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EFFECTS OF PEBULATE AND PEBULATE SULPHOXIDE ON VERY LONG CHAIN FATTY ACID BIOSYNTHESIS

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Key Word Index—*Hordeum vulgare*; Graminae; barley; *Pisum sativum*; Leguminosae; pea; thiocarbamate herbicides; pebulate; pebulate sulphoxide; fatty acid elongation.

Abstract—The effects of the thiocarbamate herbicide, pebulate (*S*-propylbutylethylthiocarbamate) and its sulphoxide were investigated *in vivo*. The sulphoxide caused greater inhibition of very long chain fatty acid (VLCFA) synthesis from [1-^{14}C]acetate than pebulate, suggesting that the former was the physiologically-active form of the herbicide. In contrast, to fatty acid elongation, *de novo* synthesis was insensitive to pebulate or its sulphoxide *in vivo*. The greater sensitivity of elongation to the sulphoxide was confirmed by *in vitro* assays which measured stearoyl-CoA or arachidoyl-CoA elongation with [2-^{14}C]malonyl-CoA. The results confirm suggestions that thiocarbamates are oxidised to their sulphoxide derivatives for full herbicidal activity and it is the latter which inhibit VLCFA and, hence, surface wax synthesis.

INTRODUCTION

Treatment of susceptible plants with thiocarbamate herbicides has been suggested to have physiological effects on the epicuticular layer and its wax components [1, 2]. These effects may alter the quality and/or the quantity of this protective layer and, hence, give rise to some of the herbicidal features of thiocarbamates. It has been hypothesised that the alterations in the epicuticular layer are caused by a disruption in the synthesis of the individual constituents of the wax. This is believed to occur due to a specific inhibition of the elongation reactions responsible for the synthesis of very long chain (> C18) fatty acids (VLCFAs) [3] which are precursors of many components in the plant surface layers, wax, cutin and suberin [4].

Harwood and Stumpf [3] reported that CDEC, diallate and EPTC (at 10^{-5} M) inhibited the incorporation of radioactivity from [^{14}C]acetate into the VLCFAs of germinating pea seeds. At these concentrations no effects upon *de novo* fatty acid synthesis were apparent. Further studies by Bolton and Harwood [5] confirmed these findings when 10^{-5} M diallate, triallate and EPTC reduced the detectable level of radiolabelled C20–C26 fatty acids in a different experimental system, aged potato slices. Again, it was proposed that the reduction in VLCFAs occurred as a result of a rather specific inhibition of fatty acid elongases which are responsible for the extra-chloroplastic synthesis of VLCFAs [6]. In contrast, thiocarbamates were less selective *in vitro* where incorporation of radioactivity from [^{14}C]malonyl-CoA into both long chain (made *de novo*) and VLCFAs were inhibited [7]. Two possible explanations were proposed for this difference in selectivity between *in vivo* and *in vitro* systems. First, the thiocarbamate could have been metabolised to a more potent and selective inhibitor of VLCFA synthesis (e.g. a sulphoxide derivative) *in vivo*. Alternatively, the difference in results from the two systems could have been due to the fact that VLCFA synthesis is concentrated in the extraplastidial compartment of the outer epithelial cells and, hence, would be likely to receive higher levels of the applied herbicide than the chloroplasts of the inner mesophyll cells where *de novo* synthesis is concentrated [see 8].

Sulphoxide derivatives are thought to be active metabolites of thiocarbamates [9]. Therefore, in order to directly assess whether sulphoxide derivatives are more active than the parent thiocarbamates against VLCFA synthesis, we have carried out experiments with pebulate (*S*-propylbutylethylthiocarbamate) and its sulphoxide on fatty acid synthesis in two experimental systems—germinating pea seeds and young barley leaves. These tissues were chosen because they are both active in VLCFA formation [3, 10]. The effects of pebulate and pebulate sulphoxide were also

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investigated further by testing individual elongase systems using microsomal fractions (from germinating pea seeds) *in vitro*. Our results are consistent with the proposal that it is the sulphoxide metabolite which herbicidally active and which is a potent inhibitor of fatty acid elongation.

RESULTS AND DISCUSSION

Effects in vivo

Two systems (germinating pea seeds and young barley leaves) were chosen as they had each been shown previously to be capable of good rates of fatty acid elongation [3, 10]. Challenge of each tissue with pebulate or its sulphoxide resulted in inhibition of growth and alterations to the structure of the epicuticular wax layer, as viewed by scanning electron microscopy and determined by chemical analysis [11]. Thus, we were confident that the two systems, which had been studied with other thiocarbamates, would be appropriate for work with pebulate (*S*-propylbutylethylthiocarbamate) and its sulphoxide.

We monitored the labelling of fatty acids from [1-^{14}C]acetate by the two preparations (Tables 1 and 2). The percentage of total radiolabel in the VLCFAs was higher in barley, where docosanoic (behenic; 22:0) and tetracosanoic (lignoceric; 24:0) acids were significant components. Similar percentage labelling of arachidic acid (20:0) was apparent for both peas and barley. The lower labelling of 22C and 24C fatty acids in germinating pea seeds was compensated for by a relative increase of acids made *de novo*, such as palmitate. In peas it has been demonstrated that, during the first 24 h of germination (in contrast to barley) fatty acid desaturases are inactive [3, 12]. Therefore, no radiolabelled oleate or polyunsaturated fatty acids were produced during this time. In contrast, barley leaves were able to label significant quantities of unsaturated fatty acids [10]. Since the 18C unsaturated fatty acids are labelled from [1-^{14}C]acetate *de novo* [8, 13], we did not separate them but continued to use a non-polar GLC column, which was more suitable for the analysis of labelled elongation products.

Preliminary experiments investigated the time course of the effect of pebulate on fatty acid labelling in the two systems. The herbicide or its sulphoxide was included within the incubation medium for pea seeds. Pebulate was found to be effective when used simultaneously with [1-^{14}C]acetate in the standard 24 h germination period. No further decrease in the relative labelling of VLCFAs was achieved by longer incubations of up to 48 h [11]. For young barley leaves, pre-incubation with pebulate for 8 h was found to be sufficient for a consistent effect on fatty acid labelling and, especially, VLCFAs to be seen [11].

The two experimental systems were incubated with increasing concentrations of pebulate or its sulphoxide (Tables 1 and 2). For peas, neither pebulate nor pebulate sulphoxide affected the total labelling of fatty acids. However, 0.5 μM pebulate reduced the labelling of both docosanoate and tetracosanoate (Table 1). No radiolabel was detected in either of these VLCFAs following treatment with 25 μM pebulate. In contrast, arachidate labelling was unaffected even though this acid is also made by elongation. This result is con-

Table 1. The effect of different concentrations of pebulate and its sulphoxide on the labelling of fatty acids from [1-^{14}C]acetate by germinating pea seeds

Treatment	Fatty acid labelling (d.p.m. $\times 10^{-4}$)	Fatty acids (% total ^{14}C-fatty acids)				
		16:0	18:0	20:0	22:0	24:0
Expt. 1						
None*	13.0	25.9	51.2	7.6	9.1	6.1
0.1 μM Pebulate	11.7 $\pm$ 0.5	16.7 $\pm$ 0.6	64.6 $\pm$ 4.2	8.7 $\pm$ 0.2	7.2 $\pm$ 3.2	2.9 $\pm$ 0.8
0.5 μM Pebulate	11.9 $\pm$ 0.6	20.1 $\pm$ 2.9	66.3 $\pm$ 3.7	6.4 $\pm$ 0.2	4.0 $\pm$ 0.7	3.2 $\pm$ 1.9
5.0 μM Pebulate	12.4 $\pm$ 1.1	19.5 $\pm$ 4.2	70.9 $\pm$ 2.3	5.9 $\pm$ 3.0	3.2 $\pm$ 1.8	0.5 $\pm$ 0.8
10.0 μM Pebulate	11.1 $\pm$ 1.1	17.9 $\pm$ 4.5	70.2 $\pm$ 3.5	8.3 $\pm$ 1.2	3.6 $\pm$ 3.2	n.d.
25.0 μM Pebulate	12.5 $\pm$ 2.1	23.1 $\pm$ 3.2	66.7 $\pm$ 5.1	10.2 $\pm$ 2.8	n.d.	n.d.
Expt. 2						
None	4.3 $\pm$ 2.0	22.6 $\pm$ 0.3	61.1 $\pm$ 2.8	6.5 $\pm$ 0.2	5.6 $\pm$ 2.1	3.7 $\pm$ 1.6
0.1 μM pebulate sulphoxide	3.3 $\pm$ 0.8	15.5 $\pm$ 1.5	69.2 $\pm$ 0.7	8.5 $\pm$ 0.3	4.7 $\pm$ 0.7	2.0 $\pm$ 1.4
0.5 μM pebulate sulphoxide	2.9 $\pm$ 0.8	16.4 $\pm$ 1.8	77.8 $\pm$ 1.9	5.3 $\pm$ 0.1	0.9 $\pm$ 0.9	0.3 $\pm$ 0.6
5.0 μM Pebulate sulhoxide	4.9 $\pm$ 0.9	19.3 $\pm$ 4.7	74.8 $\pm$ 2.1	5.9 $\pm$ 3.1	n.d.	n.d.
10.0 μM pebulate sulphoxide	3.9 $\pm$ 0.6	16.4 $\pm$ 2.6	81.7 $\pm$ 3.7	1.9 $\pm$ 1.8	n.d.	n.d.
25.0 μM pebulate sulphoxide	4.6 $\pm$ 0.8	19.3 $\pm$ 3.4	77.8 $\pm$ 3.8	2.3 $\pm$ 2.6	0.7 $\pm$ 1.3	n.d.

Results are means $\pm$ S.D.s ($n = 3$). * Denotes single sample. n.d. = none detected. Pea seeds were germinated in the presence of different concentrations of pebulate or pebulate sulphoxide and [1-^{14}C]acetate (1 μCi).

Table 2. The effect of different concentrations of pebulate and its sulphoxide on the labelling of fatty acids from [1-^{14}C]acetate in young barley leaves

Treatment	Fatty acid labelling (d.p.m. × 10^{-4})	Fatty acids (% total ^{14}C-fatty acids) 16:0	*18C	20:0	22:0	24:0
Expt. 1						
None	6.5±1.2	27.8±3.9	29.1±3.2	6.3±0.9	19.5±1.6	17.2±6.3
0.1 μM Pebulate	3.2±1.5	31.9±7.9	30.6±6.1	5.0±3.3	17.1±3.4	15.4±4.6
0.5 μM Pebulate	2.2±0.3	38.9±4.2	34.7±3.1	5.0±2.0	12.8±2.9	8.6±2.9
5.0 μM Pebulate	1.4±0.1	39.8±2.4	35.0±4.5	4.3±2.3	10.9±0.5	10.0±4.7
10.0 μM Pebulate	2.7±0.9	42.7±2.4	35.0±1.5	4.6±0.5	11.6±0.8	6.1±0.5
Expt. 2						
None	15.6±1.4	23.8±5.2	41.0±6.8	4.3±0.7	18.9±4.2	12.0±1.4
0.1 μM pebulate sulphoxide	9.6±1.1	31.7±7.3	41.2±6.9	4.3±2.4	12.2±4.1	10.2±1.8
0.5 μM pebulate sulphoxide	10.8±8.0	23.8±4.1	53.4±10.2	4.6±0.5	10.8±2.2	7.4±3.6
5.0 μM pebulate sulphoxide	4.0±0.6	25.3±2.2	62.9±2.3	3.1±0.3	6.6±1.9	2.9±2.7
10.0 μM pebulate sulphoxide	3.2±1.6	38.7±6.5	49.6±5.8	2.2±0.9	5.3±3.8	4.2±3.9

Results are means±S.D.s (n = 3). * The 18C acids were not resolved from each other on the non-polar column used (see Experimental) but other experiments showed that oleate was the major radiolabelled component. Young barley leaves (10 days old) were incubated with different concentrations of pebulate or pebulate sulphoxide prior to incubation with 2 μCi [1-^{14}C]acetate.

sistent with the presence of different enzyme systems for stearate and arachidate elongation. That separate elongases were involved in the production of different VLCFAs was first suggested by genetic experiments [14]. Later, by the use of protein synthesis inhibitors during the induction of elongation enzymes in aged potato slices, direct biochemical evidence was provided [15]. In recent years, the isolation of chain-length specific elongases from leek has confirmed these conclusions [6]. Moreover, the leek elongases have slightly different properties as well as distinct subcellular locations [16]. For peas, assay of fatty acid elongation by microsomal fractions has also revealed the presence of separate stearoyl-CoA and arachidoyl-CoA elongases [17]. Thus, a difference in inhibition of these two systems by pebulate (Table 1) is not surprising.

The sulphoxide derivative of pebulate was incubated with germinating pea seeds under the same conditions as for the parent herbicide. Inhibition of fatty acid elongation was significantly greater than for comparable concentrations of pebulate (Table 1). Thus, no detectable radiolabelled docosanoate or tetracosanoate was found for 5 μM pebulate sulphoxide and, moreover, the labelling of arachidate was also inhibited at 10 or 25 μM concentrations. These results are consistent with the idea that the sulphoxide derivative is the herbicidally active chemical and that pebulate is converted to its sulphoxide *in vivo*.

For young barley leaves, pebulate reduced total labelling of fatty acids at all concentrations used (Table 2). This result may be connected with the fact that grasses tend to be more susceptible physiologically than dicotyledons such as pea [18]. However, again, the proportional labelling of VLCFAs was reduced, indicating that the elongases were specially sensitive. Significant reductions in docosanoate and tetracosanoate labelling were seen with 0.5 μM pebulate. As with pea, there was no statistically significant reduction in the proportion of arachidate labelling at concentrations of pebulate up to 10 μM. For pebulate sulphoxide the inhibition seen was more pronounced. Total fatty acid labelling was reduced by up to 80%, while the proportion of VLCFAs declined from 35 to 12%. Labelling of arachidate was halved while in docosanoate and tetracosanoate labelling was reduced by about 70% (Table 2). Again, as with peas, the data show clearly that the sulphoxide is more active and suggest that pebulate is activated to its sulphoxide *in vivo*.

From the data given in Tables 1 and 2 and equivalent experiments, we were able to calculate I_{50} values for the inhibition of fatty acid elongation in the two experimental systems. The results (Table 3) showed that pebulate sulphoxide was always more effective than its parent compound. The relative effectiveness of the sulphoxide varied from 5- to 20-times that of pebulate, depending on the elongation product measured and the experimental system used. The data agrees with the hypothesis, based on indirect experiments, that thiocarbamates are activated to sulphoxides which are the more effective inhibitors of fatty acid elongation [7].

Sulphoxidation of thiocarbamate compounds is generally considered to be mediated by microsomal NADPH-dependent cytochrome P_{450} monoxygenases [19]. Experiments with 1-aminobenzotriazole, an inhibitor of mixed function oxidase activity, resulted

Table 3. I_{50} values (μM) for the inhibition of VLCFA labelling from [1-^{14}C]acetate by barley leaves or germinating pea seeds by pebulate or its sulphoxide

	Pea seeds		Barley leaves	
Fatty acid product	Pebulate	Pebulate sulphoxide	Pebulate	Pebulate sulphoxide
C20	> 100	8.7 ± 2.1	n.a.	n.a.
C22	3.9 ± 2.0	0.2 ± tr.	15.0 ± 5.9	1.8 ± 0.8
C24	0.5 ± 0.2	0.1 ± tr.	15.0 ± 6.0	1.0 ± 0.4

I_{50} values (μM) given as means ± S.D.s (n = 3). n.a. = not available (no significant inhibition of arachidate labelling in barley). tr. = trace (< 0.05).

in the blocking of the specific inhibitory effect of the thiocarbamate, diallate, on fatty acid elongation [7]. This result supported the proposal that oxidation of diallate involved mixed function oxidases. However, 1-aminobenzotriazole was not effective against pebulate [11] which may be oxidised by the action of peroxidases [20] or an unusual enzyme, the peroxygenase [21], both of which have also been implicated in (sulph)oxidation.

Effects in vitro

The effects of pebulate and its sulphoxide on VLCFA synthesis were investigated in more detail using microsomal fractions from germinating peas. Specific elongase assays were developed for the elongation of stearate and arachidate, respectively [11, 17]. The assay conditions were optimised (see Experimental) and acyl-CoAs were used as exogenous substrates in common with other standard assays for fatty acid elongation [see 16]. The assays were carried out in the presence of pebulate or its sulphoxide and analysis of the radiolabelled products by reverse-phase HPLC revealed that stearoyl-CoA elongation was unaffected by pebulate even at a relatively high concentrations (Table 4). However, pebulate at 2.5×10^{-5} M did inhibit the arachidoyl-CoA elongation significantly. These results supported the data from *in vivo* experiments (Table 1) where behenate formation was more sensitive to inhibition by pebulate than stearate elongation to arachidate. Both elongations were more sensitive to inhibition by the sulphoxide which gave progressive inhibition with increasing concentrations (Table 5) with statistically significant differences being apparent at 1 μM and above. It will also be noted that for both assays there was some further elongation of the direct product of the enzymatic reaction. This was expected since a limited amount of further elongation is consistently observed for elongase assays using [^{14}C]malonyl-CoA [22].

The results from these *in vitro* experiments support our interpretation of the experiments *in vivo* and our previous conclusions [7] that (a) sulphoxides are more effective inhibitors of fatty acid elongation than the parent thiocarbamate and (b) the elongation of arachidate is more easily inhibited than that of stearate. Except at high concentrations (above 5×10^{-4} M), pebulate sulphoxide had no effect on *de novo* fatty acid synthesis, again supporting the idea that sulphoxide metabolites are responsible for the effectiveness and specificity of thiocarbamates with whole plants. Of course, it should also be borne in mind that the rapid inactivation of thiocarbamate derivatives *in vivo* [23] will also have a strong influence on the selectivity of herbicidal activity in the field.

Conclusions

Our experiments have shown clearly that fatty acid elongation is much more sensitive *in vivo* to inhibition by pebulate and its sulphoxide than is *de novo* fatty acid synthesis. Moreover, the sulphoxide derivative is considerably more potent than its parent compound, confirming the suggestion that the sulphoxide is the active herbicidal compound. Experimental results *in vitro* also suggest that pebulate sulphoxide is relatively selective towards elongation reactions, thus providing an explanation as to why its precursor, pebulate, is selective *in vivo* but less so *in vitro*. The reason for the specific effect on elongation and the nature of the interaction between pebulate sulphoxide and the proteins involved in elongase reactions, remain important topics for future research.

EXPERIMENTAL

Materials

Barley (*Hordeum vulgare*, cv. Maris Otter) and pea (*Pisum sativum* cv. Feltham First) seeds were obtained from Plant Breeding Int., Cambridge and Asmer Seeds, Leicester, U.K. [1-^{14}C]Acetate, Na salt (sp. act. 1.85 GBq mmol$^{[mom01]}$) and [2-^{14}C]malonyl-CoA (sp. act. 1.85 GBq mmol$^{[mom01]}$) were from Amersham. Fatty acid Me ester (FAMEs) standards were from Nu-Check Prep. Inc. (P.O. Box 172, Elysian, Mn 56028, U.S.A.) and silica gel G plates from Merck. Pebulate and pebulate sulphoxide were kind gifts from C. J. Spillner (Stauffer, I.C.I. Americas, Richmond, Calif., U.S.A.). To keep pebulate and its sulphoxide

Table 4. The effects of pebulate and pebulate sulphoxide on fatty acid synthesis by pea seed microsomal fractions in fatty acid elongase assays

Treatment	Stearoyl-CoA elongase assay (fatty acid labelling–d.p.m. × 10^{-3})				Arachidoyl-CoA elongase assay (fatty acid labelling–d.p.m. × 10^{-3})			
	Total fatty acids	⩽ C18	C20	Total VLCFAs	Total fatty acids	⩽ C18	C22	Total VLCFAs
None	2.2 ± 0.2	0.7 ± tr.	1.3 ± 0.2	1.5 ± 0.3	4.3 ± 0.3	0.8 ± tr.	2.9 ± 0.2	3.6 ± 0.3
P	3.3 ± 0.4	1.1 ± 0.4	1.6 ± 0.1	2.1 ± tr.	3.1 ± 0.2*	0.7 ± tr.	2.0 ± 0.1*	2.4 ± 0.1*
PS	1.6 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.2 ± 0.2*	0.2 ± tr.*	0.8 ± 0.1*	1.0 ± 0.1*

Results = means ± S.D.s ($n = 2$). $P = 2.5 \times 10^{-5}$ M pebulate, $PS = 2.5 \times 10^{-5}$ M pebulate sulphoxide, tr. = < 20 d.p.m. *Significantly different from untreated controls by Student's *t*-test ($p < 0.05$). Microsomes were incubated with exogenous CoA derivatives and the necessary incubation medium in the presence of pebulate or its sulphoxide derivative (see Experimental).

derivative in suspension it was necessary to have EtOH present at a final concn of 2.5% or 0.5% for whole tissue or *in vitro* studies, respectively. These concns of solvent were shown to have no significant effect on either the total labelling of fatty acids or the pattern of incorporation in either of the systems used [11]. The exact conditions for herbicide treatment are shown in the legends to the tables.

Labelling experiments in vivo. Barley seeds were germinated in damp vermiculite for 10 days at 20° in a 16 h day/8 h night cycle with 650 μE s^{-1} m^{-2} illumination in a Gallenkamp illuminator. Barley leaves were detached from their seeds under water to prevent an air gap in the transpiration stream. They were incubated with 2 μCi [1-^{14}C]acetate per three leaves, essentially under conditions described previously [24]. Pea seeds were germinated with 1 μCi [1-^{14}C]acetate in 1 ml H_2O [3] at 20°. At the end of the incubation period, tissues were rinsed in H_2O to remove unimbibed radiolabel and reactions terminated by the addition of 1.25 ml of *iso*-propanol and heating at 70° for 30 min. Further lipid extraction [25] and the preparation of FAMEs were as previously described [26].

Microsomal incubations. Microsomal fractions were prepared from germinating pea seeds [26]. Microsomal membranes (ca 2 mg protein) were incubated for 3 h at 25° in the presence of 0.05 μCi [2-^{14}C]malonyl-CoA, 20 mM K-Pi buffer, pH 7.0 and 9 μM stearoyl- or archidoyl-CoA in a final vol. of 1 ml. The final concn of exogenously added cofactors were 0.2 mM NADPH and 3 mM ATP or 0.5 mM NADPH and 5 mM ATP for stearoyl-CoA and archidoyl-CoA elongation, respectively. These concns were optimal [11]. Herbicides were added in EtOH. Control tubes contained the equivalent concn of EtOH (0.5% v/v, final conc.). Reactions were terminated by the addition of 0.1 ml of 60% KOH and heating at 70° for 30 min. After cooling, the reaction mixture was acidified with 0.3 ml 20% (v/v) H_2SO_4 and the non-esterified fatty acids extracted into $CHCl_3$.

Lipid analysis. FAMEs were prepared as previously [26] and analysed using a Pye-Unicam GCD gas chromatograph connected via an effluent splitter to a Lab-Logic RAGA (LabLogic, Sheffield, U.K.) gas flow proportional counter. Glass columns (1.5 m × 4 mm int. diam.) packed with 5% SP-2100 on 100/120 Supelcoport (Supelco, Walden, Essex) were employed. A temp. programme (initial temp. 210° for 10 min, then 4°/min to 250°) was routinely used for separations.

Fatty acid phenacyl derivatives were prepared [27] and separated on a reverse-phase HPLC column (5 μM, 25 cm × 4.6 mm) (Spherisorb, Phase Separations Ltd., Clwyd, U.K.) using a Varian 9050 UV detector operating at 242 nm. A gradient elution programme of MeCN/H_2O (4:1, v/v) initially, increasing linearly over 20 min to 100% MeCN (flow rate 2 ml/min) was used. Fatty acid identifications were made routinely by comparison with authentic standards but fuller checks had been made as previously described [28].

Table 5. Effect of different concentrations of pebulate sulphoxide in the arachidoyl-CoA elongation assay with pea microsomes

Conc. of pebulate sulphoxide (μM)	Fatty acid labelling (d.p.m.)		
	≤C18	22:0	24:0
0.0	383 ± 94	2802 ± 143	3106 ± 134
0.1	302 ± 21	1954 ± 548	2159 ± 633
0.5	296 ± 23	1686 ± 454	1873 ± 465
1.0	217 ± 9	1613 ± 99*	1732 ± 126*
5.0	98 ± 50	1343 ± 2*	1471 ± 32*

Results show means ± S.D.s ($n = 3$). Labelling was from [2-^{14}C]malonyl-CoA under conditions as described in Experimental. * Significantly different from controls (no pebulate sulphoxide) as analysed by Students t-test ($p < 0.05$).

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