Similarly as in the non-saponified and glycosidic fraction, 2-^{14}C-DL-mevalonate is most effective precursor of sterols, methylsterols, β-amyrin and oleanolic acid at illumination of 3000 lx. The incorporation of $^{14}CO_2$ into individual triterpenoids at 3000 lx is less than the incorporation of 1-^{14}C-acetate into these compounds.

The graphs presented above show that maximal labelling of sterols after administration of $^{14}CO_2$ and 1-^{14}C-acetate, at higher illumination appears earlier (27 hr) than at lower illumination (81 hr). Maximal radioactivity derived from 2-^{14}C-DL-mevalonate occurs in sterols also after 27 hr at 3000 lx. Similar effect of earlier maximal labelling of methylsterols

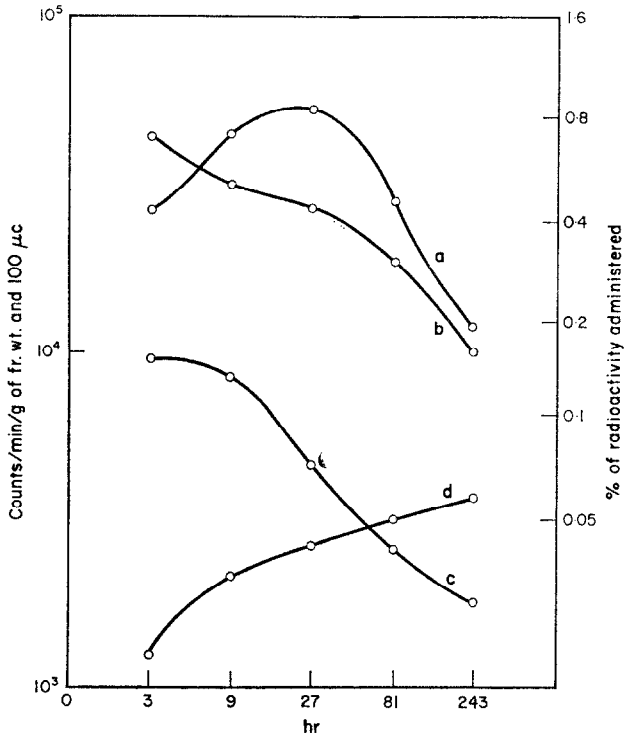

FIG. 4. THE DYNAMICS OF INCORPORATION OF 1-^{14}C-ACETATE INTO: (a) STEROLS, (b) METHYLSTEROLS, (c) β-AMYRIN, (d) OLEANOLIC ACID IN THE SHOOTS OF *C. officinalis* AT ILLUMINATION OF 40,000 lx.

and β-amyrin is observed in the case of 1-^{14}C-acetate. An increase of intensity of illumination causes a rise in radioactivity derived from $^{14}CO_2$ in all triterpenoids, being most signicant for the triterpenes β-amyrin and oleanolic acid. Increased illumination after administration of 1-^{14}C-acetate does not significantly affect the level of incorporation of radioactivity into sterols and methylsterols but causes its decrease in β-amyrin and oleanolic acid.

The above results indicate that each precursor is metabolized to fatty acids and triterpenoids according to the way in which precursors are introduced into the leaves of *Calendula*. $^{14}CO_2$ penetrates to the chloroplast in a natural photosynthetic pathway whereas 1-^{14}C-acetate and 2-^{14}C-DL-mevalonate, drawn through the stem, penetrate to the exochloroplastic area in a way which is unnatural for the transport of the organic compounds in the plant. Whereas acetate is able to pass through the chloroplastic membrane, mevalonate is not. Fatty acids are synthesized in the chloroplasts and in the cytoplasm but the synthesis of sterols and triterpenes (the so-called non-plastidic terpenoids) is restricted to the cytoplasm.[6,7]

The high incorporation of 2-^{14}C-DL-mevalonate into sterols and triterpenes is explained by the fact that mevalonate is a closer precursor of these compounds than CO_2 and acetate.

[6] A. R. WELLBURN and F. W. HEMMING, *Biochem. J.* **104,** 173 (1967).
[7] J. B. PRIDHAM and T. SWAIN, *Biosynthetic pathways in Higher Plants* (proceedings of the Plant Phenolics Group Symposium Leeds, April 1964), p. 57, Academic Press, London (1965).

TABLE 2. DYNAMICS OF INCORPORATION OF RADIOACTIVE PRECURSORS INTO FRACTIONS OF: NON-SAPONIFIABLE COMPOUNDS (STEROLS AND β-AMYRIN), FATTY ACIDS AND GLYCOSIDIC COMPOUNDS IN SHOOTS OF *C. officinalis* AT ILLUMINATION OF 3000 AND 40,000 lx

Hours	Radioactive precursor	3000 lx			40,000 lx		
		Non-saponifying fraction	Fatty acids	Glycosidic fraction	Non-saponifying fraction	Fatty acids	Glycosidic fraction
3	$^{14}CO_2$	0·75	1·75	0·12	1·60	2·60	0·40
	1-^{14}C acetate	1·25	11·50	2·25	2·20	3·00	0·30
	2-^{14}C DL MVA	10·80	1·44	2·50			
9	$^{14}CO_2$	1·05	2·15	0·15	1·75	3·10	0·50
	1-^{14}C acetate	1·40	12·50	2·25	2·30	3·50	0·50
	2^{14}C DL MVA	16·40	1·28	2·80			
27	$^{14}CO_2$	1·25	7·00	0·18	2·90	3·90	0·71
	1-^{14}C acetate	2·50	17·25	2·10	4·30	9·50	0·75
	2^{14}C DL MVA	14·40	3·60	3·20			
81	$^{14}CO_2$	1·27	1·60	0·21	1·75	5·75	0·71
	1-^{14}C acetate	3·75	9·00	2·12	2·80	7·00	0·75
	2^{14}C DL MVA	14·00	4·80	3·90			
243	$^{14}CO_2$	0·75	1·00	0·25	1·70	4·25	0·75
	1-^{14}C acetate	1·75	5·50	3·50	2·50	5·00	0·73
	2^{14}C DL MVA	13·80	2·40	4·70			

The results are presented in percentage of radioactivity administered.

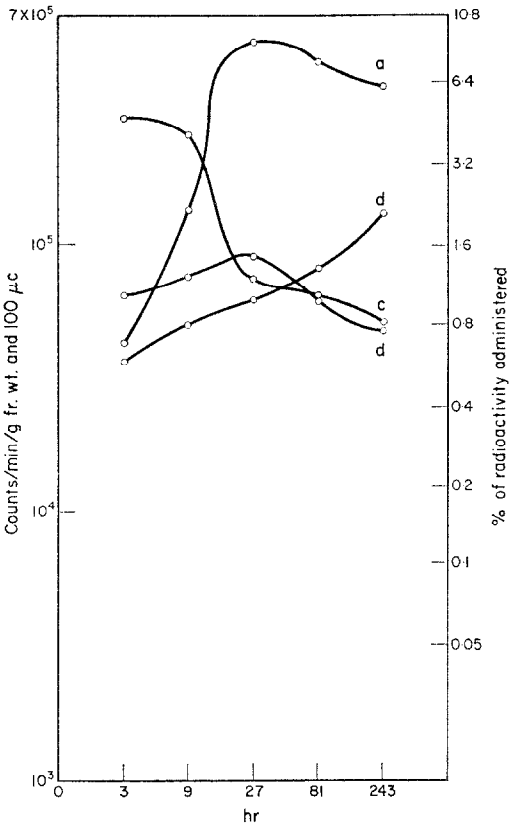

FIG. 5. THE DYNAMICS OF INCORPORATION OF 2-^{14}C-DL-MEVALONATE INTO: (a) STEROLS, (b) METHYLSTEROLS, (c) β-AMYRIN, (d) OLEANOLIC ACID IN THE SHOOTS OF *C. officinalis* AT ILLUMINATION OF 3000 lx.

Mevalonate, introduced into the cell, remains in the cytoplasm where it is utilized for the synthesis of sterols and triterpenes. Fatty acids to which mevalonate may be incorporated only after degradation to acetate, are poorly labelled by this precursor. Light intensity does not affect the incorporation of mevalonate which represents a purely enzymatic process taking place in the cytoplasm.

1-^{14}C-acetate penetrating to the cytoplasm is first of all utilized in the synthesis of fatty acids and then in the synthesis of triterpenoids. As acetate enters the chloroplasts the incorporation of this precursor is affected by the intensity of light. The labelling of fatty acids and triterpenes by acetate is much lower at 40,000 than at 3000 lx, the incorporation of this precursor into sterols is less affected by the intensity of light. The utilization of the greater part of acetate in photosynthetic syntheses in chloroplasts is clearly intensified by the high intensity of light.

$^{14}CO_2$ at higher illumination is incorporated into fatty acids less intensively and into triterpenoids more intensively than at lower illumination. The intensity of light influences then the synthesis of fatty acids from CO_2 in the same way as the synthesis of fatty acids from acetate, but the reverse is true for triterpenoids. In the conditions of intensive photosynthesis, at high concentration of $^{14}CO_2$ the entire metabolism of the cell is increased and

the whole cell is supplied with greater quantities of metabolities as well as of energy-carrying and reduced cofactors needed for the synthesis of mevalonate and triterpenoids also outside the chloroplasts. This explains the high rate of synthesis of triterpenoids (belonging to the group of non-plastidic terpenoids) from CO_2 at high light intensity. The low labelling of triterpenes by the 1-^{14}C-acetate at high illumination is explained if one assumes that in these conditions the greater part of triterpenes is formed from a non-radioactive CO_2 of the air assimilated by the plant during 243 hr of the experiment.

The above results suggest that there is a direct relation between the intensity of photosynthesis and the rate of the synthesis of the triterpenoids, especially of triterpenes, in the cytoplasm. The nature of the precursor of these compounds penetrating from chloroplasts to the cytoplasm is not known. It is probably not acetate because fatty acids for which acetate is a closer precursor than for triterpenoids are poorly labelled in the conditions of intensive photosynthesis. It may be acetol acetate which, as suggested by Whistance and Threlfall,[8] is an effective biosynthetic precursor of terpenoids but not of fatty acids.

EXPERIMENTAL

Material

The plants of *Calendula officinalis* variety Radio were cultivated in a lumistat and then incubated under conditions of stabilized illumination (3000, 20,000 or 40,000 lx during a 9 hr day) and constant temperature (26° during the day and 18° in the night). The lamps NARVA served as a source of light.

Administration of Radioactive Precursors

Radioactive compounds were administered to the cut shoots of 6–8 weeks old plants with removed inflorescence buds (wt 2·5 g ± 10%) by the method described previously.[4] Twenty μc of 1-^{14}C-sodium acetate was given per g fr. wt. (about 0·43 mg CH_3COONa). 2-^{14}C-DL-sodium mevalonate obtained from the lactone of 2-^{14}C-DL-mevalonic acid was administered in proportion 4 μc/g fr. wt. (about 0·09 mg of sodium mevalonate). Acetate and mevalonate were given in water solution which was absorbed completely during 1 hr. $^{14}CO_2$ in quantity of 100 μc/g fr. wt. (about 9·5 mg of CO_2) was administered into plants in a plexiglass chamber illuminated with light of intensity 40,000 lx. $^{14}CO_2$ was evolved by treating $Na_2\,^{14}CO_3$ with 1% aqueous H_2SO_4. After administration the air from the chamber was passed through a trap with 1% KOH. The radioactivity of precipitated $^{14}CO_2$ in the form of $Ba^{14}CO_3$ was counted on aluminum planchettes. Time of administration of the total dose of radioactive precursor was not longer than 1 hr.

Fractionation of the Material and Counting of Radioactivity

Fractionation of the material and isolation of free compounds and the method of determination of radioactivity were described previously.[4]

[8] G. R. WHISTANCE and D. R. THRELFALL, *Biochem. J.* **109**, 482 (1968).