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

EFFECTS OF SEVERAL SUBSTRATES, COFACTORS AND CATIONS

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Abstract—Studies were made of the effects of several substrates, cofactors and cations on the production of ethylene by a subcellular fraction from ripening tomatoes. Compounds found to be effective were ethanol, aspartic acid, glutamic acid, serine, γ-aminobutyric acid, propionic acid and galactose. Those found to exert little influence on ethylene evolution were β-alanine, α-ketoglutaric acid and xylose. Inhibitory to ethylene production were glycolaldehyde, pyruvate, acrylic acid, and glyoxylate. None of the added cations stimulated ethylene evolution, and copper, zinc and cobalt reduced it. Cofactors found to be stimulatory were: reduced α-lipoate, nicotinamide adenine dinucleotide phosphate, and thiamine pyrophosphate. Coenzyme A, reduced glutathione, nicotinamide adenine dinucleotide, bovine serum albumin, flavin adenine dinucleotide, flavin mononucleotide and pyridoxal phosphate, were either inhibitory or had little effect on ethylene production.

INTRODUCTION

ALTHOUGH the pathway of biosynthesis of ethylene has been intensively investigated in recent years,[1–5] considerable uncertainty remains. A subcellular fraction that produces the olefin presents a biological system less complex than the intact cell or organism, yet yields information that could not be obtained with purified enzymes. This series of papers† reports experiments with such a system from tomatoes, in which the effects of various inhibitors, substrates, cofactors, and cations, were evaluated and used to suggest and assess pathways for the biogenesis of ethylene.

RESULTS AND DISCUSSION

While only two results are given for each factor in these papers, they are representative of several that have been obtained. Quantitative comparisons are made only within each run because current methods do not permit the isolation of subcellular fractions with identical biochemical properties from one preparation to the next.

Total ethylene values in the tables are given for the 0–3 hr and 22–24 hr collection periods. The initial (0–3 hr) production probably includes both preformed ethylene liberated upon disruption of the particles and also newly formed ethylene.[6] High yields of ethylene were invariably obtained after resonication of the suspension at the end of the 3–22 hr period, as

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† Part II, p. 545; Part III, p. 551.

[1] C. H. WANG, D. W. JACOBSEN and F. S. TANAKA, *Fed. Proc.* **23**, 224 (1965).
[2] D. W. JACOBSEN and C. H. WANG, *Plant Physiol.* **40**, xix (supp.) (1965).
[3] S. P. BURG and E. A. BURG, *Science* **148**, 1190 (1965).
[4] M. GIBSON, *The Biogenesis of Ethylene*, Doctoral Dissertation, Purdue University (1963).
[5] J. THOMPSON and M. SPENCER, *Nature* **210**, 595 (1966).
[6] G. R. CHANDRA, M. SPENCER and M. MEHERIUK, *Nature* **199**, 767 (1963).

previously reported.[7] Reference to the 3–22 hr collection period is made only when ethylene production was significant.

Yields of ethylene are not expressed on a per milligram basis because most nitrogenous components of the subcellular fraction may have little or no influence on ethylene production. Instead, the mean and standard deviation (in mg nitrogen) of the differences between the control and experimental samples of each run was calculated. Values found for Tables 1–3 were: $0{\cdot}08 \pm 0{\cdot}67$; $0{\cdot}32 \pm 0{\cdot}43$; $0{\cdot}19 \pm 0{\cdot}18$ mg, respectively.

Section 1. Substrates (Table 1)

(*a*) *Carbohydrates.* A severe inhibitory effect on total ethylene production was exhibited by glycolaldehyde (Runs 1, 2). This effect could have resulted from the production of γ-hydroxy-α-ketoglutaric acid,[8] a tricarboxylic acid cycle inhibitor, or glycolaldehyde itself may have inhibited α-carboxylase activity.[9] (The requirement for thiamine pyrophosphate and α-carboxylase activity for the production of ethylene will become evident in Section 2 of this paper.)

Little effect on ethylene production was observed when glucose (Runs 3, 4) was supplied and it is presumed that either extensive metabolism of the hexose failed to occur, or metabolic intermediates produced did not influence ethylene evolution. Since glucose has been reported to sustain ethylene production in whole tissue,[10,11] and is also known to label ethylene,[10,12,13] it is likely that the metabolism of glucose to ethylene is dependent upon enzymes not present in the particulate fraction.

Significant elevation of ethylene production was observed with galactose (Runs 5, 6). Epimerization to glucose is not suspected as the mechanism, since glucose itself had little effect on ethylene production. Production of ethylene from galactose by *Penicillium digitatum* has been reported.[14,15]

Addition of xylose (Runs 7, 8) did not increase the total yield of ethylene. Tracer studies by Burg and Burg[13] disclosed no incorporation of label from xylose-^{14}C into ethylene. Furthermore, xylose does not normally occur in the free state.[16]

On the whole, carbohydrates were found to be ineffective in stimulation of ethylene production. Two probable reasons may be advanced for this observation; first, that carbohydrates are distant precursors of ethylene, or second, that they do not undergo extensive metabolism because the necessary enzymes are lacking in the system used.

(*b*) *Alcohols.* An appreciable increase in ethylene production was evident when ethanol (Runs 9, 10) was supplied to the particulate fraction. The alcohol was regarded by Phan-Chan-Ton[10] as a major precursor of ethylene. Gibson[4] detected a relatively high level of radioactivity in ethylene when ethanol-2-^{14}C was administered to *P. digitatum.* (Metabolism of ethanol to acetyl-CoA[17] and its entry into the tricarboxylic acid cycle is a reasonable possibility although a more direct pathway may be available.)

[7] M. SPENCER and M. MEHERIUK, *Nature* **199**, 1077 (1963).
[8] B. PAYES and G. G. LATIES, *Biochem. Biophys. Res. Commun.* **10**, 460 (1963).
[9] E. JUNI, *J. Biol. Chem.* **236**, 2302 (1961).
[10] PHAN-CHAN-TON, *Contribution à l'étude de la production d'éthylène par le* Penicillium digitatum, *Sacc.*, Doctoral Dissertation, l'universit de Paris (1961).
[11] D. H. SPALDING and M. LIEBERMAN, *Plant Physiol.* **38**, lviii (1963).
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[14] C. L. FERGUS, *Mycologia* **46**, 543 (1954).
[15] W. C. HALL, *Botan. Gaz.* **113**, 55 (1952).
[16] J. BONNER, *Plant Biochemistry*, p. 22. Academic Press, New York (1950).
[17] E. A. COSSINS and H. BEEVERS, *Plant Physiol.* **38**, 375 (1963).

TABLE 1. EFFECTS OF SEVERAL SUBSTRATES ON ETHYLENE PRODUCTION BY A PARTICULATE FRACTION FROM TOMATOES*

Run No.	Substrate (0·05 M)†	C_2H_4 (mμl) 0–3 hr	% Diff.‡	22–24 hr	% Diff.‡
1	Glycolaldehyde	93	−64	878	−77
2	Glycolaldehyde	157	+28	407	−55
3	Glucose	270	+15	928	+8
4	Glucose	301	−12	1226	−13
5	Galactose	276	−18	675	+64
6	Galactose	201	+2	438	+34
7	Xylose	277	+34	851	−5
8	Xylose	219	—	772	−20
9	Ethanol	170	—	2120	+193
10	Ethanol	223	−35	3767	+167
11	Propionate	321	+111	1684	+85
12	Propionate	494	+97	731	+260
13	Pyruvate (Na salt)	64	−26	889	−37
14	Pyruvate (Na salt)	177	+40	618	−22
15	α-Ketoglutarate	272	+5	2203	+11
16	α-Ketoglutarate	220	−15	1682	+8
17	Glyoxylate (Na salt)	171	−10	604	−38
18	Glyoxylate (Na salt)	163	−7	478	−25
19	Acrylate	227	+1	413	−45
20	Acrylate	155	−8	438	−12
21	Aspartic acid	180	+28	6649	+24
22	Aspartic acid	347	+1	2757	+95
23	Aspartic acid	73	−48	638	+14
24	Aspartic acid	326	+11	3264	+16
25	Glutamic acid	197	−12	1252	+94
26	Glutamic acid	293	+68	1895	+43
27	Glutamic acid	248	−25	1783	+39
28	Glutamic acid	152	+38	450	+15
29	γ-Aminobutyric acid	332	−7	1141	+41
30	γ-Aminobutyric acid	219	+8	1325	+39
31	β-Alanine	97	−17	506	+21
32	β-Alanine	182	−5	562	+85
33	Serine	236	−9	2756	+39
34	Serine	338	+67	2562	+16
35	Glycine	124	−19	688	+12
36	Glycine	182	−21	547	−7

* Basic reaction mixture: 0·5 M sucrose, 0·125 M KH_2PO_4, pH 7·2, $1·9 \times 10^{-3}$ M ATP added to each flask after initial sonication. Particles were sonicated for 4 min at 1·2 A at the beginning of collection periods 0–3 hr and 22–24 hr respectively.

† Except runs 21 and 22, and 27 and 28 in which the substrate concentration was 0·01 M.

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

(c) *Organic acids.* A pronounced increase in ethylene production was observed with propionate (Runs 11, 12). The metabolism of propionic acid to acrylate[18–23] is especially

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[19] J. GIOVANELLI and P. K. STUMPF, *J. Am. Chem. Soc.* **79**, 2652 (1957).
[20] N. P. MAHLER and F. M. HUENNEKINS, *Biochim. Biophys. Acta* **11**, 575 (1953).
[21] G. RENDINA and M. J. COON, *J. Biol. Chem.* **225**, 523 (1956).
[22] E. R. STADTMAN, *Fed. Proc.* **15**, 30 (1956).
[23] P. K. STUMPF, *Nature* **194**, 1158 (1962).

significant to the pathways proposed by Wang *et al.*[1] and Thompson and Spencer,[5] for both pathways include acrylic acid as an immediate precursor of ethylene. Labelling of ethylene when propionate-2(3)-^{14}C was supplied has been reported by Burg and Burg[13] and Jacobsen and Wang.[2]

Significant inhibition was evident with pyruvate (Runs 13, 14) although labelled ethylene was produced when pyruvate-2(3)-^{14}C was administered to *P. digitatum*.[4] It is possible that in the particulate fraction from tomatoes, pyruvate is converted to a metabolic product inhibitory to ethylene biogenesis.

Little effect was exhibited by α-ketoglutarate (Runs 15, 16). Either the Kreb's cycle intermediate is not involved in ethylene production or it may be poorly metabolized in the particulate fraction. It is interesting to note that succinate-2(3)-^{14}C was found to be poor in the labelling of ethylene.[1,4]

Inhibition of ethylene production occurred in the presence of glyoxylate (Runs 17, 18). Condensation with oxalacetate to form γ-hydroxy-α-ketoglutaric acid,[8] an inhibitor of the Kreb's cycle, provides a tentative explanation for the inhibitory effect.

Acrylate (Runs 19, 20) produced some inhibition of ethylene evolution. The result is apparently inconsistent with the proposals of Wang *et al.*[1] and Thompson and Spencer[5] that acrylic acid is an immediate precursor of ethylene. However, it is conceivable that acrylate added entered other reactions more quickly than the one leading to its utilization in ethylene synthesis. Also, the level of the acid used may have been deleterious to the ethylene-producing system, for acrylic acid is a very reactive compound.

(*d*) *Amino acids.* Marked stimulation of ethylene production by the particulate fraction was observed with the higher levels of aspartate supplied (Runs 21–24). Whether aspartic acid participates in ethylene biosynthesis through entry into the Kreb's cycle is not known, although the work of Wang, Persyn and Krackov[12] implied the cycle to be the primary pathway involved.

Similar results to those obtained with aspartic acid were observed with glutamate (Runs 25–28) at the same concentrations. Moreover, both aspartic-3-^{14}C acid and glutamic-3(4)-^{14}C have been shown to label ethylene.[12] Not only may both amino acids contribute their carbons to ethylene but they may promote ethylene production by virtue of adenosine triphosphate formation through their metabolism in the tricarboxylic acid cycle. Stimulation of ethylene evolution by adenosine triphosphate has been reported by Chandra *et al.*,[6] with this subcellular fraction from tomatoes.

γ-Aminobutyrate (Runs 29, 30), a decarboxylation product of glutamic acid,[24–26] also gave a significant elevation in ethylene production. Conversion to form glutamic acid[27] is a likely mode of action for the amino acid. A small augmentation in the total yield of ethylene was noted with β-alanine (Runs 31, 32). The amino acid is known to label ethylene[1,5] and constitutes a precursor in the pathway proposed by Thompson and Spencer.[5]

An appreciable stimulatory effect was noted with serine (Runs 33, 34). Lieberman *et al.*[28] obtained labelled ethylene from methionine-3(4)-^{14}C, and since serine participates in methionine metabolism it is possible that serine is involved in ethylene production through the latter process. Since glycine (Runs 35, 36) had little effect on ethylene production it is evident that the effect of serine is not dependent upon its conversion to glycine.

[24] Y. Y. CHENG, P. LINKO and M. MILNER, *Plant Physiol.* **35**, 68 (1960).
[25] O. SCHALES and S. S. SCHALES, *Arch. Biochem. Biophys.* **11**, 155 (1946).
[26] A. W. NAYLOR and N. E. TOLBERT, *Physiol. Plantarum* **9**, 220 (1956).
[27] M. DIXON and E. C. WEBB, *Enzymes*, Longmans, Green, London (1964).
[28] M. LIEBERMAN, A. T. KINISHI, L. W. MAPSON and D. A. WARDALE, *Plant Physiol.* **40**, 19 (*Supp.*) (1965).

Most of the carbohydrates evaluated were rather ineffective in the promotion of ethylene production by the particulate fraction from tomatoes. It would, therefore, appear that the fraction is unable to metabolize them to the necessary intermediates for conversion to ethylene, or perhaps that these sugars are involved in the biosynthesis of ethylene only as distant precursors. Galactose, on the other hand, did stimulate production of the olefin, as did amino acids. Propionic acid promotion of ethylene production raises the possibility of a relationship between fatty acid metabolism and ethylene biosynthesis.

Section 2. Cofactors (Table 2)

Addition of bovine serum albumin (Runs 1, 2) decreased the overall production of ethylene. It would seem that the functions associated with the protein, namely, the binding of inhibitory cations and of long-chain fatty acids, are not essential to the ethylene-producing activity of the particulate fraction. The small decrease in ethylene evolution in the presence of the albumin may be a result of interference with enzyme-substrate proximity.

Coenzyme A (Runs 3, 4) also exhibited an inhibitory effect with the particulate fraction. The cofactor may have promoted another reaction detrimental to ethylene biogenesis, or it may have given rise to an acyl intermediate that is metabolized to a product other than ethylene.

The two flavins (Runs 5–8) both reduced the total yield of ethylene. However, a substantial production of ethylene was noted with flavin mononucleotide during the 3–22 hr collection period (an increase of 100 per cent over the control sample), at which time ethylene evolution is normally low. One possible explanation would be that a complex necessary to ethylene synthesis undergoes reconstitution in the presence of the prosthetic group.

A pronounced decrease in ethylene production by the particles was observed when reduced glutathione (Runs 9, 10) was added to the suspension. It is probable that disulfide bonds are reduced by glutathione, a result reported with wheat protein.[29] Conformational states of enzymes would be altered by the reduction of these bonds.

Slight increases in total ethylene evolution were measured with the fraction to which α-lipoate (Runs 11, 12) was added. The cofactor is associated with the oxidative decarboxylation of α-keto acids.

Nicotinamide adenine dinucleotide (NAD, Runs 13–16) exerted no stimulatory effect on ethylene production but nicotinamide adenine dinucleotide phosphate (NADP, Runs 17–20) caused some increase in production at both concentrations studied. An NADP-linked dehydrogenase may be involved in the biosynthesis of ethylene.

The addition of both coenzyme A and NAD to the suspension (Runs 21, 22) resulted in a slight elevation of ethylene production, in comparison with the depression obtained with coenzyme A alone. The synergistic effect is consistent with the involvement of an enzyme complex or enzyme sequence in ethylene biogenesis. Coenzyme A and NADP added together had little effect (Runs 23, 24).

Neither addition of pyridoxal hydrochloride (Runs 25, 26) or of pyridoxal phosphate (Runs 27, 28) had much effect on total ethylene production, nor did concomitant addition of Fe^{+++} (Runs 29, 30).

TPP at a level of 2×10^{-4} M (Runs 31, 32) had little effect, but at a concentration of 2×10^{-3} M (Runs 33, 34) an increase in production was noted. Addition of 1×10^{-3} M Mg^{++} with either level of thiamine pyrophosphate (Runs 35–38) did not alter the effects of the factors added by themselves.

[29] R. Frater and F. J. R. Hird, *Biochem. J.* **88**, 100 (1963).

TABLE 2. EFFECT OF SEVERAL COFACTORS ON ETHYLENE PRODUCTION BY A PARTICULATE FRACTION FROM TOMATOES*

Run No.	Cofactor†	C_2H_4 (mμl) 0–3 hr	% Diff.*	22–24 hr	% Diff.*
1	BSA (1 mg/ml)	208	+7	2145	−12
2	BSA (1 mg/ml)	231	−26	824	−13
3	Coenzyme A (7×10^{-5} M)	144	−40	1227	−14
4	Coenzyme A (7×10^{-5} M)	270	−2	2013	−18
5	FAD ($1{\cdot}2\times10^{-4}$ M)	264	+47	1095	−17
6	FAD ($1{\cdot}2\times10^{-4}$ M)	182	+54	1220	−28
7	FMN (2×10^{-4} M)	177	−2	1066	−19
8	FMN (2×10^{-4} M)	101	−14	1083	−36
9	GSH (1×10^{-2} M)	174	−21	1671	−49
10	GSH (1×10^{-2} M)	287	+30	1384	−58
11	α-Lipoic acid (1×10^{-3} M)	621	+51	3742	+3
12	α-Lipoic acid (1×10^{-3} M)	329	−8	1057	+11
13	NAD ($1{\cdot}5\times10^{-4}$ M)	244	+8	698	−7
14	NAD ($1{\cdot}5\times10^{-4}$ M)	137	+16	1696	—
15	NAD ($3{\cdot}0\times10^{-4}$ M)	293	−7	534	−14
16	NAD ($3{\cdot}0\times10^{-4}$ M)	227	−4	489	—
17	NADP ($1{\cdot}3\times10^{-4}$ M)	343	+22	1283	+17
18	NADP ($1{\cdot}3\times10^{-4}$ M)	203	+2	1027	+17
19	NADP ($4{\cdot}0\times10^{-4}$ M)	124	—	956	+5
20	NADP ($4{\cdot}0\times10^{-4}$ M)	264	+74	1325	+16
21	NAD (3×10^{-4} M)+CoA (7×10^{-5} M)	231	−2	540	+11
22	NAD (3×10^{-4} M)+CoA (7×10^{-5} M)	225	+3	1022	+6
23	NADP (4×10^{-4} M)+CoA (7×10^{-5} M)	153	−39	1710	+1
24	NADP (4×10^{-4} M)+CoA (7×10^{-5} M)	126	−34	1026	+5
25	Pyridoxal hydrochloride (5×10^{-4} M)	253	+66	1188	+4
26	Pyridoxal hydrochloride (5×10^{-4} M)	253	+10	691	+18
27	Pyridoxal phosphate (5×10^{-4} M)	225	−26	715	+11
28	Pyridoxal phosphate (5×10^{-4} M)	244	−21	1417	−10
29	Pyridoxal phosphate** (5×10^{-4} M), Fe^{+3} (1×10^{-4} M)	360	−2	1569	−1
30	Pyridoxal phosphate** (5×10^{-4} M), Fe^{+3} (1×10^{-4} M)	281	+15	1450	+2
31	TPP (2×10^{-4} M)	242	−21	691	+7
32	TPP (2×10^{-4} M)	233	−23	723	+12
33	TPP (2×10^{-3} M)	355	−16	704	+30
34	TPP (2×10^{-3} M)	618	+37	973	+21
35	TPP (2×10^{-4} M),‡ Mg^{+2} (1×10^{-3} M)	129	+54	934	+1
36	TPP (2×10^{-4} M),‡ Mg^{+2} (1×10^{-3} M)	141	−17	641	+14
37	TPP (2×10^{-3} M),‡ Mg^{+2} (1×10^{-3} M)	191	−24	1548	+11
38	TPP (2×10^{-3} M),‡ Mg^{+2} (1×10^{-3} M)	101	−37	1647	+35

* See Table 1.

† BSA, bovine serum albumin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GSH, reduced glutathione; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; TPP, thiamine pyrophosphate.

** = control contains pyridoxal phosphate.

‡ = control contains TPP.

The stimulatory effects observed with TPP and Mg^{++} are indicative of decarboxylation, but whether it is oxidative or non-oxidative cannot be stated on the basis of these results. Decarboxylation of acrylic acid had been proposed as the terminal step in the pathways

proposed by Wang *et al.*[1] and Thompson and Spencer.[5] The likelihood of an enzyme complex or a sequence is suggested by the results obtained with FAD, and NAD and CoA.

Section 3. Cations (Table 3)

Total ethylene production decreased in the presence of magnesium (Runs 1, 2). It is interesting to note that a stimulatory effect for magnesium in the presence of thiamine pyrophosphate (TPP) is dependent upon the concentration of the latter cofactor (Table 2, Runs 35, 36).

TABLE 3. EFFECT OF SEVERAL CATIONS ON ETHYLENE PRODUCTION BY A PARTICULATE FRACTION FROM TOMATOES*

Run No.	Cation	C_2H_4 (mμl) 0–3 hr	% Diff.*	22–24 hr	% Diff.*
1	Mg^{++} ($MgSO_4$) (1×10^{-3} M)	88	−25	857	−21
2	Mg^{++} ($MgSO_4$) (1×10^{-3} M)	362	+9	1017	−14
3	Mn^{++} ($MnSO_4$) (1×10^{-3} M)	127	−15	763	−6
4	Mn^{++} ($MnSO_4$) (1×10^{-3} M)	354	+7	1361	−2
5	Fe^{++} ($FeSO_4$) (1×10^{-3} M)	198	+28	359	−8
6	Fe^{++} ($FeSO_4$) (1×10^{-3} M)	107	−9	676	−37
7	Cu^{++} ($CuSO_4$) (1×10^{-3} M)	99	−16	507	−53
8	Cu^{++} ($CuSO_4$) (1×10^{-3} M)	298	+32	382	−31
9	Zn^{++} ($ZnSO_4$) (1×10^{-3} M)	104	+20	900	−36
10	Zn^{++} ($ZnSO_4$) (1×10^{-3} M)	194	−22	1238	−27
11	Co^{++} ($CoCl_2$) (1×10^{-3} M)	115	−35	362	−43
12	Co^{++} ($CoCl_2$) (1×10^{-3} M)	100	+3	110	−72
13	Al^{+++} ($Al_2(SO_4)_3$) (1×10^{-3} M)	141	−20	627	−2
14	Al^{+++} ($Al_2(SO_4)_3$) (1×10^{-3} M)	207	+34	409	+5

* See Table 1.

Addition of manganese ions at 1×10^{-3} M (Runs 3, 4) resulted in decreased ethylene production, an effect also observed by Abeles and Rubenstein[30] with a pea enzyme preparation. Some suppression of ethylene production during the 22–24 hr period was noticed on the addition of ferrous ions (Runs 5, 6), possibly as a result of chelation with thiol groups.

Inhibition was evident when 1×10^{-3} M Cu^{++} (Runs 7, 8) was added to the particulate fraction. While the cation avidly binds thiol groups, it can also complex with imidazole and amino groups[31] and thus affect the availability of an active site on an enzyme necessary for ethylene production. Like copper, it chelates with thiol groups on proteins.[31] Although cobalt is reported to have lower affinity for thiol groups than either copper or zinc, the cation exhibited marked inhibition of ethylene production (Runs 11, 12).

Little effect on total ethylene production was observed with aluminium (Runs 13, 14), a result similar to that obtained with ferric ions in the presence of pyridoxal phosphate (see Runs 27, 28, Table 2). It would appear that a trivalent cation, often essential for amino acid decarboxylation and transamination, need not be added to the system.

[30] F. B. ABELES and B. RUBENSTEIN, *Biochim. Biophys. Acta* **93**, 675 (1964).
[31] R. M. HOCHSTER and J. H. QUASTEL, *Metabolic Inhibitors*, Vol. 2. Academic Press, New York (1963).

EXPERIMENTAL

Tomatoes, either the V121 variety grown in the university greenhouse or commercial varieties obtained from a local source, were frozen at −15° and used routinely in all of the experiments, although fresh tomatoes were occasionally used to check the procedure. Approximately 1 kg of fruit at the climacteric maximum in respiration was ground at low speed in a Waring Blendor with 1 l. of ice-cold buffer (0·5 M sucrose, 0·5 M KH_2PO_4, pH adjusted to 8·0 with NaOH) to give a homogenate of pH 6·8–7·5. The homogenate was then strained through four layers of cheesecloth and the filtrate centrifuged at 5000 *g* for 5 min to remove the cellular debris and other coarse particulate matter. The resulting supernatant was then centrifuged at 35,000 *g* for 15 min to give an orange pellet constituting the subcellular fraction used in the ethylene studies.[32] The pellet was dispersed in 75 ml of sucrose phosphate buffer (0·5 M sucrose, 0·125 M KH_2PO_4, pH 7·2 with NaOH), then sonicated at 0° for 4 min at 1·2 A in a Raytheon DF 101 10 kc sonic oscillator. Temperature of the suspension during sonication was in the range of 0–4°. Into each reaction vessel (50 ml extraction type) containing the necessary factors was pipetted 25 ml of the sonicated suspension. Ethylene evolved by the suspension, kept at room temperature, was collected by the method described by Chandra and Spencer.[33] Collection periods used were 0–3, 3–22, 22–23 and 23–24 hr and at the end of the 3–22 hr period the suspension was resonicated for 4 min at 1·1 A. The ethylene samples were analyzed with a hydrogen flame ionization gas chromatograph[32] employing a column filled with Burrel activated alumina containing 2·5% silicone 550 (dimensions of 3 ft × $\frac{5}{16}$ in. i.d.) or according to the method of Chandra and Spencer.[32] Nitrogen was determined by a microkjeldahl method.[34]

The possibility that the process of sonication could generate free radicals leading to ethylene[35] was evaluated in a series of experiments. Previous results[7] disclosed that evacuation and aeration, followed by boiling and sonication of the particulate suspension resulted in a markedly reduced yield of ethylene to that produced by unboiled particles. Furthermore, the boiled particles failed to produce ethylene during a 20 hr period. Sonicated particles that were boiled, evacuated and aerated produced little or no ethylene but unboiled particles continued to produce the volatile for a 20 hr period. A particulate suspension that had been denaturated initially (100° for 15 min), allowed to age overnight (3–22 hr) and then sonicated was found to produce a considerably smaller quantity of the gas, in comparison to unboiled particles sonicated after ageing[7]. Also the differential responses to various levels of arsenite,[36] and the influence of temperature on ethylene production by the subcellular particles under the same conditions as used in the present experiments, are consistent with the major ethylene production being enzymatic.

In addition, sonication blanks were determined with several substrates and cofactors. Since in normal runs these factors were not sonicated initially but only after the ageing of the suspension, the factors were allowed to age for 22 hr in 25 ml of the usual buffer (see above), then the mixture was sonicated under the usual conditions (see above) and the volatiles collected in mercuric perchlorate reagent[37] for 2 hr. Sonication of the cofactors and substrates resulted in low to non-detectable amounts of ethylene at the gas chromatographic

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[33] G. R. CHANDRA and M. SPENCER, *Biochim. Biophys. Acta* **69**, 423 (1963).
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sensitivities used in our experiments. The one exception was 0·05 M ethanol, which gave a value of 300 mμl, and this was subtracted from the value obtained for the 22–24 hr ethylene collection period (where 0·05 M ethanol was added to the reaction mixture). Ethanol determinations by use of the Sigma Ethanol Kit 330 (Sigma Chemical Co., St. Louis, Miss.) disclosed that the ethyl alcohol content of the particulate suspension at all times (except when ethanol was added) was considerably below 0·05 M and that contribution from the sonication of the ethanol present would be insignificant to the total yield of ethylene. (The ethanol content was determined from the absorptivity of reduced nicotinamide adenine dinucleotide, formed from the reduction of nicotinamide adenine dinucleotide by alcohol dehydrogenase, read at 340 mμ.)

Recently, Burg and Burg[13] stated that ethylene could be formed from the dehydration of ethanol in mercuric perchlorate. The significance of this observation to the total yield of ethylene by the particulate fraction was evaluated. The ethanol content of the volatiles was determined by the Sigma method, and ten times this amount was added to mercuric perchlorate and the mixture was aged for the periods that samples were normally stored before analysis. Gas chromatography of the volatiles after liberation from the mercury complex[32] revealed no peaks for ethylene at the sensitivities used in the determination.

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