Phytochemistry, 1976, Vol. 15, pp. 1501–1506. Pergamon Press. Printed in England.

FATTY ACID SYNTHESIS IN AGED POTATO SLICES

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(*Received* 11 *March* 1976)

Key Word Index—*Solanum tuberosum*; Solanaceae; potato; aged potato; slices; fatty acid synthesis; inhibitor action; complex lipid labelling.

Abstract—Synthesis of fatty acids has been studied in aged potato slices. Formation of the very long chain fatty acids was inhibited by the presence of fluoride or by high incubation temperatures. Arsenite caused an increase in the percentage incorporation of radioactivity from acetate-[^{14}C] into palmitic acid, apparently by inhibiting further elongation. The results indicate that the aged potato contains at least three enzymes responsible for saturated fatty acid synthesis. At short incubation times, the newly formed fatty acids were mainly unesterified but later become incorporated into phospholipids. Phosphatidylcholine contained the greatest proportion of radioactive fatty acids. Newly formed polyenoic fatty acids were principally transacylated into phosphatidylcholine and phosphatidylethanolamines. The very long chain fatty acids, on the other hand, were mainly located in the wax ester and unesterified fatty acid fractions, from which they can easily be converted into suberin components.

INTRODUCTION

Synthesis of saturated fatty acids has been studied in a number of higher plant systems [1]. Recently it has become clear that a number of different enzymes are involved, namely fatty acid synthetase (which produces palmitic acid) and at least two elongases [2].

Fatty acid synthetase, which has been demonstrated in several plant systems [2], has been partially purified from potato by Huang and Stumpf [3] who showed that the chain length of the product could be altered by the incubation conditions, particularly the concentration of acyl carrier protein. In contrast to the tightly bound yeast and animal synthetase complexes [4,5], the plant enzymes require exogenous acyl carrier protein (ACP) for full activity [6].

The first of the elongation enzymes is that which converts palmitic into stearic acid. Evidence from labelling studies indicated the presence of this enzyme in leaves from the *Gramineae* [7], in germinating pea [8,9] and in avocado mesocarp [10]. It has recently been studied using avocado [10,11] and developing safflower seeds [12] where palmityl-ACP is the substrate and malonyl-CoA the 2C donor. In the case of avocado mesocarp, the palmityl-ACP elongase probably arises from the plastid [11,13] whereas the fatty acid synthetase is present in the cytosol [11].

Harwood and Stumpf [8] suggested that the pea contains an additional elongase(s) which converts stearate into the very long chain fatty acids. The enzyme is apparently most active in the microsomal fraction [8,9] and may be analogous to mammalian microsomal elongating systems [14,15]. The second pea elongase was sensitive to fluoride, thiocarbamate weed-killers and raised temperature in contrast to the palmitate elongase which is inhibited by arsenite [8]. The inhibition of very long chain fatty acid synthesis by thiocarbamates was later confirmed by Kolattukudy and Baker [16].

In order to study the elongation reactions further, we have used the aged potato slice—a system which is capable of particularly high rates of very long chain fatty acid synthesis [17]. Results of such studies are now reported.

RESULTS AND DISCUSSION

Extraction and methylation of ^{14}C-labeled fatty acids

A number of methods for extraction of ^{14}C-fatty acids, formed during incubation of slices with acetate-[^{14}C], were studied (see Experimental). Of these, methods (c), (d) and (e) were the most efficient. All the methods gave a similar pattern of ^{14}C-labeled fatty acids. Although method (d) gave a somewhat higher extraction of ^{14}C-fatty acids than method (c), it also gave rise to a number of unidentified side products. Methods (c) and (e) gave comparable results except that the latter was somewhat more reproducible and was, therefore, adopted as the method of choice.

We also studied methylation procedures including acid and alkaline methanol, in the presence or absence of benzene, as well as the method described. Of these, BF_3–methanol gave the most reproducible and efficient results and was, therefore, utilised.

Time course of ageing

Willemot and Stumpf [17] have reported a rapid increase in the ability of potato slices to synthesise fatty acids and this is reflected in the total counts in Table 1. Total incorporation increased for about 6 hr, in agreement with previous data [17], and then declined. The distribution of counts in fatty acids synthesised by potato

Table 1. Effect of ageing on fatty acid synthesis by potato slices

Ageing time (hr)	Total fatty acids (dpm × 10^{-3})	Distribution of radioactivity (% Total counts in fatty acids) C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	Other
0	8.9 ± 0.5	25.7 ±1.9	74.3 ±1.3	n.d.	n.d.	n.d.	tr
2	21.5 ± 3.1	45.3 ±1.0	52.7 ±1.3	0.7 ±0.2	0.5 ±0.1	n.d.	0.8 ±0.5
4	25.9 ± 2.6	36.7 ±0.3	51.8 ±3.8	4.3 ±2.1	7.2 ±1.3	tr	tr
6	32.1 ± 0.5	40.7 ±2.0	46.7 ±6.1	4.3 ±1.4	8.3 ±0.8	tr	tr
16	24.9 ± 0.6	23.0 ±4.7	40.7 ±4.7	11.5 ±0.8	12.8 ±1.2	12.0 ±0.3	tr
20	23.4 ± 0.3	20.5 ±0.8	53.0 ±1.9	9.3 ±0.3	8.7 ±0.9	8.5 ±0.2	tr
24	22.6 ± 1.1	21.2 ±0.5	68.0 ±1.8	4.1 ±0.6	3.9 ±2.1	2.8 ±0.7	tr

Results represent the means ± S.D. (4 expts). For experimental details see Experimental. Analysis was in columns containing SE-30 which separates fatty acids on the basis of chain length. n.d. = not detected.

slices as a function of the period of ageing is also shown. There was a marked decline in the proportion of palmitic acid synthesised and an increase in the very long chain fatty acids with time. Eicosanoic, docosanoic and tetracosanoic acids were similarly affected. These results agree with Willemot and Stumpf [17] who indicated that the proportion of label in total very long chain fatty acids increased and that in palmitic acid declined with time. The maximal percentage of counts in the very long chain fatty acids was after about 18 hr of ageing when they accounted for up to 35% of the total radioactivity in fatty acids. Although we usually separated fatty acids on the basis of chain length only, additional experiments indicated that the C_{18} fraction (Table 1) showed similar proportions of stearic, oleic and linoleic acids to results previously reported [17]. Therefore, there seems to be little difference with regard to the ageing characteristics of the different potato varieties that have been used to synthesise fatty acids.

Since the supply of plant material during the year is somewhat variable, we felt it important to be aware of any changes in the proportions of very long chain fatty acids produced. Mature potatoes (which had been stored) produced a significantly higher proportion of very long chain fatty acids than young (new) potatoes (Table 2). This is most probably due to the age of the potato at harvesting since no change was found in the pattern of acids produced by a given batch of potatoes on storage. It is noticeable however (Table 2), that young potatoes gave higher rates of total incorporation than mature tissue.

Table 2. Effect of tissue maturity on fatty acid synthesis

Ageing time (hr)	Total fatty acids (dpm × 10^{-3}) Old	New	% counts as C_{20}–C_{24} acids Old	New
4	2.6 ±0.3	9.9 ±1.0	13.5 ±2.5	3.2 ±1.3
24	8.9 ±1.9	19.4 ±2.0	12.7 ±0.8	4.2 ±2.1

Analysis of fatty acids was by reversed phase TLC. For experimental details see Experimental. "New" potatoes were the early crop, harvested in July and "old" potatoes were the normal, fully mature, crop. Results represent means ± S.D. (3 expts).

Effect of temperature

The elongation steps to produce very long chain fatty acids in germinating pea proved to be rather heat-labile [8]. We, therefore, examined the effect of incubation temperature on the production of ^{14}C-labeled fatty acids. As Table 3 shows, slices incubated at 30° showed the highest rates of acetate-[^{14}C] incorporation into fatty acids. The amount of label in very long chain fatty acids declined from 25% at 25° to only 6% at 40°. Thus, as in pea, the elongase(s) which are responsible for forma-

Table 3. Effect of temperature on fatty acid synthesis by aged potato discs

Incubation Temperature	Number of expts	Fatty acids (dpm × 10^{-3})	Distribution of radioactivity* (% total counts in fatty acids) C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	Other
20°	3	7.2 ± 1.9	21.7 ± 2.5	56.7 ± 6.7	9.3 ± 3.1	7.1 ± 2.7	4.5 ± 1.5	0.7 ± 0.3
25°	4	8.9 ± 1.8	22.6 ± 3.4	52.2 ± 4.9	8.0 ± 2.3	10.1 ± 3.1	7.1 ± 1.6	tr
30°	3	15.5 ± 2.8	28.0 ± 6.8	57.1 ± 8.3	4.1 ± 1.7	5.4 ± 1.7	5.4 ± 1.1	tr
35°	1	10.3	39.3	52.9	3.8	4.0	tr	tr
40°	3	7.9 ± 0.3	34.6 ± 3.2	59.5 ± 0.3	4.3 ± 0.6	0.5 ± 0.4	1.1 ± 1.1	tr

Results are expressed as means ± S.D. Slices were aged for 18 hr at 25° in the dark. For incubation, extraction and analysis see Experimental. *See Table 1.

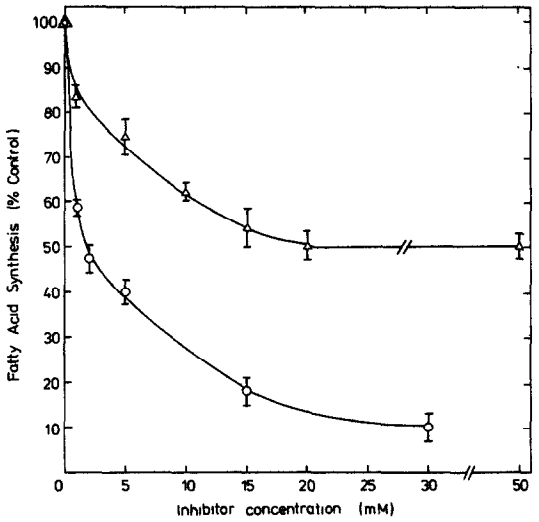

Fig. 1. Effect of inhibitors on acetate-[^{14}C] incorporation into fatty acids. Means ±S.D.s are shown. For experimental details see Experimental. All slices were aged for 18 hr at 25°: O——O synthesis in presence of fluoride; Δ----Δ synthesis in presence of arsenite.

tion of these acids in potato seem to be somewhat heat labile. Synthesis of eicosanoic, docosanoic and tetracosanoic acids are all reduced and there is no evidence, in these data, for more than one elongase.

Effect of metabolic inhibitors

Use of the inhibitors, arsenite and fluoride, in pea (8) produced evidence for at least three enzyme systems synthesising saturated fatty acids. Although arsenite has consistently inhibited palmitate elongation in a number of systems[8,10,12,13] the effect of fluoride was less consistent[8]. Both inhibitors reduced the incorporation by potato discs of acetate-[^{14}C] into fatty acids (Fig. 1). Fluoride had a larger effect than arsenite, as had been previously observed with germinating pea and safflower [8]. The percentage inhibition was similar, at comparable concentrations, to that produced in pea.

Increase in the concentration of arsenite resulted in a progressive increase in the relative proportion of palmitic acid (Table 4). Even allowing for the overall reduction in counts, this meant that incorporation of radioactivity into palmitic acid was unimpaired. Elongation to stearic and very long chain fatty acids was, therefore, inhibited by arsenite. Total inhibition of synthesis of C_{18} fatty acids by arsenite was less than the highest figures for pea [8] and avocado [10]. This may be due to unsaturated acids representing a high proportion of the C_{18} peak since their synthesis is less affected [18]. If both stearate and the very long chain fatty acids in potato slices are formed by elongation reactions involving malonyl CoA, such as has been suggested in other plant tissues [2,8,10–13,19], the mechanism of 2C addition may be similar for the two processes and, hence, arsenite will inhibit both.

Fluoide caused an increase in the proportion of label incorporated into C_{18} fatty acids at the expense of the very long chain fatty acids (Table 5). These results are similar to the germinating pea system [8] and provide additional evidence for separate elongation enzymes. Since fluoride will form complexes with a variety of

Table 4. Effect of arsenite on the distribution of radioactivity from acetate-[^{14}C] in fatty acids

Arsenite conc. (mM)	No. of Expts	Distribution of radioactivity* (% Total in fatty acids) C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	Others
0	8	21 ± 2	42 ± 2	11 ± 1	15 ± 1	11 ± 2	tr
1	4	40 ± 4	35 ± 4	6 ± 4	11 ± 2	8 ± 3	tr
5	6	42 ± 1	54 ± 3	1 ± 1	3 ± 1	tr.	tr
10	3	55 ± 2	38 ± 7	2 ± 1	2 ± 1	3 ± 1	tr
15	2	58 ± 6	42 ± 4	n.d.	n.d.	n.d.	tr
20	3	70 ± 6	30 ± 6	n.d.	n.d.	n.d.	tr
50	2	69 ± 7	29 ± 3	n.d.	n.d.	n.d.	2 ± 1

Slices were aged for 18 hr at 25°. Values represent means ± S.D. and are corrected to whole numbers trace = <0.5%. n.d. = not detected. *See Table 1.

Table 5. Effect of fluoride on fatty acids synthesised by aged potato slices

Fluoride conc. (mM)	No. of expts	Distribution of radioactivity* (% Total in fatty acids) C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	Others
0	6	25 ± 3	62 ± 4	5 ± 1	5 ± 2	3 ± 1 (11)	tr
1	2	12 ± 3	80 ± 4	3 ± 1	5 ± 2	n.d. (8)	tr
5	4	17 ± 3	82 ± 5	tr	1 ± tr.	n.d. (1)	tr

Slices were aged for 18 hr at 25° and then incubated at 30° for 2 hr. Figures represent means ± S.D. and are corrected to whole numbers. tr. = trace (<0.5%). n.d. = not detected. *See Table 1.

Table 6. Time course of acetate-[^{14}C] incorporation into lipid classes

Incubation (hr)	Total incorp. (dpm × 10^{-5})	Distribution of counts (% Total) PI	PC	PE	PA	Other polar lipids	Neutral
½	13.2	3.3	7.0	4.8	8.6	1.1	75.2
1	23.7	7.8	27.5	11.7	11.7	1.7	39.6
2	34.3	5.4	30.0	9.8	12.1	1.8	40.9
4	37.0	7.8	28.8	12.0	12.2	1.2	38.0

Abbreviations: PI = phosphatidylinositol, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PA = phosphatidic acid. Slices were aged for 18 hr at 25° and incubated with 4 μCi of acetate-[^{14}C]/ml of medium at 25°. For extraction and separation of lipids see Experimental.

metal containing enzymes, including those dependent on Fe, Ca, and Mg [20] it is tempting to speculate if divalent cations are involved in the elongation of stearic acid. Alternatively, the reason for fluoride inhibition of C_{20}–C_{24} fatty acid synthesis may be due to an indirect effect on metabolism and, hence, supply of cofactors. In this connection it is worth noting that the subcellular location of the two elongation systems in pea is probably different [8–10].

Complex lipid labeling

Newly synthesised fatty acids are usually rapidly incorporated into complex lipids by plant tissues (see [21]). This is true both for *in vivo* experiments such as with developing [22] or germinating seeds [23–25] as well as for subcellular fractions [26–28]. In studies with microsomes prepared from aged potato slices, it has been noted that newly synthesised linoleic acid is 80% localised in phosphatidylcholine [28]. This may be because phosphatidylcholine is the actual substrate for oleate desaturation [27] or because there is a specific and very active linoleyl transacylase [26].

When the incorporation of acetate-[^{14}C] into complex lipids was followed (Table 6) we found that phospholipids became rapidly labeled. ^{14}C-Labeled fatty acids were a major component of the neutral lipid fraction. Although total incorporation increased over a 2 hr incubation period, the proportion of counts in the various fractions reached equilibrium after 1 hr. Because mitochondrial [29] and microsomal [30] membrane formation is rapid in ageing potato slices and because phospholipids are the major lipid components of such membranes [31] we paid particular attention to separation of this lipid class. As expected, phosphatidylcholine contained the highest amount of radioactivity but phosphatidylinositol and phosphatidylethanolamine, the two other major phospholipids located outside chloroplasts [31], were also highly labeled. Phosphatidic acid, a key intermediate in phospholipid synthesis [32], has a particularly high sp. act., as would be expected in a system where membrane synthesis is so rapid.

These results can be compared with those obtained with other plant systems where membrane synthesis is important. Thus, Hawke, Rumsby and Leech [33] noted that phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were rapidly labeled by acetate-[^{14}C] in the leaves of developing maize. Katayama and Funashi [34] measured orthophosphate-[^{32}P] labeling of phospholipids in *Phaseolus radiatus* seedlings. They noted high labeling of phosphatidylcholine and phosphatidylethanolamine in the cotyledons and of phosphatidylethanolamine in the hypocotyls and radicles. *Pisum sativum* gave rapid incorporation of acetate-[^{14}C] into phosphatidylinositol. At longer germination times, phosphatidylcholine accounted for more total radioactivity [23]. In *Corylus avellana* phosphatidylcholine and phosphatidic acid were rapidly labelled [25]. The main difference between the incorporations obtained using aged potato and those with germinating seeds or developing leaves is the high sp. act. of the important chloroplast phospholipid, phosphatidylglycerol, in the latter two systems [23–25,33]. Phosphatidylglycerol is only present in trace amounts in aged potato slices, which is typical of non-photosynthetic tissue [31].

It is clear from the results obtained by a number of workers that the fatty acid contents of the various plant complex lipids are different and, to some extent, characteristic of a given lipid [21,31]. Thus it could be antici-

Table 7. Distribution of ^{14}C-fatty acids within major lipid classes

Lipids	% Total radioactivity in fatty acids 16.0	16.1	18.0	18.1	18.2	18.3	20.0	22.0	24.0	Others
Wax ester	23.5	0.2	23.9	32.6	6.5	tr	6.1	6.1	tr	0.1
Triacylglycerol	23.4	0.4	25.7	39.9	4.3	tr	4.2	1.5	tr	0.6
Un-esterified fatty acids	32.7	0.4	22.6	30.7	1.6	n.d.	8.0	3.8	tr	0.2
Phosphatidylethanolamine	23.9	tr	16.6	41.6	16.7	0.7	tr.	tr.	n.d.	0.5
Phosphatidylcholine	29.1	tr	9.0	35.6	22.9	3.4	tr.	tr.	n.d.	tr
Phosphatidylinositol	44.4	4.6	21.5	24.5	tr	n.d.	5.0	tr.	n.d.	tr
Total	24.2	0.5	24.0	29.0	6.3	0.8	7.2	7.0	1.0	tr

Slices were aged for 18 hr at 25°. Incubation with acetate-[^{14}C] was for 2 hr at 25°.

For extraction and lipid separation see Experimental. Fatty acids are indicated with the number before the colon indicating the chain length and the figure after denoting the number of double bonds. The position of the double bonds was determined and 16:1 was found to be palmitoleic acid, 18:1 was oleic acid, 18:2 was linoleic acid and 18:3 was α-linolenic acid.

pated that the incorporation of newly synthesised fatty acids into glycerolipids will vary according to the types concerned. In Table 7, it can be seen that the major complex lipids which were labeled in potato slices showed differences in the percentage distribution of radioactivity between fatty acids. A few features can be emphasised. Palmitic acid-[^{14}C], although present in all lipid classes, was highest in phosphatidylinositol. This has been observed in several other plant systems [23–25]. At the same time stearic acid-[^{14}C] was lowest in phosphatidylcholine as was also the case in germinating soya bean [24]. It has been noted by a number of workers [26–28] that newly synthesised linoleic acid is present in high amounts in phosphatidylcholine. When examining desaturation of oleyl-[^{14}C] CoA by microsomes prepared from aged potato slices, Abdelkader *et al.* [28] also observed that the product linoleate-[^{14}C] was preferentially esterified in phosphatidylcholine. Phosphatidylethanolamine was the only other phospholipid containing any. Other experiments, with a variety of plant tissues (e.g. [24,33]) have confirmed that the nitrogenous phospholipids, phosphatidylcholine and phosphatidylethanolamine, are rapidly labeled with newly synthesised linoleic acid. The data presented in Table 7 are in agreement with these results.

The very long chain fatty acids were found mainly in the neutral lipid fraction, the wax esters and unesterified fatty acids being particularly rich. In germinating *Pisum sativum* [23], also, the phospholipids contained little of the newly synthesised very long chain fatty acids. In contrast, experiments with *Zea mays* [33] showed transacylation of C_{20} and C_{22} fatty acids onto all major phospholipids. Since the very long chain fatty acids in aged potato slices are probably precursors of suberin [35] and hence are not required for internal membrane synthesis, it is hardly surprising that they should be localised in approriate lipid classes such as wax esters and unesterified fatty acids. Dicarboxylic fatty acids, which are major components of suberin, were only present in trace amounts in the present experiments. This is in agreement with the data of Kolattukudy and his coworkers [35] who showed that longer ageing times are required for maximal deposition of suberin, as measured by its content of ω-hydroxyoctadec-9-enoic and octadec-9-ene dioic acids.

Since the C_{20}–C_{24} fatty acids have been formed *in vitro* by microsomal preparations from pea [8,9,19] we prepared a similar fraction from aged potato discs. Under the usual assay conditions (see Experimental), these preparations incorporated radioactivity from malonyl-[1,3-^{14}C] CoA into vary long chain fatty acids. However, total rates of synthesis of fatty acids were poor. Because the true substrate for palmitate elongase is, apparently, palmityl-ACP [11,12] it may be that stearyl–ACP and analogous substrates are necessary. Exogenous ACP is frequently ineffective in plant subcellular fractions because of their inability to esterify it to fatty acids [1,2].

In conclusion, we have shown that the synthesis of saturated fatty acids by aged potato slices, like pea, involves at least three enzyme systems. The first elongation step is sensitive to arsenite and the second to fluoride and heat. Until rapid formation of very long chain fatty acids can be achieved with a subcellular fraction, the exact nature of the second elongation reaction will remain unknown.

EXPERIMENTAL

Materials. Acyl carrier protein was isolated from *E. coli* [36]. Potatoes (variety King Edward) were obtained from the local supermarket. Slices were cut 1 mm thick and 5 mm diameter and aged at 25° [17].

Incubation and lipid extraction. Aged slices were incubated for various periods in 0.1 M K Pi buffer (pH 5.5)–chloramphenicol (50 μg/ml). Usually 10 slices/ml were used. At the end of incubation, lipids were extracted from the rinsed tissue by one of the following methods: (a) 0.2 ml of 60% KOH was added and the incubation tubes sealed and heated at 60° for 30 min. After cooling, 0.7 ml of 20% H_2SO_4 were added, followed by 4 ml $CHCl_3$ and the fatty acids extracted for 10 hr at 15°. (b) The method of ref. [37] was used. (c) The method of ref. [17] was used. (d) 5 ml of 0.5 M NaOH in MeOH were added and the mixture heated at 70° for 30 min. Methylation was carried out directly on the extract with 14% BF_3–MeOH. (e) The reaction was stopped with 0.2 ml 20% H_2SO_4, the acid removed and the slices heated at 60° for 30 min in 2.5 ml MeOH. After addition of 5 ml $CHCl_3$, extraction took place for 18 hr. The tissue was filtered off and re-extracted with $CHCl_3$–MeOH (2:1). Method (e) was routinely used (see Results and Discussion). Subcellular fractions were prepared from tissue homogenised as previously [3]. Centrifugation was carried out at 6000 *g* for 10 min, 10000 *g* for 20 min, and 105000 *g* for 60 min. Pellets were resuspended and fractions incubated as in refs. [8,38].

Chromatography. Lipids were separated by TLC on Si gel G using $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:7) or petrol–Et_2O–HOAc (90:10:1) and visualized with Rhodamine [24]. Lipid elution and identification was as previously [24]. Fatty acid Me esters were separated by GLC on 3% SE-30 or 15% DEGS columns using both isothermal and temp programmed operation. Identification was based on comparison of R_t with authentic standards on at least to different columns, and by $AgNO_3$–TLC. Confirmation was provided by hydrogenation [39] and oxidation [40] of individual fractions. GC–RC was used to quantitate ^{14}C-fatty acids. Fatty acids were also separated by reversed-phase TLC [11].

Measurement of radioactivity. Radioactive samples were counted in a scintillant consisting of PCS (Amersham-Searle)–xylene (2:1). Counts were corrected for quenching by the channels ratio method.

Acknowledgements—The authors are grateful to the S.R.C. for financial support.

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