A MECHANISM OF THE INHIBITION OF PLANT GROWTH BY MALEIC HYDRAZIDE

By

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INTRODUCTION

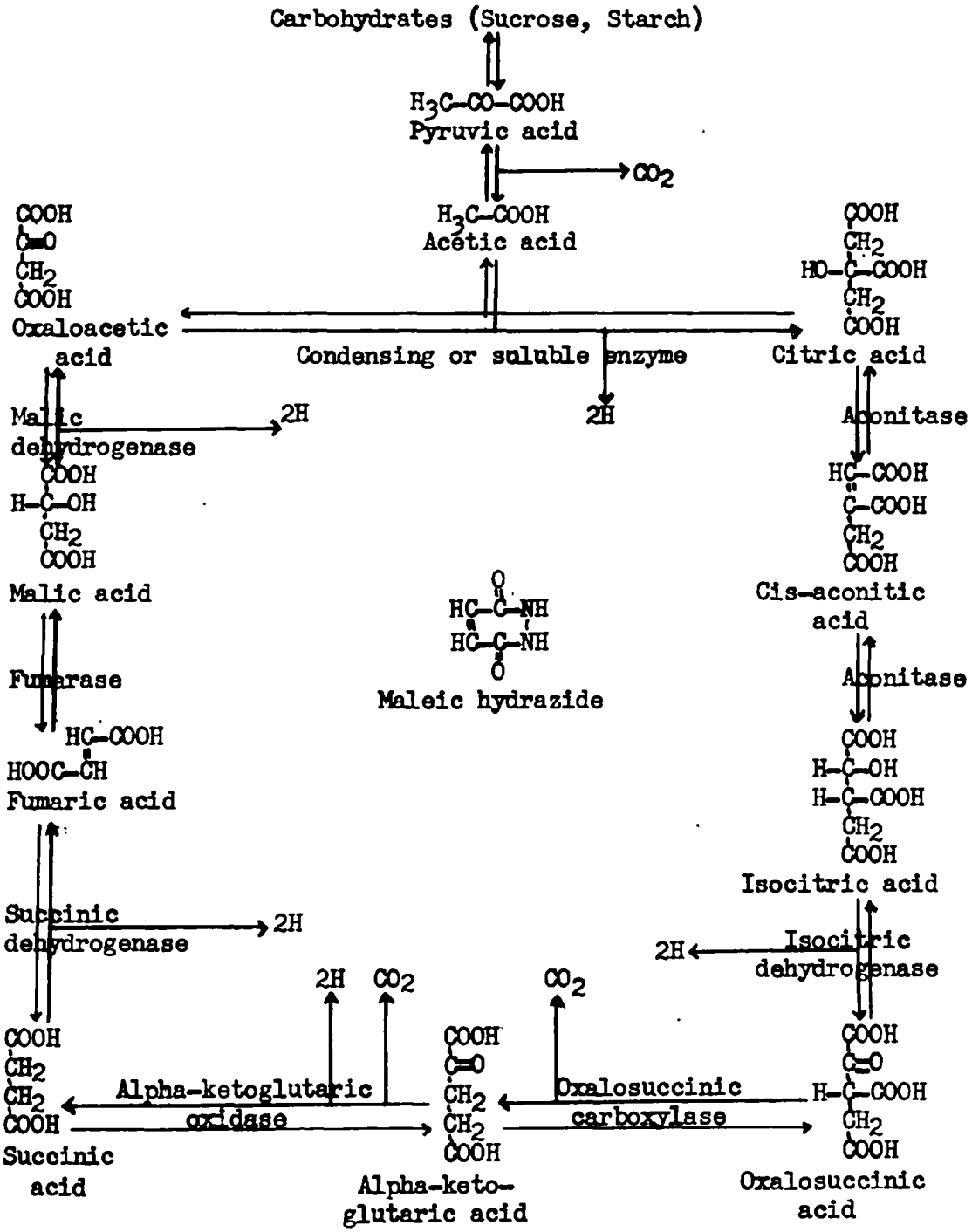
Early work by Schoene and Hoffman (28) and others has shown that maleic hydrazide (MH) has an effect on plant growth, development, and flowering. Although many workers have studied the effects of MH on the morphology of plants, the mechanism of its action is not well understood.

Leopold and Klein (16, 17), Greulach and Haesloop (10), and others suggest that MH acts as an antiauxin in plants. Isenberg et al. (11) and Naylor and Davis (25), assert it affects dehydrogenases. Phouphas (26) suggests it disturbs the enzyme balance. Greulach and Atchison (9), Greulach (8), and Darlington and McLeish (4) report that MH is antimitotic in action and does not effect cell elongation. Work of Currier et al. (3), Greulach (7), Moore (20), Naylor (23), Naylor and Davis (24), Zukel (33), and others established the fact that MH causes the accumulation of carbohydrates and the appearance of anthocyanins.

Respiration is generally considered to be one of the processes associated with growth (2) although the proportion of respiratory energy used in growth is small (6, 31). Since MH inhibits growth of plants, Naylor and Davis (25) performed experiments to demonstrate that MH inhibits respiration, and therefore growth. The MH provided inhibition of aerobic respiration and they speculated along with others (11, 26, 29) that MH prevents dehydrogenases from functioning normally.

If such is the case MH will prevent the activity of dehydrogenases evolved in the formation of malic acid and certain other essential respiratory metabolites. Addition of these metabolites might restore respiration and therefore growth.

The experiments reported here concern the mechanism of the effect of MH on plant respiration. The results indicate that MH, a derivative of maleic acid (5), the cis-isomer of fumaric acid, inhibits plant respiration by competing for the enzymes fumarase and aconitase (Figure 1). Fumarase normally acts upon fumaric acid to form malic acid, and aconitase acts upon cis-aconitic acid to form isocitric acid (12, 14). In the presence of Krebs cycle intermediates, individually or in combination, MH induced inhibition is not completely circumvented. Partial restoration is achieved, however, by the addition of malic and isocitric acids. MH appears to inhibit respiration by competitive inhibition, although this is not conclusively proved. Also, the data suggest that MH inhibits respiration through some other mechanism, possibly by non-competitive inhibition of the thiol groups of certain dehydrogenases of the Krebs cycle. This conclusion is in agreement with McRae and Bonner (22) who showed that MH is not a strict competitive inhibitor of growth according to kinetic treatment of MH growth inhibition data.



General outline of the Krebs cycle

Figure 1

METHODS AND MATERIAL

Mung bean, Phaseolus aureus, was used because preliminary experiments showed that MH inhibits respiration of this plant, and because Millerd, Bonner, Axelrod, and Bandurski (19) isolated mitochondria from mung bean seedlings which were capable of carrying out all reactions of the Krebs cycle (Figure 1). Seeds for all the experiments were from a uniform stock supply of field grown mung beans obtained from the Department of Agronomy of the University of Arizona.

Mung bean seeds were washed and soaked in tap water for two hours. During this period the seeds were rinsed three or four times until the rinse water was clear. No difference in quality or quantity of seedlings was observed between unsterilized seeds and seeds sterilized in a 0.006 per cent solution of Roccal[1] as recommended by Addicott et al. (1), or with various concentrations of Purex. Therefore sterilization was omitted. At the end of two hours the soaked seeds were placed on one thickness of absorbent cheesecloth. A Petri dish cover was placed over the seeds, the cheesecloth was fastened tightly to the cover, and the whole was placed over a deep-well Petri dish filled with distilled water. The seeds were germinated in a dark cabinet for 48 hours. At the end of 24 to 30 hours the distilled water was discarded, the Petri dish washed,

1/ Supplied by Sterwin Chemicals, Inc., New York, New York.

and a fresh supply of distilled water added. After 48 hours the roots had grown 2 to 3 cm. straight down in the water. They were then removed from the cabinet, again rinsed in distilled water, and the terminal 1 cm. section of root tip removed. Cutting of root tips was facilitated by a special scissors which had a 1 cm. piece of wire fastened at a right angle to a blade 1 cm. from the tip of the blade. The cut sections were placed in distilled water in a covered Petri dish.

This cheesecloth-water culture method was used because it provided a dependable supply of straight roots of uniform thickness and length in a minimum of time. A minimum of handling reduced injury to the roots. Another advantage was that the medium surrounding the roots was always aqueous since the roots were cultured in water and root respiration was measured while the roots were in water solutions.

Root tips were selected at random and carefully placed into Warburg vessels containing the test solutions. Respiration was measured in standard, single-sidearm Warburg vessels of approximately 16 ml. capacity provided with a central well. These vessels were attached to manometers, placed in a constant 30.0°C. water bath, and allowed to equilibrate for 1.5 hours before respiration measurements were begun. Measurements were then made once an hour until the limits of the manometers were reached, usually 4 to 6 hours later. The machine used was of the circular type and had a shaking rate of approximately 120 oscillations per minute. Total volume of liquid in each vessel in every experiment was 2.3 ml. This

included 0.2 ml. of 20 per cent KOH in the center well for the absorption of respiratory CO_2. All measurements of oxygen consumption were made using the direct method of Warburg as described by Umbreit, Burris, and Stauffer (32), and all results are expressed as µl. O_2 consumption/10 roots/unit time. To maintain constant pH, phosphate buffer (KH_2PO_4-K_2HPO_4) was used in all experiments. Experiments to be discussed in detail later showed that 10 root tips and pH 7.0 were most satisfactory.

MH[2] was used in the experiments rather than its commercial sodium or diethanolamine salts (which are 40 and 30 per cent active MH, respectively) in order that all results obtained could be attributed directly to MH. The nearly pure MH as supplied was further purified by recrystallization from water (5). The purified MH was white and had no apparent impurities or foreign matter. It was found that MH thus purified gave greater inhibition than the nearly pure MH.

MH solutions were prepared to provide MH concentrations of 0.05 and 0.075 molarity. This was done by dissolving crystalline MH in hot buffer of a molarity greater than the MH being prepared. Because MH is so strongly acid and the buffer is weak by comparison, the solution was cooled and adjusted to the desired pH of 7.0 with KOH. Buffer was again added to bring the solution to volume. It should be emphasized that more buffer was added after the pH had been adjusted with KOH

2/ Maleic hydrazide used in this work was generously supplied in the nearly pure form by the Naugatuck Chemical Division, U. S. Rubber Co., Naugatuck, Connecticut.

in order to provide a reserve of buffer to maintain the pH of the solution at 7.0. If this step was omitted the final pH of solutions after 5 to 6 hours in the Warburg vessels varied between 5 and 7.0. Solutions of other compounds were prepared in a similar manner. Although the addition of KOH to solutions of MH, and to other compounds not available as a potassium salt, increased the total molarity of the solution to an unknown degree, these solutions were used uniformly in any one experiment so that the effect of the increased molarity would not alter the respiratory measurements. Total molarity of the solution being tested varied slightly among experiments, but in any one experiment test solutions had uniform molarity.

Before the actual study of MH inhibition in the presence of Krebs cycle intermediates, certain preliminary experiments were made to determine the number of roots to be used per vessel, the optimal pH, molarity limits, concentration of MH, length of time to make measurements, and length of time to equilibrate the vessels and roots before beginning oxygen uptake measurements. These will be discussed individually later.

Occasional experiments which for unknown reasons yielded data of a precision less than desirable were discarded.

EXPERIMENTAL RESULTS

Manometric Procedure

It was necessary to determine the number of roots to be used per vessel, equilibration period required, and number of replications of tests necessary for accuracy. This was done by adding 10, 15, or 20 mung bean root tips to separate manometer vessels containing 0.05 M buffer at pH 6.0. Following a 5 minute equilibration period respiration measurements were made every 30 minutes. Eight vessels contained 10 roots, four vessels held 15 roots, and four vessels received 20 roots. It was desired to use as many roots as possible in order to minimize variation between samples of roots and to obtain maximum respiration. However, within three hours the flasks with 20 roots and within five hours flasks with 15 roots had so greatly respired that measurements could no longer be made. Because MH usually requires a 2 to 3 hour period to attain maximum inhibition, and because roots in the presence of many of the Krebs cycle intermediates respire much more rapidly than do roots in plain buffer, a 4 to 5 hour measurement of respiration was desired. Therefore, 10 root tips were used in all experiments. Measurements from flask to flask were not consistent for the first 90 minutes so an equilibration period of 90 minutes was established.

From this and subsequent experiments it seemed obvious

that replication of treatments in each experiment was required. Averages of measurements of flasks in groups of three provided values which did not deviate significantly from the mean established by eight flasks. Therefore experiments were designed to provide three replications of treatments. However, because only 17 manometers were available for experimentation, one treatment occasionally received only two replications. Measurements of three flasks receiving the same treatment provided measurement of oxygen absorption of 30 root tips. These three sets of 10 roots provided measurements which, when totaled, averaged, and expressed on a 10 root basis, eliminated most of the variation single measurements furnished.

An attempt was made to express oxygen uptake on a dry root weight basis, but the dry weight of the roots was small and rather large variation of root weights existed between samples. Expression of oxygen consumption on a mg. nitrogen basis, as often recommended, was discarded because of the nitrogen added to the roots in the form of MH. For these reasons, oxygen uptake was expressed on the number of roots used.

Hydrogen Ion Concentration (pH): To determine the optimum pH for mung bean root respiration, oxygen uptake was measured in 0.10 M solutions of buffer at pH 5, 6, 7, 8, and 9. Results (Table I) show that respiration is highest at pH 7, and that it rapidly declines at pH 8 and 9. Although pH values of 5 and 6 were almost as satisfactory for respiration as pH 7, they were not used since (a) more MH will go into solution at a high rather than a low pH, (b) mung bean

Table I. Effect of pH on respiration of mung bean root tips in 0.10 M phosphate buffer. Experiment 3.

pH	µl. O_2/10 roots/5 hours			
	Sample			
	1	2	3	Mean
5	172	155	163	163
6	169	175	189	178
7	201	195	211	203
8	140	7	12	53
9	135	95	70	100

Table II. Effect of molarity on respiration of mung bean root tips. Roots in phosphate buffer, pH 7.0. Experiment 6.

Molarity	µl. O_2/10 roots/5 hours				
	Sample				
	1	2	3	4	Mean
0.05	263	234	256	243	249
0.10	245	274	276	263	265
0.15	229	239	238	---	235
0.20	215	228	218	217	220

Table III. Effect of MH (nearly pure) concentration on respiration of mung bean root tips. Experiment 7.

Molarity MH	Molarity buffer	Total molarity	pH	µl. O_2/10 roots/4 hours				
				Sample			Mean	% of control
				1	2	3		
0.00	0.125	0.125	7.0	225	230	175	210	100%
0.025	0.10	0.125	7.0	153	197	140	163	78
0.05	0.075	0.125	7.0	26	16	39	27	13
0.075*	0.05	0.125	7.	6	6	2	5	2
0.10*	0.025	0.125	7.	8	-2	1	3	1

* = Some MH precipitate.

mitochondria respire rapidly at pH 7.1 (19), and (c) it will be shown that mung bean root respiration is effectively inhibited by MH at pH 7.0.

Molarity: To determine the effect of total molarity of the test solution on respiration, root tips were exposed to 0.05, 0.10, 0.15, and 0.20 M solutions of buffer at pH 7.0. Results (Table II) show that most rapid respiration occurs at 0.10 M. However, because of the high concentration of MH required for inhibiting respiration, and therefore the concentration of buffer needed to maintain controlled pH, the molarity used was generally 0.20.

Maleic Hydrazide

MH Concentration: Because most previous work with MH has been limited to its commercial preparations, the inhibitory concentrations of pure MH had to be determined. To do this MH solutions, in water rather than buffer, of 0.20, 0.15, 0.10, and 0.05 M were prepared. These were diluted half and half with 0.05, 0.10, 0.15, and 0.20 M buffer (pH 7.0) respectively. Each resulting solution had a total molarity of 0.125. Because in the presence of high concentrations of MH low molar buffer can not maintain the pH, some MH precipitated out of the 0.15 and 0.20 M MH solutions. Therefore, actual MH concentration and pH of these two treatments are not known. This experiment was performed before the need for a concentration of buffer equal to that of the MH had been recognized, otherwise precautions would have been used to avoid the concentration and pH changes. However, the experiment

as it was conducted provided the necessary information (Table III). Some of the inhibition at the 0.15 and 0.20 M concentrations must be attributed to a low pH, however, at these concentrations MH inhibition might have been considerably greater if all of the MH had been in solution. The results indicate 0.05 M MH to be the minimum concentration for significant inhibition.

Effect of MH Purity: The MH used in the previous experiment had not been purified by recrystallization from water (5). Therefore a comparison was made between inhibitory effect of the nearly pure MH as used above and purified MH. In this experiment 0.10 M MH was dissolved in 0.20 M buffer, pH 7.0, rather than water. One ml. of the MH was pipetted into a Warburg vessel and one ml. of 0.10 M buffer added. The resulting solution contained 0.05 M MH and 0.15 M buffer, total molarity in the vessel being 0.20. It is readily seen (Table V) that the purified MH, both alone and in combination with a Krebs cycle intermediate (citrate), inhibits respiration to a greater degree than does nonpurified or stock MH under the same conditions. Purified MH was therefore used in all other experiments.

Variation of Results: It should be noted that throughout this study MH does not always inhibit respiration to the same degree. Variable inhibition with MH may be explained on the basis of varying permeability of tissue to MH, different tissue susceptibility to MH due to variation in enzyme content, and different susceptibility due to variation in

auxin content (16, 29). However, since each experiment deviates with the same general respiratory trend, the variable inhibition with MH was not considered further.

Effect of Krebs Cycle Intermediates on MH Inhibition

Citrate: In order to determine the effect of MH on roots in the presence of citrate, a concentration of citrate was determined which stimulated root respiration. This was determined by two experiments. The first compared a control against concentrations of 0.05, 0.10, and 0.15 M potassium citrate. These were set up in the following manner.

Molarity of potassium citrate	Molarity of phosphate buffer	Total molarity of solution
0.00	0.15	0.15
0.05	0.10	0.15
0.10	0.10	0.20
0.15	0.05	0.20

This experiment (Table IV, Experiment 8) indicates that low concentrations are not very inhibitory to respiration.

The second of these concentration experiments used 0.01, 0.005, 0.001, and 0.0005 M potassium citrate. These were set up in the following manner.

Molarity of potassium citrate	Molarity of phosphate buffer	Total molarity of solution
0.00	0.10	0.10
0.0005	0.10	0.1005
0.001	0.10	0.101
0.005	0.10	0.105
0.01	0.10	0.11

Results of this experiment (Table IV, Experiment 9) show that potassium citrate solutions less than 0.005 M stimulate mung

Table IV. Effect of various concentrations of potassium citrate on respiration of mung bean root tips. Experiments 8, 9.

Molarity citrate	Total molarity	µl. O_2/10 roots/4 hours Sample 1	2	3	Mean	% of control
			Experiment 8			
0.00	0.15	160	241	211	204	100%
0.05	0.15	182	172	122	159	78
0.10	0.20	80	78	78	79	39
0.15	0.20	82	60	47	63	32
			Experiment 9			
0.00	0.10	226	256	253	245	100
0.0005	0.1005	270	243	294	269	110
0.001	0.101	289	286	310	295	120
0.005	0.105	265	297	299	287	117
0.01	0.11	245	253	234	244	100

Table V. Effect of nearly pure stock MH (S MH) and purified MH (P MH) and citrate on respiration of mung bean root tips. Experiment 10.

Vessel contents	Total molarity	µl. O_2/10 roots/4 hours Sample 1	2	3	Mean	% inhibition due to MH
Buffer (Control)	0.20	83	120	112	105	
0.05 M S MH	0.20	58	85	77	73	31
0.05 M P MH	0.20	58	76	55	63	40
0.005 M citrate	0.205	95	97	110	100	
" " 0.05 M S MH	0.205	71	67	--	69	31
" " 0.05 M P MH	0.205	73	29	69	59	41

bean root respiration.

The effect of MH on respiration in the presence of citrate was determined using 0.005 M citrate. Solutions of higher total molarity resulted upon the introduction of MH and its accompanying buffer. Molarities of the various solutions used are shown in Table V. The results from this experiment (Table V) indicate MH-inhibited respiration is not restored within 4 hours. Therefore there is no action of MH in the Krebs cycle on the enzyme immediately preceding citrate. These results, in conjunction with the results of isocitrate (Table XI) and cis-aconitate (Table XII) experiments, indicate that this MH action is on the enzyme aconitase, which maintains the citrate, cis-aconitate, and isocitrate equilibrium.

Malate: Previous experiments with malic acid indicated that 0.005 M malate was stimulatory to respiration. Therefore experiments to measure malate effect on oxygen uptake were set up using this concentration. Results from these experiments (Table VI) indicate that malate partially restores MH inhibited respiration. The respiratory inhibition due to MH has been calculated as the per cent decrease in respiration between a test solution without MH and a similar test solution with MH. Inhibition as thus calculated shows that malate partially restores MH reduced respiration. These experiments show that one action of MH in respiration is on an enzyme which preceded the formation of malate in the Krebs cycle. Results from the fumarate experiments (Table VII) show this enzyme to be fumarase, which yields malate as a reaction

Table VI. Effect of MH and malate on respiration of mung bean root tips. Experiments 11, 15, 23.

Vessel contents	Total molarity	µl. O_2/10roots/4hrs. Sample 1	2	3	Mean	% inhibition due to MH	% restoration
Experiment 11							
Buffer (Control)	0.20	141	152	150	148		
0.05 M MH	0.20	93	144	102	113	24	
0.005 M malate	0.205	257	240	--	249		
" , 0.05 M MH	0.205	207	182	180	189	24	0
Experiment 15							
Buffer (Control)	0.20	154	147	--	151		
0.075 M MH	0.2125	82	96	74	84	44	
0.005 M malate	0.205	231	220	206	219		
" , 0.075 M MH	0.2175	168	156	156	160	27	17
Experiment 23							
Buffer (Control)	0.20	197	206	--	201		
0.075 M MH	0.20	98	147	144	130	35	
0.005 M malate	0.205	277	248	267	264		
" , 0.075 M MH	0.205	199	182	209	196	26	9

product. It may also be inferred that MH action is not on malic dehydrogenase.

Fumarate: The effect of fumarate (0.005 and 0.05 M) on restoration of MH-inhibited respiration was studied with 0.05 and 0.075 M MH. Results (Table VII) show that fumarate does not restore MH inhibition. In Experiment 12, 0.05 M fumarate in the presence of 0.05 M MH after 4 hours had apparently restored respiration 7 per cent, but if one abnormally high sample is discarded, the inhibition caused by MH is 33 per cent, or no apparent restoration. In this same experiment 0.05 M fumarate in 5 hours did not restore respiration. It can be concluded from this and the malate experiments that one action of MH is to inhibit the enzyme fumarase.

Succinate: Concentrations of 0.005 and 0.05 M succinate do not restore respiration retarded with MH (Table VIII). Succinic dehydrogenase oxidatively converts succinate to fumarate. Therefore the addition of succinate to mung bean roots stimulates respiration. However, in the presence of MH this increase in respiration did not occur and it may be assumed that MH inhibits succinic dehydrogenase.

alpha-Ketoglutarate: This Krebs cycle intermediate, at concentrations of 0.005 and 0.05 M, did not restore MH-caused inhibition within 4 hours (Table IX). Because alpha-keto-glutarate is oxidatively decarboxylated by alpha-ketoglutaric oxidase to succinate, an addition of alpha-ketoglutarate to mung bean root tips stimulates respiration. However, in the

Table VII. Effect of MH and fumarate on respiration of mung bean root tips. Experiments 12, 14.

Vessel contents	Total molarity	µl.O_2/10 roots Per 4 hours Sample 1	2	3	Mean	Per 5 hours Sample 1	2	3	Mean	% inhibition due to MH Per 4 hours	Per 5 hours
Experiment 12											
Buffer (Control)	0.20	198	209	171	193	269	260	213	247		
0.05 M MH	0.20	122	140	131	131	192	216	195	201	32	19
0.05 M fumarate	0.20	219	194	228	214	349	323	347	340		
" , 0.05 M MH	0.20	193	150	137	160(143)*	255	243	212	237	25(33)	30
0.005 M fumarate	0.205	219	263	=	241	--	--	--	--		
" , 0.05 M MH	0.205	153	150	144	149					38	
Experiment 14											
Buffer (Control)	0.20	209	188	--	199						
0.075 M MH	0.2125	150	169	141	153					23	
0.05 M fumarate	0.18	271	270	267	269						
" , 0.075 M MH	0.2175	159	203	188	183					32	

* = Mean of samples 2 and 3.

Table VIII. Effect of MH and succinate on respiration of mung bean root tips. Experiments 13, 14.

Vessel contents	Total molarity	µl. O_2/10 roots/2 hours* Sample 1	2	3	Mean	% inhibition due to MH
		Experiment 13				
Buffer (Control)	0.20	89	95	114	99	
0.05 M MH	0.20	87	81	79	82	17
0.05 M succinate	0.20	118	113	98	110	
" " 0.05 M MH	0.20	92	94	94	93	16
0.005 M succinate	0.205	124	115	--	120	
" , 0.05 M MH	0.205	99	96	110	102	15
		ul. O_2/10 roots/4 hours				
		Experiment 14				
Buffer (Control	0.20	209	188	--	199	
0.075 M MH	0.2125	150	169	141	153	23
0.005 M succinate	0.18	292	277	271	280	
" , 0.075 M MH	0.2175	194	201	198	198	29

* = Equilibration period of 3.5 hours used.

presence of MH this alpha-ketoglutarate stimulation is reduced (Table IX). Therefore it may be assumed that MH inhibits the normal function of alpha-ketoglutaric oxidase.

Oxalosuccinate: This compound was in limited supply and only one experiment was conducted using a concentration of 0.0125 M. This concentration was not able to restore MH-inhibited respiration (Table X). No oxidation is involved in the conversion of oxalosuccinic acid to alpha-ketoglutarate. Therefore the manometric techniques employed could not measure this reaction. However, because oxalosuccinate is converted to alpha-ketoglutarate which is further oxidatively converted to succinate, an increase in respiration should be noted unless MH inhibits (a) oxalosuccinic carboxylase which converts oxalosuccinate to alpha-ketoglutarate, or (b) alpha-ketoglutaric oxidase which converts alpha-ketoglutarate to succinate. The latter effect has been noted previously, while the former action needs further investigation.

Isocitrate: Measurements of oxygen consumption indicate that 0.0125 M isocitrate will partially restore respiration reduced with 0.075 M MH (Table XI). Therefore, MH does not act upon isocitric dehydrogenase because an increase in respiration did occur. This and a previous experiment with citrate indicate that MH inhibits aconitase (Figure 1).

Cis-aconitate: This intermediate at 0.0125 M did not restore MH-reduced respiration (Table XII). Only one experiment was conducted because of the limited supply of cis-aconitate. This experiment, in conjunction with the fact

Table IX. Effect of MH and alpha-ketoglutarate (a-KG) on respiration of mung bean root tips. Experiment 16.

Vessel contents	Total molarity	µl. O_2/10 roots/4 hours Sample 1	2	3	Mean	% inhibition due to MH
Buffer (Control)	0.20	200	203	--	201	
0.075 M MH	0.2125	179	144	154	159	21
0.05 M a-KG	0.20	297	258	295	284	
0.05, 0.075 M MH	0.2375	179	145	203	175	38
0.005 M a-KG	0.205	272	275	306	284	
" , 0.075 M MH	0.2175	148	149	130	142	50

Table X. Effect of MH and oxalosuccinate (OS) on respiration of mung bean root tips. Experiment 19.

Vessel contents	Total molarity	µl. O_2/10 roots/4 hours Sample 1	2	3	Mean	inhibition due to MH
Buffer (Control)	0.20	231	247	--	239	
0.075 M MH	0.20	211	178	200	196	18
0.0125 M OS	0.20	239	250	249	246	
" , 0.075 M MH	0.20	179	178	197	185	25

Table XI. Effect of MH and isocitrate on respiration of mung bean root tips. Experiments 19, 20.

Vessel contents	Total molarity	µl. O_2/10 roots/4hrs Sample 1	2	3	Mean	% inhibition due to MH	% restoration
Experiment 19							
Buffer (Control)	0.20	231	247	==	239		
0.075 M MH	0.20	211	178	200	196	18	
0.0125 M isocitrate	0.20	217	245	195	219		
" , 0.075 M MH	0.20	218	183	177	193	12	6
Experiment 20							
Buffer (Control)	0.20	216	220	==	218		
0.075 M MH	0.20	134	144	143	140	36	
0.0125 M isocitrate	0.20	172	165	178	172		
" , 0.075 M MH	0.20	143	129	124	132	23	13

Table XII. Effect of MH and cis-aconitate (CA) on respiration of mung bean root tips. Experiment 20.

Vessel content	Total molarity	µl. O_2/10 roots/4 hours Sample 1	2	3	Mean	% inhibition due to MH
Buffer (Control)	0.20	216	222	==	218	
0.075 M MH	0.20	134	144	143	140	36
0.0125 M CA	0.20	183	194	210	196	
" , 0.075 M MH	0.20	87	114	128	110	44

that citrate did not restore respiration while isocitrate was able to, indicates that MH inhibits aconitase.

Oxaloacetate: One experiment with this intermediate indicates (Table XIII) that oxaloacetate probably does not restore respiration. This is not proved conclusively, however, since there was one very low value of MH inhibition (Experiment 23). If oxaloacetate does not restore respiration MH does not inhibit malic dehydrogenase. MH may inactivate the condensing or soluble enzyme involved in the condensation of acetate and oxaloacetate, but this cannot be determined since when citrate is added no restoration of respiration occurs because aconitase is inhibited.

Isocitrate and Malate: Action of MH has been located between cis-aconitate and isocitrate, and fumarate and malate. Since the addition separately of malate or isocitrate partially restored respiration, this experiment was to determine the effect of isocitrate and malate in combination. This was done with 0.005 M malate, 0.0125 M isocitrate, and 0.075 M MH. Results (Table XIV) show that MH reduced respiration is partially restored but this restoration is not greater than either malate (Table VI) or isocitrate (Table XI) is able to produce alone.

Effect of MH on Glycolysis and Pyruvate Oxidation

Yeast: Since MH may also influence growth of plants by the inhibition of enzymes elsewhere than in the aerobic respiratory cycle, the effect of MH on yeast fermentation was studied. Yeast is strictly anaerobic and does not carry out

Table XIII. Effect of MH and oxaloacetate (OA) on respiration of mung bean root tips. Experiment 23.

Vessel content	Total molarity	µl. O_2/10 roots/4 hours Sample 1	2	3	Mean	% inhibition due to MH
Buffer (Control)	0.20	197	206	--	201	
0.075 M MH	0.20	98	147	144	130(146)*	35(27)
0.0125 M OA	0.20	197	193	156	183	
" , 0.075 M MH	0.20	132	156	124	137	25

* = Mean of sample 2 and 3.

Table XIV. Effect of MH, malate, and isocitrate on respiration of mung bean root tips. Experiment 22.

Vessel content	Total molarity	µl. O_2/10 roots/4 hrs. Sample 1	2	3	Mean	% inhibition due to MH	% restoration.
Buffer (Control)	0.20	204	185	--	195		
0.075 M MH	0.20	130	116	125	124	36	
0.005 M malate	0.205	242	228	213	228		
0.0125 M isocitrate	0.20	170	151	161			
0.005 M malate, and 0.0125 M isocitrate	0.205	203	187	200	197		
" , 0.075 M MH	0.205	140	144	162	149	24	12

any Krebs cycle reactions. Yeast was grown in the presence of sucrose as a substrate, and carbon dioxide evolution was measured for: (a) yeast in a phosphate buffer-sucrose control, (b) yeast in the presence of buffer, sucrose, and 0.05 M MH, and (c) yeast in buffer and sucrose to which 0.05 M MH was added after 45 minutes of measured respiration. The latter treatment was accomplished by tipping MH from the sidearm of a Warburg vessel into the main chamber after 45 minutes. Yeast fermentation was rapid so only a 20 minute equilibration period was used. All solutions contained 0.10 M buffer at a pH of 5.0, and 0.30 M sucrose. MH used was 0.05 M. Solutions had a total molarity of 0.40 without MH present, and 0.45 with MH present. The amount of yeast added to each solution was 0.73 mg. of dry, granulated, Fleischmann's "active dry" yeast. Carbon dioxide evolution is expressed as ul. CO_2/0.73 mg. yeast/45 minutes.

As is shown in Table XV, MH did not exert an inhibitory effect upon yeast respiration. Since yeast does not have an aerobic cycle, it is likely that inhibition of respiration by MH in mung bean roots is also limited to the aerobic respiratory cycle.

Pyruvate and Acetate: Experiments to further locate MH inhibition as to aerobic respiration were initiated using pyruvate and acetate as substrates. If MH inhibits the Krebs cycle the addition of pyruvate to roots treated with MH should partly restore oxygen uptake because pyruvate will form

Table XV. Effect of MH on respiration (anaerobic) of yeast. Experiment 30.

$\mu l. CO_2$/ 0173 mg. yeast/time	Buffer (Control) Sample 1	2	Mean	0.05 M MH Sample 1	2	Mean	0.05 M MH tipped in at 45 minutes Sample 1	2	Mean
1st 45 minutes	38	40	39.	44	42	43	42	43	42
2nd 45 minutes	33	34	33	32	30	31	33	31	32
3rd 45 minutes	24	29	26	19	26	22	27	25	26

Table XVI. Effect of MH and pyruvate on respiration of mung bean root tips. Experiments 17, 18.

Vessel contents	Total molarity	$\mu l. O_2$/10roots/4hrs. Sample 1	2	3	Mean	% inhibition due to MH	% restoration
		Experiment 17					
Buffer (Control)	0.20	192	219	--	206		
0.075 M MH	0.20	190	129	154	158	23	
0.025 M pyruvate	0.20	265	270	289	275		
" , 0.075 M MH	0.20	219	272	256	249	9	14
0.005 M pyruvate	0.20	233	312	253	266		
" , 0.075 M MH	0.20	218	177	283	226	15	8
		Experiment 18					
Buffer (Control)	0.20	226	232	--	229		
0.075 M MH	0.20	203	178	193	191	17	
0.0125 M pyruvate	0.20	271	247	235	251		
" , 0.075 M MH	0.20	228	219	211	219	13	4

acetate with the loss of one molecule of carbon dioxide and the uptake of one atom of oxygen. Further, addition of acetate to an MH-inhibited system should not restore respiration (Figure 1).

As shown in Table XVI, pyruvate partially restored MH inhibited respiration, which indicates that MH does not inhibit the oxidation of pyruvate. When acetate was added to MH inhibited respiration no increase in oxygen uptake occurred (Table XVII). These results, in conjunction with the results of the effects of oxaloacetate, citrate, cis-aconitate, and isocitrate on MH-inhibited respiration, and the effect of MH on yeast fermentation, indicate that MH partly inhibits aerobic respiration by inhibition of aconitase.

Competitive Inhibition of Fumarase by MH

The previous experiments indicate that MH inhibits the aerobic Krebs cycle by specifically inhibiting the enzymes fumarase and aconitase. Inhibitors of enzymatic reactions are traditionally classified as competitive or noncompetitive. In competitive inhibition the inhibitor is regarded as competing with the substrate for specific sites on the enzyme so that the decrease in activity of the enzyme depends on the relative concentration of both the substrate and inhibitor. In noncompetitive inhibition the inhibitor inactivates the enzyme by combining directly with enzymatic groups. Hence inactivation depends only on concentration of inhibitor. Because of the structural similarities of MH, fumarate, and cis-aconitate, it is probable that MH competitively inhibits

Table XVII. Effect of MH and acetate on respiration of mung bean root tips. Experiment 18.

Vessel content	Total molarity	µl. O_2/10roots/4hrs. Sample 1	2	3	Mean	% inhibition due to MH	% restoration
Buffer (Control)	0.20	226	232	--	229		
0.075 M MH	0.20	203	178	193	191	17	
0.0125 M acetate	0.20	288	317	310	305		
" , 0.075 M MH	0.20	164	150	165	159	48	≈35 (none)

fumarase and aconitase.

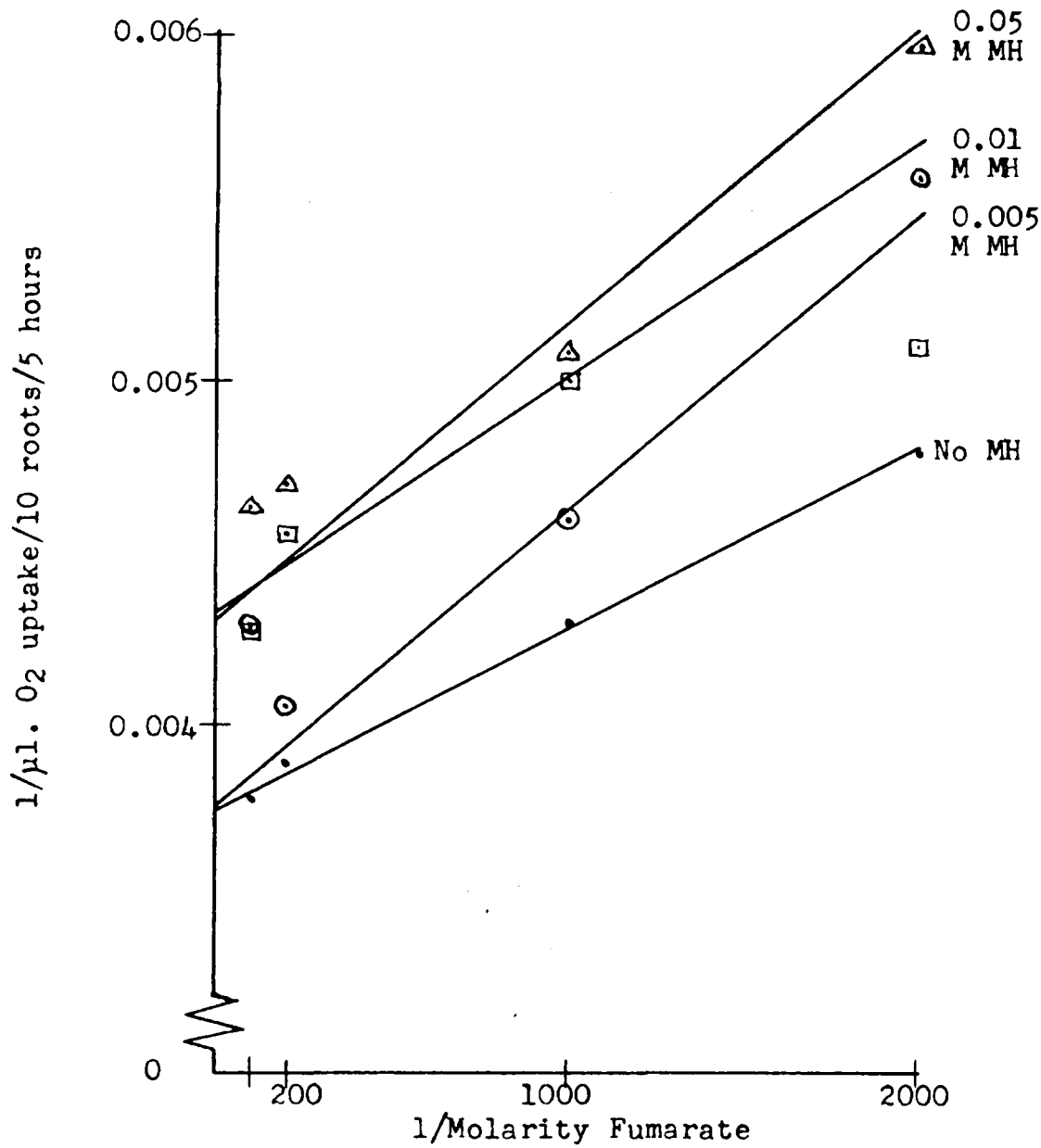
To test the hypothesis that MH is a competitive inhibitor, Lineweaver and Burk's (18, 30) kinetic treatment of inhibition was applied to the inhibition of fumarase by MH (Table XVIII). The inhibition of respiration at 0.00, 0.005, 0.01, and 0.05 M MH was studied in the presence of 0.00, 0.0005, 0.001, 0.005, and 0.01 M fumarate.

It is apparent that MH inhibits fumarate-induced respiration and that inhibition increases with higher concentrations of MH. Further, at most concentrations of MH the inhibition decreases with increasing concentration of fumarate. However, MH is not shown conclusively to be a competitive inhibitor of fumarate (Table XVIII) as shown by the double reciprocal plot (1/v against 1/(S)) of these data (Figure 2). The requirements, as described by Lineweaver and Burk (18) for competitive inhibition, are not fully met because the resultant curves are not straight, do not possess increasing slopes, and do not have a common ordinate intercept.

It should be noted that more replications of the MH-fumarate experiment might yet prove MH to be a competitive inhibitor of the enzyme fumarase. That is, if four positions on the graph (Figure 2) are ignored because they do not fit the general trends, the curves then become almost straight and nearly pass through a single origin.

Table XVIII. Effect of different MH concentrations on various concentrations of fumarate on respiration of mung bean root tips. Experiments 24 through 27.

Molarity of MH	Samples (Exp. No.)	Molarity of fumarate				
		0.00	0.0005	0.001	0.005	0.01
		µl. O_2 uptake/10 roots/5 hours				
0.00	24	185	228	221	262	265
	25	213	202	243	306	288
	26	155	180	209	218	251
	27	178	222	252	253	249
	Mean	183	208	231	260	263
0.005	24		197	223	194	188
	25		155	261	301	252
	26		164	174	282	243
	27		200	212	211	243
	Mean		179	218	249	232
0.01	24		213	161	182	233
	25		213	208	267	268
	26		185	217	231	196
	27		174	214	202	242
	Mean		196	200	220	234
0.05	24		183	202	159	208
	25		176	204	259	275
	26		147	200	219	187
	27		167	181	210	189
	Mean		168	197	212	215



Inhibition of fumarate stimulated mung bean root tip respiration by MH. Means from Table XVIII plotted according to the Lineweaver-Burk treatment as a test of competitive inhibition.

Figure 2

DISCUSSION AND CONCLUSIONS

Because MH was thought to inhibit aerobic or Krebs cycle respiration, intermediates in this cycle were added to MH inhibited roots. If MH inhibition occurs in the Krebs cycle, the addition of intermediates directly following the site of inhibition should at least partially restore respiration. It was found that low concentrations of malate and isocitrate were able to partially restore MH-inhibited respiration, whereas the addition of other intermediates of the Krebs cycle did not restore respiration. MH may therefore act upon the enzymes fumarase and aconitase.

Data from yeast, pyruvate, and acetate experiments indicate that MH action is limited to respiration in the Krebs aerobic cycle. Data from MH-fumarate inhibition experiments indicate that MH inhibits respiration by competing with the substrate for the enzyme. While this inhibition is probably competitive, data obtained are not conclusive. More study is needed concerning the effect of MH on the enzyme fumarase and it should also be determined whether MH competitively inhibits aconitase.

If MH is a competitive inhibitor of fumarase and aconitase, it is likely that it is so because of its structural similarity to the substrates of these enzymes, fumarate and cis-aconitate. MH is a derivative of maleic acid, which is

the cis- form of fumaric acid. The structure of cis-aconitate is also similar to MH.

MH inhibition of fumarase and aconitase may also be due to a concomitant noncompetitive inhibition of thiol (sulfhydryl or -SH) groups of these enzymes. It has been shown that iodoacetate, a known thiol acceptor, will cause partial inhibition of fumarase (15). It has similarly been shown that copper and mercury ions, also thiol competitors, will inhibit aconitase in relatively low concentrations (13). The presence of thiol groups in these enzymes is therefore indicated. Although MH has not been shown to be a thiol competitor, it is known that maleic acid will compete for thiol groups of glyoxalase and succinic dehydrogenase (21). It is a possibility, therefore, that MH may also noncompetitively act upon fumarase and aconitase for thiol groups which they contain. However, since the MH-fumarate competitive inhibition study indicates that MH is a structural competitor, the possibility of superimposed noncompetitive inhibition is not too likely.

MH may also noncompetitively act on oxalosuccinic carboxylase, alpha-ketoglutaric oxidase, and succinic dehydrogenase (Tables VII, VIII, IX, and XI). It is seen from the Krebs cycle (Figure 1) that if MH inhibits respiration by acting on isocitric acid dehydrogenase, then isocitrate added to the system should not restore respiration. If isocitric acid dehydrogenase was inactivated, the oxidation of isocitrate to oxalosuccinate could not be measured. Because this isocitrate oxidation has been shown to occur (Table XI), MH must not act

on isocitric acid dehydrogenase. The action of MH on oxalosuccinic carboxylase was not determined since oxidation does not occur between oxalosuccinate and alpha-ketoglutarate. The possibility therefore exists that MH may inactivate oxalosuccinic carboxylase. Since MH may act on this enzyme, addition of alpha-ketoglutarate should restore respiration. However, restoration was not demonstrated (Table VIII) and MH must therefore block the oxidation of alpha-ketoglutarate by inactivating alpha-ketoglutaric oxidase. Succinate is oxidized to fumarate in the Krebs cycle. Therefore the addition of succinate should measureably restore respiration to an MH inhibited system, since MH acts on alpha-ketoglutaric oxidase and possibly on oxalosuccinic carboxylase. However, this does not occur (Table VII) and it can be assumed that MH acts on succinic dehydrogenase. It is known that succinic dehydrogenase contains thiol groups and is partially inactivated by thiol acceptors (27), including maleic acid (21). Again one should note the structural similarities between maleic acid and MH. Inhibitors of alpha-ketoglutaric oxidase and oxalosuccinic carboxylase are not known, nor has the presence or absence of thiol groups in the structure of these enzymes been indicated.

The mechanism of inhibition of aerobic respiration by MH therefore is two-fold since MH appears to be a competitive inhibitor of fumarase and aconitase and a noncompetitive inhibitor of alpha-ketoglutaric oxidase and succinic dehydrogenase. The effect of MH on the condensing or soluble enzyme

and oxalosuccinic carboxylase can not be determined from the experiments reported here. It should be noted that this mechanism of inhibition by MH accounts for the usual gross effects of this herbicide, such as accumulation of fructose, sucrose, and starch (3, 7, 10, 20, 23, 24, 33).

SUMMARY

A culture technique for growing straight and uniform mung bean roots that were easily cut and rapidly measured was developed. Root tips obtained by this technique were subjected to respiration measurements. Because the respiration of these mung bean root tips is dependent upon pH of the medium, pH 7.0 was used. Concentrations of 0.05 and 0.075 M MH were used to inhibit respiration.

Data from the experiments show the locations of respiratory responses of mung bean root tips to MH. These locations appear limited largely to the Krebs cycle, and within this cycle to specific areas. The mechanism of inhibition may be two-fold since MH appears to be a competitive inhibitor of fumarase and aconitase and a noncompetitive inhibitor of alpha-ketoglutaric oxidase and succinic dehydrogenase. The effect of MH on the condensing or soluble enzyme and oxalosuccinic carboxylase can not be determined from the experiments reported here.

LITERATURE CITED

1. Addicott, F. T., Lynch, R. S., Livingston, G. A., and Hunter, J. K. A method for the study of foliar abscission *in vitro*. Plant Physiol. 24: 537-539. 1949.

2. Bonner, J. Relations of respiration and growth in the Avena coleoptile. Amer. Jour. Bot. 36: 429-436. 1949.

3. Currier, H. B., Day, B. E., and Crafts, A. S. Some effects of maleic hydrazide on plants. Bot. Gaz. 112: 272-280. 1951.

4. Darlington, C. D., and McLeish, J. Action of maleic hydrazide on the cell. Nature 167: 407-408. 1951.

5. Flett, L. H., and Gardner, W. H. Maleic Anhydride Derivatives. p. 130. John Wiley and Sons, Inc., New York. 1952.

6. Goddard, D. R., and Meeuse, B. J. D. Respiration in higher plants. Ann. Rev. of Plant Physiol. 1: 207-232. 1950.

7. Greulach, V. A. Growth inhibition and injury of plants by maleic hydrazide. Texas Jour. of Sci. 2: 219-221. 1950.

8. Greulach, V. A. The effect of maleic hydrazide on tomato plants in relation to their age at the time of treatment. Plant Physiol. 26: 848-852. 1951.

9. Greulach, V. A., and Atchison, E. Inhibition of growth and cell division in onion roots by maleic hydrazide. Bull. Torr. Bot. Club 77: 262-267. 1950.

10. Greulach, V. A., and Haesloop, J. G. Some effects of maleic hydrazide on internode elongation, cell enlargement, and stem anatomy. Amer. Jour. Bot. 41: 44-50. 1954.

11. Isenberg, F. M. R., Odland, M. L., Popp, H. W., and Jensen, C. O. The effect of maleic hydrazide on certain dehydrogenases in tissues of onion plants. Science 113: 58-60. 1951.

12. Krebs, H. A. Intermediary stages in the biological oxidation of carbohydrates. Advances in Enzymology 3: 191-252. 1943.

13. Krebs, H. A., and Eggleston, L. V. The effect of citrate on the molybdate complexes of malate, citramalate and isocitrate. Biochem. Jour. 37: 334-338. 1943.

14. Krebs, H. A., and Johnson, W. A. Citric acid in intermediate metabolism in animal tissues. Enzymologia 4: 148-156. 1937.

15. Laki, K. Inactivation and reactivation of fumarase. Enzymologia 9: 141. 1941.

16. Leopold, A. C., and Klein, W. H. Maleic hydrazide as on antiauxin in plants. Science 114: 9-10. 1951.

17. Leopold, A. C., and Klein, W. H. Maleic hydrazide as an antiauxin. Physiol. Plant. 5: 91-99. 1952.

18. Lineweaver, H., and Burk, D. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 56: 658-666. 1934.

19. Millerd, A., Bonner, J., Axelrod, B., and Bandurski, R. Oxidative and phosphorylative activity of plant mitochondria. Proc. Nat. Acad. Sci. 37: 855-862. 1951.

20. Moore, R. H. Several effects of maleic hydrazide on plants. Science 112: 52-53. 1950.

21. Morgan, E. J., and Friedman, E. Interaction of maleic acid with thiol compounds. Biochem. Jour. 32: 733-742. 1938.

22. McRae, D. H., and Bonner, J. Chemical structure and antiauxin activity. Physiol. Plant. 6: 484-510. 1953.

23. Naylor, A. W. Accumulation of sucrose in maize following treatment with maleic hydrazide. Arch. Biochem. and Biophys. 33: 340-342. 1951.

24. Naylor, A. W., and Davis, E. A. Maleic hydrazide as a plant-growth inhibitor. Bot. Gaz. 112: 112-126. 1950.

25. Naylor, A. W., and Davis, E. A. Respiration response of root tips to maleic hydrazide. Bull. Torr. Bot. Club 78: 73-79. 1951.

26. Phouphas, M. C. Action of maleic hydrazide on content of glucide substances in Jerusalem artichoke tuber tissue cultivated <u>in vitro</u>. Bull. Soc. Chem. Biol. 34: 900-903. 1952.

27. Potter, V. R., and Du Bois, K. P. Inhibition studies with succinic dehydrogenase. Jour. Gen. Physiol. 26: 391-404. 1943.

28. Schoene, D. L., and Hoffman, O. L. Maleic hydrazide, a unique growth regulant. Science 109: 588-590. 1949.

29. Ts'o, P., and Steinbauer, G. P. Effect of maleic hydrazide on auxin-induced water uptake by pea stem segments. Science 118: 193-194. 1953.

30. Wilson, P. W. Kinetics and mechanisms of enzyme reactions. In: Respiratory Enzymes, H. A. Lardy, Editor. Burgess Publishing Company. Minneapolis, Minnesota. 1949.

31. Wohl, K., and James, W. O. The energy changes associated with respiration. New Phytologist 41: 230-256. 1942.

32. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques and Tissue Metabolism. Burgess Publishing Company. Minneapolis, Minnesota. 1949.

33. Zukel, J. W. Use of maleic hydrazide as a plant growth inhibitor. Agric. Chemicals 5: 35-36. 1950a.