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STUDIES WITH INHIBITORS AND EXOGENOUS SUBSTRATES ON DARK CO_2 FIXATION

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; dark CO_2 fixation; glycolysis; TCA cycle.

Abstract—Studies with specific metabolic inhibitors on $^{14}CO_2$ fixation by potato tuber disks indicate that an active glycolysis pathway is essential for dark CO_2 fixation. The effect of added substrates and of malonate confirmed that the TCA Cycle activity is suppressed in fresh tissue.

INTRODUCTION

DARK CO_2 fixation in potato tuber tissue has recently been reported.[1] The effects of specific metabolic inhibitors on $^{14}CO_2$ fixation by disks of tuber tissue and on the further metabolism of the products of fixation, have been investigated. Studies with exogenous substrates in the presence of CO_2 have yielded further information on the activity of the TCA Cycle in fresh and aged tuber disks.

RESULTS

The Effects of Specific Metabolic Inhibitors on Dark $^{14}CO_2$ Fixation by Disks

Fluoride. Fresh and 23 hr aged disks were incubated with $^{14}CO_2$ for 1 hr in the presence of 10^{-2} M sodium fluoride. Table 1 shows the distribution of radioactivity in the ethanol-soluble and insoluble fractions and Table 2 the activity in individual metabolites of the soluble fraction.

TABLE 1. ACTIVITY RECOVERED FROM FRESH AND AGED DISKS FED $^{14}CO_2$ FOR 1 hr IN THE PRESENCE OF 10^{-2} M FLUORIDE

	Fresh disks				Aged disks			
	Fluoride treated		Control		Fluoride treated		Control	
Fraction	(cpm/g fr. wt)	(% of total)	(cpm)	(%)	(cpm)	(%)	(cpm)	(%)
Aqueous ethanol soluble	40 100 (±6320)	98·3	1 003 320 (±58 490)	99·7	33 060 (±2320)	89·6	51 590 (±5590)	87·1
Insoluble	700 (±100)	1·7	2750 (±810)	0·3	3820 (±1000)	10·4	7630 (±800)	12·9

Mean of two replicates.

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[1] C. J. CLEGG and C. P. WHITTINGHAM, *Phytochem.* **9**, 279 (1970).

As previously reported[1,2] aged disks fix carbon dioxide at a much lower rate than fresh disks and the present experiments show that fluoride inhibited fresh disks to a greater extent than aged disks. The rate of fixation in the presence of the inhibitor was almost the same for the two types of disks. Most of the radioactivity in fresh disks is present in malate and aspartate and the effect of fluoride is to greatly reduce the activity in these two compounds.

TABLE 2. ^{14}C ACTIVITY IN ETHANOL-SOLUBLE METABOLITES FROM FRESH AND AGED DISKS FED $^{14}CO_2$ FOR 1 hr IN THE PRESENCE OF 10^{-2} M FLUORIDE

	Fresh disks				Aged disks			
	Fluoride treated		Control		Fluoride treated		Control	
	(cpm/g fr. wt)	(% of total)	(cpm)	(%)	(cpm)	(%)	(cpm)	(%)
Acidic fraction								
Citrate/isocit.	640	1·6	16 050	1·6	1320	4·0	100	0·2
Fumarate	0	0	2010	0·2	0	0	0	0
Glycollate	1080	2·7	4010	0·4	230	0·7	100	0·2
Lactate	0	0	1000	0·1	0	0	0	0
Malate	1640	4·1	200 660	20·0	10 980	33·2	23 830	46·2
PEP	120	0·3	1000	0·1	0	0	0	0
PGA	40	0·1	3010	0·3	0	0	0	0
Succinate	0	0	1000	0·1	400	1·2	520	1·0
Sugar P	1400	3·5	34 110	3·4	0	0	260	0·5
Tartarate	0	0	0	0	70	0·2	0	0
Triose P	40	0·1	5020	0·5	0	0	0	0
Total	4960	12·4	267 870	26·7	13 000	39·3	24 810	48·1
Basic fraction								
Alanine	0	0	4010	0·4	960	2·9	1190	2·3
Aspartate	14 960	37·3	594 970	59·3	6780	20·5	6040	11·7
Asparagine	4490	11·2	33 110	3·3	460	1·4	1910	3·7
Cystine/cyste.	0	0	0	0	0	0	100	0·2
Glutamate	2570	6·4	45 150	4·5	3800	11·5	6040	11·7
Glutamine	7980	19·9	16 050	1·6	2580	7·8	4690	9·1
Glycine/serine	2530	6·3	21 070	2·1	4500	13·6	2890	5·6
Proline	0	0	0	0	0	0	1030	2·0
Total	32 530	81·1	714 360	71·2	19 080	57·7	23 890	46·3

No activity was recovered in the neutral fraction (mainly sugars) of the ethanol-soluble metabolites.

Malonate. Fresh and 23 hr aged disks were incubated with $^{14}CO_2$ for 1 hr in the presence of 5×10^{-2} M malonic acid. Table 3 shows the distribution of recovered activity in the ethanol soluble and insoluble fractions from malonate-treated and control disks, and Table 4 the activity in individual metabolites of the soluble fractions.

By contrast with the effect of fluoride, malonate inhibited fixation by fresh disks less than by aged disks. There was little influence on the amount of radioactivity in aspartate but some inhibition of malate with fresh disks. On the other hand, with aged disks malonate resulted in approximately 50% inhibition in both malate and aspartate but resulted in a considerable increase in glutamate.

[2] C. J. CLEGG, Ph.D. Thesis, University of London (1969).

TABLE 3. ACTIVITY RECOVERED FROM FRESH AND AGED DISKS FED $^{14}CO_2$ FOR 1 hr IN THE PRESENCE OF 5×10^{-2} M MALONATE

Fractions	Fresh disks: Malonate treated (cpm/g fr. wt)	(% of total)	Fresh disks: Control (cpm)	(%)	Aged disks: Malonate treated (cpm)	(%)	Aged disks: Control (cpm)	(%)
Aqueous ethanol soluble	488 000 (±81 210)	99·7	593 800 (±37 980)	99·6	47 600 (±5480)	86·3	66 540 (±6670)	93·0
Insoluble	1440 (±720)	0·3	2140 (±550)	0·4	7530 (±930)	13·7	5010 (±220)	7·0

Mean of two replicates.

The Metabolism of Exogenous Substrates by Disks in the Presence of CO_2

Glucose-U-^{14}C. Fresh and 23 hr aged disks were incubated with 14·7 μg of glucose-U-^{14}C for 1 hr in air containing 0·1% $^{12}CO_2$. Table 5 shows the amount of radioactive CO_2 evolved and the radioactivity in the ethanol-soluble and insoluble fractions. The percentage of activity recovered in protein in the insoluble fraction is also shown. Table 6 gives the activity in individual metabolites of the soluble fractions.

TABLE 4. ^{14}C ACTIVITY IN ETHANOL-SOLUBLE METABOLITES FROM FRESH AND AGED DISKS FED $^{14}CO_2$ FOR 1 hr IN THE PRESENCE OF 5×10^{-2} M MALONATE

	Fresh disks: Malonate treated (cpm/g fr. wt)	(% of total)	Fresh disks: Control (cpm)	(%)	Aged disks: Malonate treated (cpm)	(%)	Aged disks: Control (cpm)	(%)
Acidic fraction								
Citrate/isocit.	9270	1·9	21 970	3·7	2050	4·3	1930	2·9
Glycollate	0	0	1190	0·2	0	0	70	0·1
Malate	143 960	29·5	226 830	38·2	18 520	38·9	35 530	53·4
Succinate	500	0·2	590	0·1	1330	2·8	1130	1·7
Sugar P	4390	0·9	8310	1·4	810	1·7	2060	3·1
Total	158 120	32·5	258 890	43·6	22 710	47·7	40 720	61·2
Basic fraction								
Alanine	2440	0·5	1780	0·3	670	1·4	330	0·5
Aspartate	284 020	58·2	280 870	47·3	7470	15·7	14 110	21·2
Asparagine	2930	0·6	2380	0·4	0	0	730	1·1
Cystine/cyste.	2930	0·6	2380	0·4	480	1·0	0	0
Glutamate	19 520	4·0	27 310	4·6	11 280	23·7	4720	7·1
Glutamine	3900	0·8	4160	0·7	3950	8·3	3260	4·9
Glycine/serine	1460	0·3	1780	0·3	0	0	470	0·7
Methionine	1460	0·3	1190	0·2	0	0	470	0·7
Total	318 660	65·3	321 850	54·2	23 850	50·1	24 090	36·2

No activity was recovered in the neutral fraction.

Aged disks absorbed approximately four times as much glucose-U-^{14}C as fresh disks. The majority of radioactivity recovered from fresh disks was present in the soluble fraction as sugars or closely related metabolites. About 50% of the activity recovered from aged disks was present in the soluble fraction as fats, amino acids and organic acids. A much larger proportion of the radioactivity fed was liberated in the form of carbon dioxide in aged disks. The activity found in soluble metabolites and insoluble protein was also much greater. Most of the activity in aged disks was present in malate and in PEP whereas in fresh disks the predominant activity was in PGA.

TABLE 5. ACTIVITY RECOVERED FROM FRESH AND AGED DISKS FED GLUCOSE U-^{14}C IN THE PRESENCE OF 0·1% $^{12}CO_2$

	Fresh disks				Aged disks			
	0·1% CO_2		Air		0·1% CO_2		Air	
	(cpm/g fr. wt)	(% of total)	(cpm)	(%)	(cpm)	(%)	(cpm)	(%)
$^{14}CO_2$ output	43 640 (±4090)	2·3	37 110 (±990)	1·8	2651 420 (±200 640)	33·2	2895 000 (±114 120)	34·3
Soluble metabolites	1 858 940 (±101 620)	97·4	1 966 750 (±97 450)	97·9	3 922 750 (±166 780)	49·2	4 250 450 (±211 440)	50·4
Insoluble fraction	5660 (±880)	0·3	5150 (±900)	0·3	1403 340 (±2280)	17·6	1295 980 (±210 100)	15·3
% protein	72·2%		69·5%		73·6%		75·1%	

Mean of two replicates. 25 μCi of glucose U-^{14}C used in each experiment.

Pyruvate. Fresh and 23 hr aged disks was incubated with $^{14}CO_2$ for 1 hr in the presence of 10^{-2} M sodium pyruvate. Table 7 shows the distribution of activity recovered in the ethanol soluble and insoluble fractions and the percentage recovered in protein. Table 8 gives the activity in individual metabolites of the soluble fraction.

The quantity of carbon dioxide fixed by both fresh and aged disks was not altered by the presence of pyruvate. Fresh disks contained 10% more activity in the acidic fraction, mostly in citrate/isocitrate, and less activity in the basic fraction of the soluble metabolites.

Malate-4-^{14}C. Fresh and 23 hr aged disks were incubated with 58·2 μg of sodium malate-4-^{14}C in air containing 0·1% $^{12}CO_2$. Table 9 shows the distribution of recovered activity in the CO_2 released and in the ethanol-soluble and insoluble fractions including the percentage activity recovered in protein. Table 10 gives the activity in metabolites of the soluble fraction.

Similar results were obtained with malate as with glucose, namely that aged disks produced far more CO_2 and also a significant radioactivity in protein. Again the proportion of radioactivity was greater than one in the basic than in the acid fraction in aged disks but less than one in the fresh disks.

DISCUSSION

Dark CO_2 fixation catalysed by the enzyme PEP carboxylase requires a supply of the substrate PEP which may be produced either by the EMP Pathway, or by the combined action of the PP and EMP Pathways. Specific inhibitors of the latter steps of the EMP

TABLE 6. ^{14}C ACTIVITY IN SOLUBLE METABOLITES FROM FRESH AND AGED DISKS FED GLUCOSE U-^{14}C IN THE PRESENCE OF 0·1% CO_2

	Fresh disks				Aged disks			
	0·1% CO_2		Air		0·1% CO_2		Air	
	(cpm/g fr. wt)	(% of total)	(cpm)	(%)	(cpm)	(%)	(cpm)	(%)
Acidic fraction								
Citrate/isocit.	3720	0·2	0	0	47 070	1·2	123 260	2·9
Fumarate	0	0	0	0	3920	0·1	4250	0·1
Glycollate	14 870	0·8	5900	0·3	0	0	0	0
Malate	27 880	1·5	11 800	0·6	639 410	16·3	769 330	18·1
PEP	1860	0·1	0	0	443 270	11·3	488 800	11·5
PGA	431 270	23·2	407 120	20·7	19 610	0·5	25 500	0·6
Pyruvate	0	0	0	0	27 460	0·7	59 510	1·4
Succinate	0	0	0	0	51 000	1·3	68 010	1·6
Sugar P	18 590	1·0	25 570	1·3	113 760	2·9	8500	0·2
Tartarate	0	0	0	0	3920	0·1	4250	0·1
Triose P	31 600	1·7	108 170	5·5	15 690	0·4	8500	0·2
Total	529 790	28·5	558 560	28·4	1 365 110	34·8	1559 910	36·7
Basic fraction								
Alanine	146 860	7·9	106 200	5·4	172 600	4·4	250 780	5·9
Aspartate	120 830	6·5	161 270	8·2	129 450	3·3	140 260	3·3
Asparagine	0	0	0	0	82 380	2·1	59 510	1·4
Cystine/cyste	0	0	0	0	19 610	0·5	8500	0·2
Glutamate	48 330	2·6	108 170	5·5	419 730	10·7	386 790	9·1
Glutamine	0	0	0	0	345 200	8·8	314 530	7·4
Glycine/serine	0	0	0	0	152 990	3·9	38 250	0·9
Lysine	0	0	0	0	35 300	0·9	4250	0·1
Methionine	0	0	0	0	251 060	6·4	416 540	9·8
Proline	0	0	0	0	23 540	0·6	17 000	0·4
Tryptophan	0	0	0	0	105 910	2·7	212 520	5·0
Valine	0	0	0	0	62 760	1·6	80 760	1·9
Fats	0	0	0	0	278 520	7·1	191 270	4·5
Total	316 020	17·0	375 640	19·1	2079 050	53·0	2120 960	49·9
Neutral fraction								
Fructose	7440	0·4	11 800	0·6	3920	0·1	12 750	0·3
Glucose	981 520	52·8	916 500	46·6	407 970	10·4	442 050	10·4
Sucrose	7440	0·4	9830	0·5	3920	0·1	4250	0·1
Total	996 400	53·6	938 130	47·7	415 810	10·6	459 050	10·8

Pathway should therefore decrease dark CO_2 fixation. Both fluoride and iodoacetate which inhibit these reactions inhibit fixation in both fresh and aged disks, although the latter to a smaller extent. The smaller inhibition in aged tissue may be correlated with their enhanced respiratory activity.[3–6] The suggestion that because fresh potato tuber tissue disks metabolise

[3] D. P. HACKETT and K. V. THIMANN, *Am. J. Bot.* **40**, 183 (1953).
[4] I. R. MACDONALD and P. C. DEKOCK, *Ann. Bot.* **22**, 429 (1958).
[5] B. C. LOUGMAN, *Plant Physiol.* **35**, 418 (1960).
[6] W. STILES and K. W. DENT, *Ann. Bot.* **11**, 1 (1947).

TABLE 7. ACTIVITY RECOVERED FROM FRESH AND AGED DISKS FED $^{14}CO_2$ FOR 1 hr IN THE PRESENCE OF 10^{-2} M SODIUM PYRUVATE ^{12}C

	Fresh disks				Aged disks			
	Pyruvate treated		Controls		Pyruvate treated		Controls	
	(cpm/g fr. wt)	(% of total)	(cpm)	(%)	(cpm)	(%)	(cpm)	(%)
Soluble metabolites	854 400 (±67 320)	99·8	963 820 (±42 200)	99·8	69 540 (±2790)	96·0	67 100 (±1840)	94·8
Insoluble fraction	1590 (±410)	0·2	1870 (±710)	0·2	2930 (±200)	4·0	3660 (±170)	5·2
% protein	86·8%		84·5%		79·6%		81·8%	

Means of two separations.

TABLE 8. ^{14}C ACTIVITY IN SOLUBLE METABOLITES FROM FRESH AND AGED DISKS FED $^{14}CO_2$ IN THE PRESENCE OF 10^{-2} M SODIUM PYRUVATE ^{12}C

	Fresh disks				Aged disks			
	Pyruvate treated		Control		Pyruvate treated		Control	
	(cpm/g fr. wt)	(% of total)	(cpm)	(%)	(cpm)	(%)	(cpm)	(%)
Acidic fraction								
Citrate/isocit.	114 490	13·4	13 490	1·4	830	1·2	1210	1·8
Fumarate	850	0·1	3850	0·4	210	0·3	130	0·2
Glycollate	8540	1·0	3850	0·4	210	0·3	600	0·9
Malate	181 990	21·3	226 500	23·5	33 310	47·9	31 270	46·6
PEP/PGA	17 090	2·0	22 170	2·3	760	1·1	740	1·1
Succinate	850	0·1	960	0·1	1460	2·1	1210	1·8
Sugar P	2560	0·3	2890	0·3	210	0·3	200	0·3
Tartarate	0	0	0	0	560	0·8	130	0·3
Triose P	850	0·1	0	0	490	0·7	330	0·5
Total	327 220	38·3	273 710	28·4	38 040	54·7	35 820	53·4
Basic fraction								
Alanine	0	0	0	0	420	0·6	200	0·3
Aspartate	367 390	43·0	620 700	64·4	7230	10·4	10 330	15·4
Asparagine	41 010	4·8	10 600	1·1	210	0·3	2010	3·0
Cystin/cysteine	0	0	0	0	210	0·3	200	0·3
Glutamate	64 080	7·5	25 060	2·6	14 260	20·5	11 000	16·4
Glutamine	19 650	2·3	4820	0·5	4870	7·0	5100	7·6
Glycine/serine	24 780	2·9	22 170	2·3	1180	1·7	870	1·3
Proline	0	0	0	0	140	0·2	330	0·5
Threonine	0	0	0	0	210	0·3	130	0·2
Tyrosine	0	0	0	0	560	0·8	130	0·2
Total	516 910	60·5	683 350	70·9	29 290	42·1	30 300	45·2

No activity was recovered in the neutral fraction.

TABLE 9. ACTIVITY RECOVERED FROM FRESH AND AGED DISKS FED MALATE-4 ^{14}-C FOR 1 hr IN THE PRESENCE OF 0·1% CO_2

	Fresh disks (cpm/g fr. wt)	(% of total)	Aged disks (cpm)	(%)
$^{14}CO_2$ output	13 990 (± 1070)	6·1	1186 880 (± 21 620)	67·4
Soluble metabolites	213 370 (± 25 220)	93·0	163 240 (± 7440)	9·3
Insoluble fraction	2060 (± 500)	0·9	409 440 (± 18 470)	23·3
% protein	85·2%		82·6%	

Means of two replicates. 10 μCi of sodium malate-4-^{14}C were used in each experiment.

exogenous ^{14}C glucose only slowly they also fail to glycolyse endogenous glucose[7] is inconsistent with the observed effect of fluoride and iodoacetate on the metabolism of fresh disks reported here. The mechanism by which, in the presence of fluoride (Table 2) and to a lesser extent, in the presence of iodoacetate,[2] a larger percentage of the activity in the soluble fraction occurred in the amides glutamine and asparagine is not clear.

TABLE 10. ^{14}C ACTIVITY IN SOLUBLE METABOLITES FROM FRESH AND AGED DISKS FED SODIUM MALATE-4-^{14}C IN THE PRESENCE OF 0·1 %CO_2

	Fresh disks (cpm/g fr. wt)	(% of total)	Aged disks (cpm)	(%)
Acidic fraction				
Citrate/isocitrate	0	0	160	0·1
Fumarate	15 360	7·2	820	0·5
Glycollate	430	0·2	0	0
Malate	189 050	88·6	5550	3·4
Total	204 840	96·0	6530	4·0
Basic fraction				
Alanine	0	0	22 200	13·6
Aspartate	4270	2·0	47 340	29·0
Glutamate	0	0	57 790	35·4
Glutamine	0	0	24 000	14·7
Total	4270	2·0	151 330	92·7

No activity was recovered in the neutral fraction.

[7] G. G. LATIES, *Austral. J. Sci.* **30**, 193 (1967).

The effect of malonate on CO_2 fixation which discriminates between aged and fresh disks is consistent with malonate specifically inhibiting succinic dehydrogenase. Aged disks are believed to have a fully functioning TCA Cycle,[8,9] whereas fresh disks do not.

It has been stated that rapid sucrose synthesis begins when potato tubers are cut[10,11] and it has been found that a substantial part of the ^{14}C glucose fed to freshly cut potato disks is incorporated into sucrose during 3 hr ageing.[9] The synthesis of radioactive sucrose in fresh disks fed glucose-U-^{14}C for only 1 hr (Table 6) was negligible. When similar tissue was aged for 24 hr in the presence of glucose-U-^{14}C 11% of the radioactivity in the soluble fraction was recovered in sucrose.[2] Changing the concentration of CO_2 from 0·03 to 0·1% in the atmosphere surrounding fresh and aged disks did not significantly alter the metabolism of exogenous glucose-U-^{14}C.

When pyruvate was present in the medium surrounding disks during dark $^{14}CO_2$ fixation (Tables 7 and 8) the percentage radioactivity recovered in citrate in the soluble fraction was greatly increased in fresh disks but not in aged. Radioactivity in malate was largely unchanged but it was reduced in aspartate. This result is in agreement with the earlier report that pyruvate is present at low concentration in fresh disks, and that when it is supplied from an exogenous source the TCA Cycle operates rapidly only as far as isocitrate.[1]

In fresh disks fed malate-4-^{14}C (Tables 9 and 10) the greater part of the absorbed activity was recovered in the soluble fraction as malate. This was in contrast with aged disks where the absorbed activity was largely evolved as $^{14}CO_2$. In the presence of full TCA Cycle activity carbon 4 of malate is evolved as CO_2 during the first turn of the Cycle. Thus the reported block in the TCA Cycle of freshly cut disks at loci between isocitrate and ketoglutarate, and its removal during 2–3 hr ageing is further confirmed.[8,12] When 23 hr aged disks were fed $^{14}CO_2$ (Tables 2 and 4) 46–53% of the activity accumulated in the soluble fraction in malate, apparently in a discrete pool physically separated from the malate concerned in the reactions of the TCA Cycle. That exogenous malate absorbed by aged potato disks is involved in the reactions of the latter pool is apparent.[13,14] That aspartate and not malate is readily accessible for metabolic interconversion was also confirmed. The present observations support the earlier work suggesting that the Krebs Cycle is not operative in freshly cut disks but becomes so during 24 hr ageing.

EXPERIMENTAL

Tissue. Potato tubers var. King Edward were obtained from commercial sources locally and were stored in the dark at 3–5° until required. Disks 1 cm × 1 mm, were cut and washed as described previously.[15]

Reagents. All chemicals were of the highest purity available, usually AR grade. Radioactive compounds were obtained from the Radiochemical Centre, except malate $^{14}C_4$, provided by D. A. Walker, Imperial College, $^{14}CO_2$ was generated from a $Na_2{}^{14}CO_3$ solution of specific activity 56 μCi/μM.

Ageing and feeding disks. Disks were aged or incubated with substrate in the dark, in groups of 10 disks (0·75–0·85 g fr. wt) resting on stretched nylon netting at the surface of 15 cm^3 of medium in a 150 cm^3 Berzelius beaker. The medium was stirred magnetically, and a moist air stream was passed over the tissue and subsequently through a CO_2 absorption tube containing 20 cm^3 of 4 N NaOH solution. The incubation medium was a 10 mM K phosphate—0·1 mM $CaCO_4$ buffer solution containing 25 μg chloramphenicol

[8] G. G. LATIES, *Plant Physiol.* **39,** 654 (1964).
[9] J. A. ROMBERGER and G. NORTON, *Plant Physiol.* **36,** 20 (1961).
[10] E. F. HOPKINS, *Bot. Gaz.* **84,** 75 (1927).
[11] J. M. NELSON and R. AUCHINCLOSS, *J. Am. Chem. Soc.* **55,** 3769 (1933).
[12] B. PAYES, *Diss. Abs. B.* **27b,** 1723 (1966).
[13] J. DANNER and I. P. TING, *Plant Physiol.* **42,** 719 (1967).
[14] S. H. LIPPS and H. BEEVERS, *Plant Physiol.* **41,** 709 (1966).
[15] C. J. CLEGG, M.Sc. Thesis, University of London (1966).

per cm^3. In each experiment the medium was finally adjusted to pH 6·0 using 4 N NaOH· $^{14}CO_2$ (200 μCi) was fed for a period of 1 hr.

Analysis of labelled metabolites. At the end of the feeding period, tissue was killed and extracted with aq. EtOH. Separation of the extract into fractions, chromatographic separation of individual metabolites and determination of their radioactivity was as described previously.[1]

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