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

EFFECTS OF SEVERAL INHIBITORS

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Abstract—A study was conducted on the effects of several inhibitors on ethylene production by a subcellular fraction from tomatoes. Results disclosed that sulfhydryl groups were essential for the synthesis of the volatile, and that a metalloprotein, or a cation, or both were involved. Carbonyl groups associated with an enzyme or substrate were also shown to be necessary for the biogenesis of the olefin. Results indicated that a considerable portion of the Kreb's cycle was not directly involved in the biosynthesis of ethylene. The study also suggested that transamination has a role in the production of the volatile.

INTRODUCTION

THE present report extends an earlier study[1] with inhibitors. The identification by use of inhibitors of individual enzymes responsible for the biosynthesis of ethylene in a subcellular fraction is not a simple task. However, application of a wide variety of inhibitors and careful interpretation of their effects was found to be of considerable assistance in the postulation of the biogenetic pathway and the kinds of enzymes involved.

RESULTS AND DISCUSSION

Results are given in Table 1, where inhibitors are grouped according to their modes of action. While two results are given for each inhibitor, they are representative of several that have been obtained. Since current methods do not permit isolation of subcellular particles with identical properties from different runs, quantitative comparisons are made only between the samples of each preparation, and not between preparations. A value of $0{\cdot}05 \pm 0{\cdot}41$ mg was found for the mean and standard deviation for the differences in nitrogen value between the control and inhibitor experiment samples. The reason for expressing nitrogen values in this manner is stated in the first paper.[2]

(*a*) *Sulfhydryl Agents*

Three levels of arsenite were employed. At 10^{-4} M (Runs 1, 2) arsenite did not inhibit ethylene production, but at 10^{-3} M (Runs 3, 4) significant inhibition occurred, and arsenite at 10^{-1} M (Runs 5, 6) effected almost total inhibition of ethylene evolution.

Reduction of ethylene production by *p*-chloromercuribenzoate (*p*-CMB) was greater at 10^{-3} M than at 10^{-4} M concentrations of the inhibitor (Runs 7–10). Reversal of the inhibition was achieved when reduced glutathione (10^{-2} M, Runs 11, 12) was added 1 hr after initial incubation of the particles with the inhibitor.

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[1] M. MEHERIUK and M. SPENCER, *Nature* **204**, 43 (1964).

[2] M. MEHERIUK and M. SPENCER, *Phytochem.* **6**, 535 (1967).

TABLE 1. EFFECT OF VARIOUS INHIBITORS ON ETHYLENE PRODUCTION BY A PARTICULATE FRACTION FROM TOMATOES*

Run No.	Inhibitor†	C_2H_4 (mμl) 0–3 hr	% Diff.‡	22–24 hr	% Diff.‡
1	Arsenite (Na salt) (1×10^{-4} M)	405	+137	624	−5
2	Arsenite (Na salt) (1×10^{-4} M)	191	+10	726	—
3	Arsenite (Na salt) (1×10^{-3} M)	169	−33	613	−28
4	Arsenite (Na salt) (1×10^{-3} M)	194	+15	230	−54
5	Arsenite (Na salt) (1×10^{-1} M)	301	+31	122	−95
6	Arsenite (Na salt) (1×10^{-1} M)	287	+199	333	−85
7	*p*-CMB (Na salt) (1×10^{-4} M)	180	−42	754	−29
8	*p*-CMB (Na salt) (1×10^{-4} M)	216	−8	889	−26
9	*p*-CMB (Na salt) (1×10^{-3} M)	216	−22	323	−59
10	*p*-CMB (Na salt) (1×10^{-3} M)	317	−12	414	−59
11	*p*-CMB (Na salt) (1×10^{-3} M); (GSH 1×10^{-2} M)**	180	−26	1369	+50
12	*p*-CMB (Na salt) (1×10^{-3} M); (GSH 1×10^{-2} M)**	216	−8	1096	−9
13	Iodoacetamide (1×10^{-4} M)	120	−22	517	−41
14	Iodoacetamide (1×10^{-4} M)	101	−20	499	−37
15	Iodoacetamide (1×10^{-2} M)	169	−25	383	−64
16	Iodoacetamide (1×10^{-2} M)	237	+45	388	−43
17	Mercuric sulfate (1×10^{-2} M)	107	−3	73	−80
18	Mercuric sulfate (1×10^{-2} M)	101	−63	45	−89
19	Silver nitrate (1×10^{-2} M)	129	+17	238	−36
20	Silver nitrate (1×10^{-2} M)	73	−73	248	−38
21	Azide (Na salt) (1×10^{-3} M)	231	—	590	−38
22	Azide (Na salt) (1×10^{-3} M)	293	−18	464	−51
23	Cyanide (K salt) (1×10^{-4} M)	118	+93	307	−10
24	Cyanide (K salt) (1×10^{-4} M)	338	−7	940	−8
25	Cyanide (K salt) (2×10^{-4} M)	332	+9	1918	+27
26	Cyanide (K salt) (2×10^{-4} M)	656	+15	2136	+6
27	Cyanide (K salt) (1×10^{-2} M)	512	+51	478	−45
28	Cyanide (K salt) (1×10^{-2} M)	310	+12	465	−35
29	DIECA (Na salt) (5×10^{-4} M)	194	+2	514	−47
30	DIECA (Na salt) (5×10^{-4} M)	261	+36	788	−17
31	DIECA (Na salt) (1×10^{-3} M)	122	−21	525	−40
32	DIECA (Na salt) (1×10^{-3} M)	62	−29	355	−73
33	Hydroxylamine–HCl (1×10^{-3} M)	186	−3	446	−53
34	Hydroxylamine–HCl (1×10^{-3} M)	271	+94	214	−56
35	Semicarbazide–HCl (1×10^{-3} M)	253	−23	1023	−21
36	Semicarbazide–HCl (1×10^{-3} M)	158	−43	709	−9
37	Malonic acid (1×10^{-3} M)	428	+17	1142	−3
38	Malonic acid (1×10^{-3} M)	175	+72	486	−10
39	Malonic acid (1×10^{-2} M)	124	−17	687	−15
40	Malonic acid (1×10^{-2} M)	227	−10	831	−2
41	Monofluoroacetate (Na salt) (1×10^{-4} M)	249	+144	400	−26
42	Monofluoroacetate (Na salt) (1×10^{-4} M)	248	+47	1147	−11
43	Monofluoroacetate (Na salt) (1×10^{-3} M)	174	−37	701	−10
44	Monofluoroacetate (Na salt) (1×10^{-3} M)	222	−6	993	−18
45	Aminooxyacetic acid (1×10^{-3} M)	250	−13	1957	−13
46	Aminooxyacetic acid (1×10^{-3} M)	96	−36	697	−14
47	D-Cycloserine (1×10^{-3} M)	175	−28	932	−35
48	D-Cycloserine (1×10^{-3} M)	253	−12	623	−42
49	Fluoride (Na salt) (1×10^{-3} M)	399	+61	1035	−6
50	Fluoride (Na salt) (1×10^{-3} M)	285	−3	1350	+2
51	Fluoride (Na salt) (1×10^{-1} M)	45	—	425	−31

TABLE 1—*continued*

Run No.	Inhibitor†	C_2H_4 (mμl) 0–3 hr	% Diff.‡	22–24 hr	% Diff.‡
52	Fluoride (Na salt) (1×10^{-1} M)	182	+5	384	−47
53	Oxythiamine (1×10^{-3} M)	292	+109	658	+34
54	Oxythiamine (1×10^{-3} M)	209	—	875	+30
55	L-Thyroxine (Na salt) (1×10^{-3} M)	259	+34	2256	−7
56	L-Thyroxine (Na salt) (1×10^{-3} M)	306	+11	2057	−17

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

† DIECA = diethyldithiocarbamate; *p*-CMB = *p*-chloromercuribenzoate.

‡ % Diff. = per cent increase or decrease in ethylene production with respect to the control sample.

** 10^{-2} M glutathione (GSH) was added 1 hr after initial sonication.

Substantial inhibition was observed with iodoacetamide at levels of 10^{-4} M (Runs 13, 14) and at 10^{-2} M (Runs 15, 16). Burg and Thimann[3] found that 10^{-2} M iodoacetamide inhibited ethylene production by apple plugs and that adenosine triphosphate (ATP) failed to reverse it. Gibson[4] noted a small reduction in ethylene evolution by *Penicillium digitatum* subjected to 10^{-4} M iodoacetate, while Abeles and Rubenstein[5] reported significant inhibition of production upon addition of 10^{-2} M iodoacetate to a pea enzyme preparation.

The effects of two heavy metals with a high affinity for thiol groups were evaluated. Mercuric sulfate at 10^{-2} M (Runs 17, 18) was pronounced in its inhibition of ethylene production but a preliminary experiment revealed that the cation complexed with ethylene in solution and therefore part of the inhibitory effect must be attributed to this phenomenon. Silver nitrate at 10^{-2} M (Runs 19, 20), although less effective than mercuric sulfate, still inhibited ethylene evolution considerably.

(*b*) *Chelating Agents*

Azide at 10^{-3} M (Runs 21, 22) exhibited appreciable inhibition of ethylene synthesis. Gibson[4] reported complete termination of ethylene production by *P. digitatum* treated with 10^{-2} M azide, and Abeles and Rubenstein[5] found marked inhibition by azide (10^{-3} M) of ethylene production by a pea enzyme preparation.

Cyanide at 10^{-2} M (Runs 27, 28) inhibited production in the 22–24 hr collection period, but was less effective at lower concentrations (Runs 23–26). This reagent not only chelates with cations, but also reacts with carbonyl groups and disulfide bonds. Hansen[6] observed no effect on ethylene production by pears when low levels of hydrogen cyanide were used, but high levels of the reagent caused irreversible damage to the tissue. Burg[7] found 10^{-2} M cyanide to be ineffective with apple plugs but Lieberman and Mapson[8] reported inhibition with cyanide at 10^{-3} M. Ethylene production by *P. digitatum* was shown to be progressively

[3] S. P. BURG and K. V. THIMANN, *Plant Physiol.* **35**, 24 (1960).
[4] MARGARET GIBSON, *The Biogenesis of Ethylene*, Doctoral Dissertation, Purdue University (1963).
[5] F. B. ABELES and B. RUBENSTEIN, *Biochim. Biophys. Acta* **93**, 675 (1964).
[6] E. HANSEN, *Botan. Gaz.* **103**, 543 (1941).
[7] S. BURG, *The Biogenesis of Ethylene*, Doctoral Dissertation, Harvard University (1958).
[8] M. LIEBERMAN and L. W. MAPSON, *Plant Physiol.* **39**, ix (1964).

inhibited by cyanide as the concentration was increased to 5×10^{-4} M[4]. A substantial decrease in evolution of the olefin was reported by Abeles and Rubenstein[5] who incubated cyanide with a pea enzyme preparation. Interpretation of results with cyanide is complicated by a penetration problem[9] and by the ability of some plants to assimilate cyanide.[10–12]

Commonly used as a chelating agent is diethyldithiocarbamate (DIECA). Inhibition of ethylene production by the tomato fraction was observed when levels of 5×10^{-4} M (Runs 29, 30) and 10^{-3} M (Runs 31, 32) DIECA were employed. In addition to its reaction with metal ions essential for activity of certain enzymes, DIECA may inhibit succinic dehydrogenase activity if it is oxidized by the cytochrome system to tetraethyldithiocarbamyl sulfide.[9] The inhibition by DIECA of ethylene synthesis by apple plugs, reported by Lieberman and co-workers,[8, 13, 14] led them to suggest that a copper enzyme was involved in the biosynthesis of ethylene, but it is now known that the reaction of DIECA is not specific for this cation.[9]

(c) Carbonyl Agents

Hydroxylamine at 10^{-3} M (Runs 33, 34) reduced production of the olefin substantially. Mild inhibition was observed with semicarbazide at 10^{-3} M (Runs 35, 36). Cyanide at 10^{-2} M, which can react with carbonyl groups to give a cyanohydrin derivative, also reduced ethylene evolution (Runs 27, 28).

(d) Citric Acid Cycle Inhibitors

Malonic acid, which competitively inhibits succinic dehydrogenase, had little effect on total ethylene production by the tomato particulate fraction when concentrations of 10^{-3} M (Runs 37, 38) and 10^{-2} M (Runs 39, 40) were used. Since malonate is metabolized by plants,[15,16] inhibition may be difficult to achieve except at high concentrations. Weak inhibition of ethylene production by *P. digitatum* was reported by Gibson[4] who employed malonate at a level of 10^{-2} M.

Monofluoroacetate, which inhibits aconitase, effected a weak inhibition at the higher level of 10^{-3} M (Runs 41–44). Burg and Thimann[3] observed progressive inhibition of ethylene production by apple plugs until a concentration of 10^{-1} M had been reached, at which level a 70 per cent inhibition occurred. Gibson[4] noted substantial inhibition of ethylene evolution by *P. digitatum* treated with 10^{-3} M monofluoroacetate.

(e) Transaminase Inhibitors

Aminooxyacetic acid, reported to inhibit a γ-aminobutyric transaminase[17] and a glutamic-alanine aminotransferase,[18] was mildly effective with the tomato fraction when used at a concentration of 10^{-3} M (Runs 45, 46).

D-Cycloserine at 10^{-3} M (Runs 47, 48) gave appreciable inhibition of ethylene production. Different transaminases, of varying importance in the biosynthesis of ethylene, may be affected by the two inhibitors.

[9] R. M. HOCHSTER and J. H. QUASTEL, *Metabolic Inhibitors*, Vol. 11. Academic Press, New York (1963).
[10] S. GOLDSCHMIDT-BLUMENTHAL, G. W. BUTLER and E. E. COON, *Nature* **197**, 718 (1963).
[11] L. FOWDEN and E. A. BELL, *Nature* **206**, 110 (1965).
[12] B. TSCHIERSCH, *Phytochem.* **3**, 365 (1964).
[13] M. LIEBERMAN and L. W. MAPSON, *Nature* **195**, 1016 (1962).
[14] M. LIEBERMAN and L. W. MAPSON, *Nature* **204**, 343 (1964).
[15] J. GIOVANELLI and P. K. STUMPF, *Plant Physiol.* **32**, 498 (1957).
[16] M. D. HUTCH and P. K. STUMPF, *Plant Physiol.* **37**, 121 (1962).
[17] D. P. WALLACH, *Biochem. Pharmacol.* **5**, 323 (1961).
[18] S. HOPPER and H. L. SEGAL, *J. Biol. Chem.* **237**, 3189 (1962).

(f) Others

Fluoride, commonly used to block enolase activity, was ineffective at 10^{-3} M (Runs 49, 50) but was inhibitory at 10^{-1} M (Runs 51, 52). However, since a high level of fluoride was required to obtain inhibition, caution must be exercised in assuming a typical mode of action. Burg and Thimann[3] noted that fluoride inhibition of ethylene production by apple plugs could be partially reversed by the addition of ATP, and Abeles and Rubenstein[5] reported a reduction of 50 per cent in ethylene evolution with 10^{-3} M NaF added to a pea enzyme preparation.

Oxythiamine, a potent inhibitor of transketolase activity, stimulated ethylene production at a concentration of 10^{-3} M (Runs 53, 54).

L-Thyroxine gave weak inhibition at 10^{-3} M (Runs 55, 56). The reagent is effective as an uncoupler of oxidative phosphorylation in intact particles but not in disrupted ones. A paper by Horvath[19] reporting that an alanine–glutamic transaminase is inhibited by thyroxine offers an explanation for the behavior of the compound with the subcellular fraction from tomatoes.

It is obvious that thiol groups are essential for ethylene biosynthesis in view of the inhibitory effects of the sulfhydryl agents, arsenite, *p*-CMB, iodoacetamide, mercuric and silver ions. Reduction of disulfide links essential to ethylene production may be involved in the inhibitory action of cyanide. Inhibition by hydroxylamine, cyanide and semi-carbazide indicates a role for carbonyl groups in ethylene production. Transaminase activity is implicated by the inhibitory action of cycloserine and aminooxyacetic acid. The lack of appreciable inhibition by malonic acid and monofluoroacetate would suggest that a considerable portion of the TCA cycle is not essential to ethylene biosynthesis. Although some other workers have detected inhibition with these reagents in whole tissue, it is possible that a lack of inhibition represents a characteristic of the tomato particulate fraction. It is conceivable that fluoride acts on an enzyme other than succinic dehydrogenase, since malonic acid was not found to be inhibitory. Azide and cyanide probably inhibit the cytochrome system with a concomitant reduction in ATP supply. Chandra, Spencer and Meheriuk[20] previously demonstrated ATP to be stimulatory to ethylene production by tomato particles and limiting its production would consequently decrease the rate of ethylene synthesis.

METHODS

The methods used in this study have been described in previous papers.[2, 21, 22] Briefly, a tomato particulate fraction, isolated at 35,000 *g*, was suspended in sucrose phosphate buffer (0·5 M sucrose, 0·125 M KH_2PO_4 adjusted to a pH 7·2 with NaOH) and sonicated to disrupt the particles. The suspension was then pipetted into reaction vessels containing the appropriate factors, and ethylene was collected from the suspensions for a period of 24 hr at room temperature. The suspensions were sonicated after the overnight ageing (3–22 hr) period.

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[19] A. Horvath, *Enzymologia* **19**, 297 (1958).
[20] G. R. Chandra, M. Spencer and M. Meheriuk, *Nature* **199**, 767 (1963).
[21] G. R. Chandra and M. Spencer, *Nature* **194**, 361 (1962).
[22] G. R. Chandra and M. Spencer, *Biochem. Biophys. Acta* **69**, 423 (1963).