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STUDIES ON ETHYLENE PRODUCTION BY A SUBCELLULAR FRACTION FROM RIPENING TOMATOES—III.

EFFECTS OF ADDITION OF β-ALANINE AND COFACTORS FOR DECARBOXYLATION

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Abstract—Studies were made of the effects on ethylene production by a subcellular fraction from tomatoes of the addition of β-alanine, and of β-alanine and cofactors for decarboxylation. Cofactors that stimulated ethylene production in the presence of β-alanine were flavin adenine dinucleotide, flavin mononucleotide, pyridoxal phosphate, and thiamine pyrophosphate and Mg^{++}. The cofactors, flavin mononucleotide, oxalacetic acid, α-lipoic acid and nicotinamide adenine dinucleotide phosphate each elevated ethylene evolution in the presence of β-alanine, thiamine pyrophosphate and Mg^{++}. Substances that stimulated production of the volatile when β-alanine, thiamine pyrophosphate, Mg^{++} and α-lipoic acid were present were flavin adenine dinucleotide, flavin mononucleotide, and α-ketoglutaric acid. On the basis of these experiments and previous ones, a tentative proposal is made for the pathways of biosynthesis of ethylene.

INTRODUCTION

THOMPSON and Spencer[1] have shown that a purified enzyme system from bean cotyledons produced labelled ethylene from β-alanine-2-^{14}C, and Wang *et al.*[2] have reported labelling of ethylene when β-alanine-2-^{14}C was supplied to *Penicillium digitatum*. With the subcellular fraction from tomatoes used in the present series of experiments, added β-alanine resulted in weak stimulation of ethylene production, and this was increased when thiamine pyrophosphate and magnesium ions were also added.[3] The present paper describes the effects of several cofactors and of two partially reconstructed decarboxylation systems on β-alanine metabolism to ethylene.

RESULTS AND DISCUSSION

Reference should be made to the first paper in this series[3] for comments regarding the presentation and interpretation of results.

The means and standard deviations of the nitrogen values for the difference between control and experimental samples were found to be: $-0{\cdot}06 \pm 0{\cdot}62$; $+0{\cdot}08 \pm 0{\cdot}61$; $0{\cdot}06 \pm 0{\cdot}62$ mg for Tables 1, 2 and 3, respectively.

(*a*) *Effects of Several Cofactors*

The effects on ethylene production of several cofactors and substrates in the presence of β-alanine are shown in Table 1. In this study the basic reaction mixture consisted of ATP, β-alanine and the particulate fraction suspended in sucrose phosphate buffer.

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[1] J. THOMPSON and M. SPENCER, *Nature* **210**, 595 (1966).
[2] C. H. WANG, D. W. JACOBSEN and F. S. TANAKA, *Fed. Proc.* **23**, 224 (1965).
[3] M. MEHERIUK and M. SPENCER, *Phytochem.* **6**, 535 (1967).

TABLE 1. EFFECT OF SEVERAL COFACTORS AND SUBSTRATES WITH β-ALANINE ON ETHYLENE PRODUCTION BY A PARTICULATE FRACTION FROM TOMATOES*

Run No.	Cofactor or substrate	C_2H_4 (mμl) 0–3 hr	% Diff.†	22–24 hr	% Diff.†
1	Coenzyme A (7×10^{-5} M)	180	+21	795	−14
2	Coenzyme A (7×10^{-5} M)	132	−13	1220	−4
3	FAD ($1{\cdot}2\times10^{-4}$ M)	113	−26	809	+9
4	FAD ($1{\cdot}2\times10^{-4}$ M)	411	+2	678	+3
5	FMN (2×10^{-4} M)	169	+11	1324	+4
6	FMN (2×10^{-4} M)	192	+25	721	−3
7	α-Ketoglutarate (1×10^{-3} M)	324	+5	2098	+8
8	α-Ketoglutarate (1×10^{-3} M)	245	+6	948	+13
9	Oxalacetate (1×10^{-3} M)	259	−16	2169	+12
10	Oxalacetate (1×10^{-3} M)	226	+34	1642	+12
11	α-Lipoic acid (1×10^{-3} M)	163	−2	1474	+6
12	α-Lipoic acid (1×10^{-3} M)	188	−19	753	+49
13	NADP ($4{\cdot}0\times10^{-4}$ M)	141	−8	885	+19
14	NADP ($4{\cdot}0\times10^{-4}$ M)	345	−14	739	+12
15	NAD ($3{\cdot}0\times10^{-4}$ M)	182	+9	1082	−22
16	NAD ($3{\cdot}0\times10^{-4}$ M)	200	−2	733	−6
17	Pyridoxal phosphate (5×10^{-4} M)	332	+7	2491	+28
18	Pyridoxal phosphate (5×10^{-4} M)	219	−5	980	+16
19	Pyridoxal phosphate (5×10^{-4} M),‡ Fe^{+3} (1×10^{-3} M)	135	−4	1242	+3
20	Pyridoxal phosphate (5×10^{-4} M),‡ Fe^{+3} (1×10^{-3} M)	96	−4	336	−3
21	TPP (2×10^{-3} M)	138	+17	845	+20
22	TPP (2×10^{-3} M)	143	−25	394	+28
23	TPP (2×10^{-3} M),†† Mg^{+2} (1×10^{-3} M)	360	+18	1711	+38
24	TPP (2×10^{-3} M),†† Mg^{+2} (1×10^{-3} M)	507	+13	1537	+40

* Basic reaction mixture: 0·5 M sucrose, 0·125 M KH_2PO_4, pH 7·2, 0·05 M β-alanine. $1{\cdot}9\times10^{-3}$ M ATP was added to each flask after initial sonication. Particles were sonicated for 4 min at 1·2 A at the beginning of the 0–3 hr and 22–24 hr collection periods, respectively.

† % Diff. = per cent increase or decrease in ethylene production compared to that of the control sample.

‡ = control also contained 5×10^{-4} M pyridoxal phosphate.

†† = control also contained 2×10^{-3} M thiamine pyrophosphate.

Coenzyme A (Runs 1, 2) caused a slight decrease in total ethylene production, as was found with CoA in the absence of an added substrate.[3] The decrease may have resulted from mediation of a reaction that withdrew an intermediate in the pathway of ethylene formation from β-alanine.

Flavin adenine dinucleotide (FAD, Runs 3, 4) and flavin monocleotide (FMN, Runs 5, 6), both increased ethylene evolution slightly. These results contrast to those observed when β-alanine was not added to the suspension,[3] when significant decreases were detected.

Two Kreb's cycle intermediates, α-ketoglutarate (Runs 7, 8) and oxalacetate (Runs 9, 10), which could act as amino group acceptors, stimulated ethylene production but weakly. α-Lipoate (Runs 11, 12), a cofactor involved in oxidative phosphorylation, increased ethylene production slightly.

NADP (Runs 13, 14) augmented production, but NAD (Runs 15, 16) caused a decrease

in the yield of the volatile. The results are analogous to the ones observed when no substrate was added to the particulate fraction.[3]

Pyridoxal phosphate (Runs 17, 18) brought about a significant increase in ethylene production, probably through stimulation of decarboxylation or transamination, or both. Addition of Fe^{+++} (Runs 19, 20) had no further effect.

A small increase was observed when thiamine pyrophosphate (TPP, Runs 21, 22) was added to the suspension. An additional increase was noted when 0·001 M Mg^{++} was also added (Runs 23, 24).

In summary, a number of substrates and cofactors in the presence of β-alanine gave small increases in ethylene evolution by the subcellular fraction, among them pyridoxal phosphate and TPP. When Mg^{++} was added to the fraction containing the latter cofactor, marked elevation of ethylene production occurred.

(b) Effects of "Decarboxylation System I"

The results of this study are presented in Table 2. The basic reaction mixture in the system consisted of β-alanine, TPP, Mg^{++} and the particulate fraction suspended in sucrose phosphate buffer.

TABLE 2. EFFECTS OF COFACTORS AND SUBSTRATES WITH β-ALANINE DECARBOXYLATION SYSTEM I ON ETHYLENE PRODUCTION BY A PARTICULATE FRACTION FROM TOMATOES*

Run No.	Cofactor or substrate	C_2H_4 (mμl) 0–3 hr	% Diff.†	22–24 hr	% Diff.†
1	Coenzyme A (7×10^{-5} M)	158	+8	1300	−19
2	Coenzyme A (7×10^{-5} M)	234	—	792	−8
3	FAD ($1{\cdot}2 \times 10^{-4}$ M)	200	+18	677	−18
4	FAD ($1{\cdot}2 \times 10^{-4}$ M)	113	−23	558	−38
5	FMN (2×10^{-4} M)	174	+27	872	+49
6	FMN (2×10^{-4} M)	377	+41	621	—
7	α-Ketoglutarate (1×10^{-3} M)	321	+4	1485	+2
8	α-Ketoglutarate (1×10^{-4} M)	270	—	2126	+8
9	Oxalacetate (1×10^{-3} M)	568	+34	2249	+54
10	Oxalacetate (1×10^{-3} M)	196	−17	2742	+37
11	α-Lipoic acid (1×10^{-3} M)	124	−37	1114	+28
12	α-Lipoic acid (1×10^{-3} M)	321	−14	2722	+36
13	NAD (3×10^{-4} M)	256	+8	2111	+5
14	NAD (3×10^{-4} M)	259	+11	871	+1
15	NADP (4×10^{-4} M)	242	+3	952	+11
16	NADP (4×10^{-4} M)	96	−21	1548	+20
17	Pyridoxal phosphate (5×10^{-4} M)	212	+25	772	−7
18	Pyridoxal phosphate (5×10^{-4} M)	349	+30	585	−7

Basic reaction mixture: 0·5 M sucrose, 0·125 M KH_2PO_4, pH 7·2; plus decarboxylation system I*: 0·05 M β-alanine, 0·002 M TPP, 0·001 M $MgSO_4$. Particles were sonicated for 4 min at 1·2 A at the beginning of the 0–3 hr and 22–24 hr collection periods, respectively. $1{\cdot}9 \times 10^{-3}$ M ATP was added to each flask after initial sonication.

† % Diff. = per cent increase or decrease in ethylene production compared to that of the control sample.

Total ethylene production decreased slightly in the presence of Coenzyme A (Runs 1, 2), as before. Addition of FAD (Runs 3, 4) resulted in a significant reduction in the evolution

of ethylene, as it did when added to the particulate suspension in sucrose-phosphate buffer alone.[3] In contrast, FMN (Runs 5, 6) augmented production.

α-Ketoglutarate (Runs 7, 8) had little effect but oxalacetate (Runs 9, 10) gave a marked increase in ethylene evolution. If transamination is an important step in the synthesis of ethylene it would appear that oxalacetic acid is the preferred amino group acceptor of the two amino acids.

The addition of α-lipoate (Runs 11, 12) in the presence of TPP and Mg^{++} resulted in an appreciable increase in total ethylene production, probably through promotion of decarboxylation reactions.

NAD (Runs 13, 14) had little effect but NADP (Runs 15, 16) stimulated production of the olefin. The latter result agrees with that of the first study (see Table 1, Runs 15, 16) and would suggest a role for NADP in the biogenesis of ethylene.

Total ethylene production decreased slightly in the presence of pyridoxal phosphate (Runs 17, 18), a result not observed in the absence of TPP and Mg^{++} (see Table 1, Runs 17, 18).

Factors found to stimulate ethylene production in the presence of β-alanine, TPP and Mg^{++} were: FMN, oxalacetate, α-lipoic acid and NADP.

(c) Effects of Decarboxylation System II

The results of this study are given in Table 3. The basic reaction mixture consisted of β-alanine, TPP, Mg^{++}, α-lipoate and the particulate fraction suspended in sucrose phosphate buffer.

TABLE 3. EFFECTS OF COFACTORS AND SUBSTRATES WITH β-ALANINE DECARBOXYLATION SYSTEM II ON ETHYLENE PRODUCTION BY A PARTICULATE FRACTION FROM TOMATOES*

Run No.	Cofactor or substrate	C_2H_4 (Mμl) 0–3 hr	% Diff.†	22–24 hr	% Diff.†
1	Coenzyme A (7×10^{-3} M)	300	−19	575	−14
2	Coenzyme A (7×10^{-3} M)	261	−5	800	−20
3	FAD ($1{\cdot}2 \times 10^{-4}$ M)	298	−25	1980	+5
4	FAD ($1{\cdot}2 \times 10^{-4}$ M)	146	−13	2122	+12
5	FMN (2×10^{-4} M)	191	+14	2159	+14
6	FMN (2×10^{-4} M)	180	+5	2075	+6
7	α-Ketoglutarate (1×10^{-3} M)	293	−2	663	+23
8	α-Ketoglutarate (1×10^{-3} M)	219	—	930	+40
9	Oxalacetate (1×10^{-3} M)	270	−4	1600	—
10	Oxalacetate (1×10^{-3} M)	225	+2	825	+24
11	NAD (3×10^{-4} M)	366	+14	2137	−21
12	NAD (3×10^{-4} M)	129	−24	1474	−24
13	NADP (4×10^{-4} M)	321	—	1980	−27
14	NADP (4×10^{-4} M)	205	+4	2401	−24
15	Pyridoxal phosphate (5×10^{-4} M)	347	−6	625	−6
16	Pyridoxal phosphate (5×10^{-4} M)	276	—	892	−11

Basic reaction mixture: 0·5 M sucrose, 0·125 M KH_2PO_4, pH 7·2; plus decarboxylation system II*: 0·05 M β-alanine, 2×10^{-3} M TPP, 1×10^{-3} M $MgSO_4$, 1×10^{-3} M α-lipoic acid. $1{\cdot}9 \times 10^{-3}$ M ATP was also added to each flask after initial sonication. Particles were sonicated for 4 min at 1·2 A at the beginning of the 0–3 hr and 22–24 hr collection periods, respectively.

† % Diff. = per cent increase or decrease in ethylene production compared to that of the control sample.

Ethylene production decreased when Coenzyme A was added (Runs 1, 2). The inhibitory effect observed previously (see Table 1, Runs 1, 2 and Table 2, Runs 1, 2) remains unchanged.

FAD (Runs 3, 4) gave a slight elevation in the yield of ethylene and thus, constitutes a result different from that observed in decarboxylation system I (see Table 2, Runs 3, 4). Apparently, the presence of α-lipoic acid reinstated the stimulatory effect of FAD observed when only β-alanine was added. Increased ethylene production was observed with FMN (Runs 5, 6) as well as an unusual elevation (greater than 100 per cent) in the overnight (3–22 hr) production period. This may reflect reconstitution of an enzyme complex.

The two amino group acceptors gave some stimulation of ethylene production. The effects were more obvious with α-ketoglutarate (Runs 7, 8) than with oxalacetate (Runs 9, 10).

Both NAD (Runs 11, 12) and NADP (Runs 13, 14) were inhibitory to ethylene evolution possibly as a result of cofactor antagonism.

No pronounced effect was evident on addition of pyridoxal phosphate (Runs 15, 16), as observed with decarboxylation system I (see Table 2, Runs 17, 18).

The results from this study indicate that only the flavin coenzymes and α-ketoglutarate were stimulatory with decarboxylation system II. Also notable are the inhibitory effects of both NAD and NADP and the significant overnight production of ethylene in the presence of FMN.

Several observations can be made from the effects on ethylene production of cofactors supplied with β-alanine and the "decarboxylation systems". FAD, which was inhibitory in system I, gave slight stimulation in system II. FMN gave increased ethylene evolution in all cases. Both NAD and NADP were significantly inhibitory in system II, as compared to no effect and slight augmentation respectively, in system I. Apparently, the addition of NAD or NADP and α-lipoic acid results in an event deleterious to ethylene biosynthesis. This combination of cofactors could promote oxidative decarboxylation reactions, and the results suggested that a simple (non-oxidative) decarboxylation is involved in the conversion of β-alanine to ethylene. The slight inhibitory effect of CoA was throughout, and a lack of effect with pyridoxal phosphate was generally observed. The two amino group acceptors, oxalacetate and α-ketoglutarate, elevated synthesis of ethylene weakly, a result also obtained with system I.

These results, along with the ones reported in the earlier papers[3] in this series and those of other workers, can be discussed in relation to Fig. 1. Although several substances were evaluated[3] in the subcellular system from tomatoes as potential precursors of ethylene only a few were demonstrated to influence ethylene production significantly. Effective ones were ethanol, propionate, aspartic acid, glutamic acid and serine. It is difficult to judge whether such substances act as actual precursors or in less direct ways, unless results from tracer studies are available. However, a recent paper by Larson and Beevers[4] has assisted in the expression of a metabolic sequence for the conversion of the effective substances to ethylene. They found that pea seedlings contain homoserine as their major amino acid and that it is not present in ungerminated seeds. Tracer studies with feeding of glutamic and aspartic acids resulted in homoserine of high activity. The fact that seedlings produce ethylene[5, 6, 7] suggests an association between homoserine metabolism and ethylene biosynthesis.

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[5] M. Spencer and A. O. Olson, *Nature* **205**, 699 (1965).
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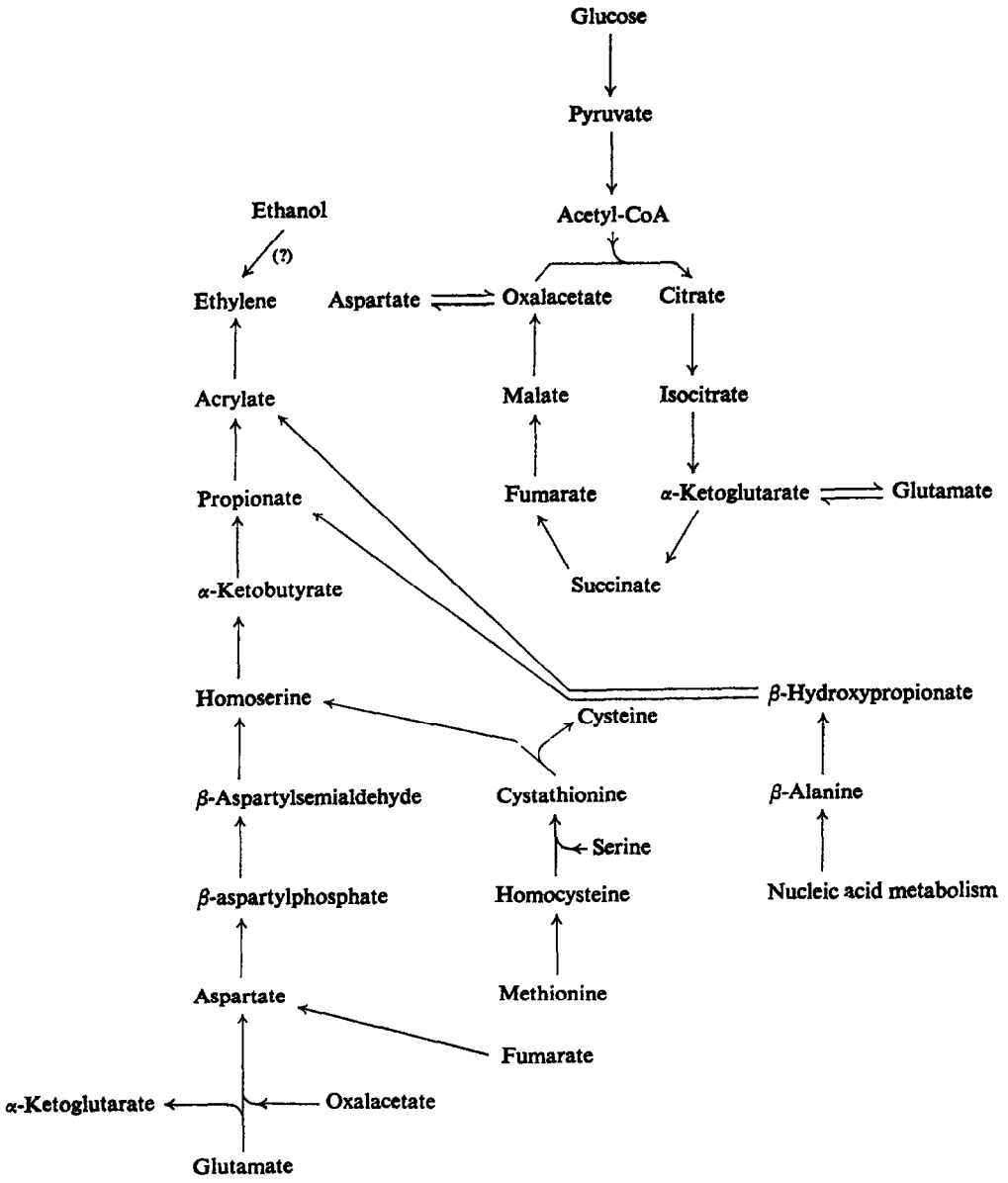

FIG. 1. SOME POSSIBLE PATHWAYS OF BIOSYNTHESIS OF ETHYLENE.

Homoserine can be deaminated to α-ketobutyrate, which subsequently can be decarboxylated to propionate, a substance found to promote ethylene production in the tomato particulate system and an intermediate included in the pathway proposed by Thompson and Spencer[1] for biogenesis of ethylene. Aspartic acid can be converted to homoserine via β-aspartylsemialdehyde.[8] The transamination, which is highly active in seedlings,[9] could

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give rise to aspartic acid from glutamic acid. Serine could participate in methionine catabolism to homoserine. Thus the effects of all the acids found to result in increased ethylene production when added to the tomato particulate fraction could be related by the proposed scheme. Ethanol has also been found to be an effective stimulant of ethylene production, both by us and other workers[10–12] but whether its conversion to the gas occurs by a pathway other than acetyl-CoA formation is unknown. L-Aspartate ammonia lyase activity has been included in the figure because fumarate has been suggested as a precursor for ethylene.[2, 13, 14] A key intermediate in the pathway is β-alanine, but when added by itself it was weak in its augmentation of ethylene evolution. Added cofactors were shown to be necessary for acceleration of its conversion to ethylene.

When a number of cofactors were studied[3] with the tomato particulate system, it was found that the highest stimulatory effect on ethylene production was induced when TPP and Mg^{++} were added together, which is consistent with the proposed decarboxylation reactions. Slight elevations in the yield of the olefin when NADP was added may indicate the involvement of an NADP-linked dehydrogenase in the biosynthesis of ethylene. A significant overnight production of ethylene in the presence of FMN suggested that reconstitution of either an enzyme complex or enzyme sequence may have been inaugurated in the presence of the prosthetic group. Moreover, support for such a reconstitution was obtained from the synergistic effect observed between CoA and NAD or NADP. Severe inhibition of ethylene production by reduced glutathione implied a need for some unreduced disulfide linkages in enzymes associated with ethylene biogenesis.

Considerable work with inhibitors was done[15] with the subcellular system from tomatoes and several interesting features emerged. The role of thiol groups in ethylene synthesis was amply demonstrated by the inhibitory effects of arsenite, iodoacetamide, *p*-CMB, mercuric ions and silver ions. The importance of carbonyl groups was disclosed by the inhibition of ethylene production with hydroxylamine, semicarbazide and cyanide. Carbonyl groups could involve proteins, cofactors and substrates. Chelation of a cation or metalloprotein was suggested by the actions of azide, cyanide, fluoride and DIECA. The necessity of transaminase activity was revealed by the inhibitory action of cycloserine and aminooxyacetic acid. Involvement of the cytochrome system is a distinct possibility in view of the inhibitory effects of azide and cyanide. Lack of substantial inhibition with monofluoracetate and malonic acid led to the proposal that a considerable portion of the TCA cycle is not essential to ethylene biosynthesis. A need for disulfide linkages was also indicated by cyanide. The effects observed with the inhibitors are consistent with the scheme suggested in Fig. 1.

No cations were found to stimulate ethylene production and, in fact, most were inhibitory, probably because of their binding of the thiol groups on essential enzymes. It is likely that sufficient endogenous supplies of the necessary cations are present in the preparations. However, Mg^{++} was found to be stimulatory in the presence of certain levels of TPP.

If β-alanine proves to be a major precursor of ethylene, as suggested by these and other[1, 2, 16] experiments, then an interesting relationship among nucleic acids and ethylene

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may exist. (β-Alanine has been sho vn to arise from nucleic acid catabolism.[17, 18]) Also, an explanation of the close relationship[19] between ethylene production and respiratory rate may involve β-alanine in a dual role, as a component of Coenzyme A and a precursor of ethylene, although other metabolic roles exist, of course, for both Coenzyme A and β-alanine.

METHODS

The procedure followed in the isolation, suspension and sonication of the particles, the collection of ethylene and the analysis of the volatile by gas chromatography have been previously described.[3, 20, 21]

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