MALONIC ACID AS AN INHIBITOR OF MAIZE ROOT RESPIRATION

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(WITH SIX FIGURES)

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Early experiments on the effects of malonate on plant respiration led to contradictory results which were largely resolved by the work of BONNER and WILDMAN (**3**), TURNER and HANLY (**18**), and LATIES (**8**) who showed that pH conditions affected the response to the inhibitor. It is now clear that for some plant tissues at least, under appropriate conditions of pH and malonate concentration, O_2 uptake is retarded and accumulation of succinic acid occurs (**2, 7**). Other evidence (**5**) has also been obtained which supports the view that the oxidation of pyruvate in plants occurs through a cycle which can be slowed down by malonate, but there are no records of the accumulation of other compounds in the presence of the inhibitor.

The present paper describes experiments in which changes in respiratory quotient (R.Q.) and the associated accumulation of some other cell components (not regarded as precursors of succinate) were followed at various malonate concentrations, as well as experiments in which the relationship between pH and the inhibitions induced by malonate was re-examined.

Methods

Hybrid corn, Wf9 × 38–11, was soaked in tap water overnight with Semesan as a fungicide, washed, drained, and placed in the dark in layers of 1 cm. thickness in large beakers. After three days at 23 to 26° C in contact with a minimum amount of water, the roots of the germinating corn had bound the grains together in mats which were then supported on small beakers placed inside the original ones and well watered. During the next two days, the roots grew rapidly downwards away from the grain; and from these roots, tips about 15 mm. long were detached, washed in distilled water, and counted into samples. In the experiments reported here, 20 root tips with phosphate buffer (0.06 M) were used in each flask; such samples had a dry weight of about 16 mg. and the average O_2 uptake per hour at 25° C was 70 cu. mm. (Q_{O_2} value of 4.4). The duration of most of the experiments was three to five hours, although other experiments showed that the O_2 uptake continued at a steady rate for considerably longer periods than this (fig. 4). It should be mentioned that the material respired equally rapidly in distilled water as in the buffered medium; there was no marked salt-induced respiration (**18**). Changes in pH in the range of 4.0 to 8.0 have very little effect on the respiratory activity; even at pH 2.9, the respiration rate was maintained at slightly over 80% of the rate at pH 6.0.

Malonate solutions were adjusted to the pH at which the individual experiment was to be carried out before making the solutions up to volume.

For experiments in which alcohol and acetaldehyde were to be measured, samples of 150 root tips with buffer and a known amount of malonate were placed in large boiling tubes and aerated. The outgoing gas-stream was passed through 2% bisulphite (**11**), and the amounts of acetaldehyde trapped were estimated from time to time in 1-ml. samples by the method of STOTZ (**14**). The alcohol estimation procedure of WILLIAMS and REESE (**20**) was applied to aliquots of a steam distillate obtained from the roots and the surrounding solution made alkaline with KOH. It was first ascertained that more than 95% of the alcohol was recovered from a solution of known concentration in the first 15 ml. of distillate; this amount was collected in each case and 5-ml. aliquots were used for the estimation, using

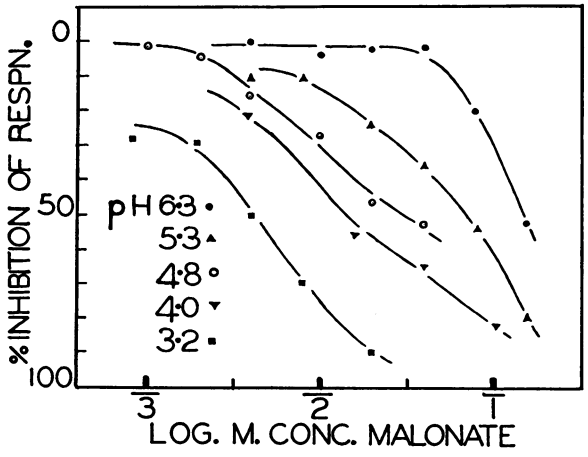

FIG. 1. Respiratory inhibitions induced by graded dilutions of malonate at five different pH levels. The points are O_2 uptake in the period of two to three hours expressed as per cent. of controls at the same pH.

half the quantities of reagents and a dichromate mixture as described by Williams and Reese. The addition of malonate or succinate in high concentrations before distillation did not alter the amount of alcohol distilling from a known sample.

Results

THE EFFECT OF pH ON MALONATE INHIBITION

In figure 1 are plotted the results of a series of experiments at pH 3.2 to 6.3 in which graded dilutions of malonate at the pH of the buffer were added to samples of roots. The per cent. inhibition was evaluated by comparing the respiration of such samples over the period of 120 to 180 minutes with that of controls at the same pH. The fact that the rates of O_2 uptake were virtually constant after the roots had been in contact with the malonate for about 90 minutes (fig. 4) justifies the adoption of this time interval.

It will be seen that a series of more or less parallel curves is obtained, and that, as the pH is raised, the curves are displaced to the right. No inhibition was obtainable at pH 6.3 until concentrations of 0.03 M were exceeded, whereas at pH 3.2 the curve was already in the descending region at 0.001 M. If the concentrations which induce a given inhibition, say 50%, are compared, there is an increase of some 50 times as the pH is raised from 3.2 to 6.5 (fig. 2). The results also show that for any given concentration of inhibitor, it is the pH which determines the extent of the inhibition, reductions in pH bringing about increases in the amount of inhibition. Thus when the per cent. inhibition induced by a given concentration of malonate is plotted against pH, a sigmoid curve is obtained, falling from zero inhibi-

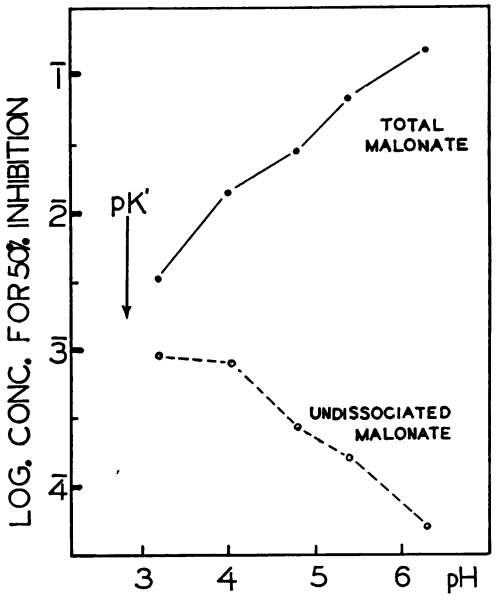

FIG. 2. The relationship between pH and the amount of malonate required to induce 50% inhibition of O_2 uptake. The continuous line shows the total applied malonate, and the points on the broken line show the calculated concentrations of undissociated acid corresponding to the applied malonate.

tion at a given pH to almost complete inhibition at a pH value a few units lower. It should be clear from figure 1 that if a series of malonate concentrations were chosen and the results plotted in this way, a series of more or less parallel curves displaced along the pH axis would result; the inhibition observed with the highest malonate concentration would be at the greater pH values. This point is made here because the placement of such a single sigmoid curve along the pH axis and the chance coincidence of its point of inflection with that of the dissociation curve for the second H of malonic acid (pH 5.3) has been interpreted by BONNER and WILDMAN (3) as indicating that the monobasic malonate ion is the major cell penetrant. Direct evidence against the monovalent ion hypothesis is given by the fact that for

any concentration of malonate, reductions in the pH below 4.5 (which would lead to a reduction of the concentration of monobasic malonate ions) induce not smaller but greater inhibitions than those at pH 4.5. The results recorded here for malonate are in good accord with those described in earlier studies (**1, 12, 13**), and there is no reason to suppose that the penetration behavior of this weak acid is markedly different from others investigated. The close similarity of the data shown in figure 2 (in which the concentrations of malonate inducing 50% inhibition are plotted against pH) to results previously described supports this view. The general interpretation of such

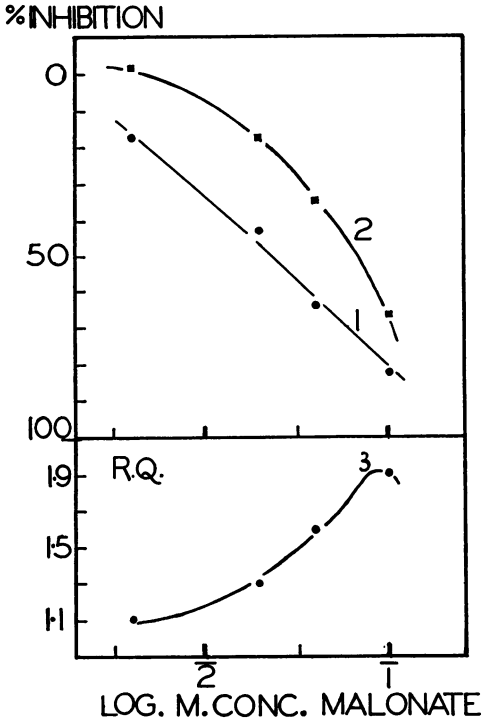

FIG. 3. The effect of malonate in different concentrations on O_2 uptake (curve 1), CO_2 output (curve 2), and R.Q. during the period of two to three hours (curve 3).

curves is that the response to a weak acid is determined largely, but not entirely, by the concentration of undissociated molecules present. The fact that the broken line in figure 2 (joining points which show the concentrations of undissociated molecules corresponding to the equi-effective concentrations of applied malonate) does not remain horizontal at pH levels higher than pK (**1, 12, 13**) bears out this statement. Subsequent experiments were carried out at pH 4.0.

EFFECT OF MALONATE ON RESPIRATORY QUOTIENT

Most workers on the effects of malonate have confined their attention to O_2 uptake although reports have been made of increases in R.Q. due to malo-

nate treatment (**3, 8**). Figure 3 shows the effects of a series of malonate concentrations on the O_2 uptake and CO_2 output of samples of roots during the period of 120 to 180 minutes after addition. At each concentration, the effect on CO_2 uptake is less than on O_2 uptake; and the R.Q. is progressively raised from the control value of 0.9. It will be seen that, although the R.Q. is highest at about 0.1 M malonate where it may be initially as high as 2.6, the malonate concentration which induces the greatest excess of CO_2 in absolute terms is somewhat lower than this, *i.e.*, where the O_2 uptake is inhibited by some 50%.

It will be apparent that if malonate inhibits aerobic respiration because of a specific interference with succinic dehydrogenase, there is no reason to suppose that anaerobic respiration would be affected since the enzyme is not

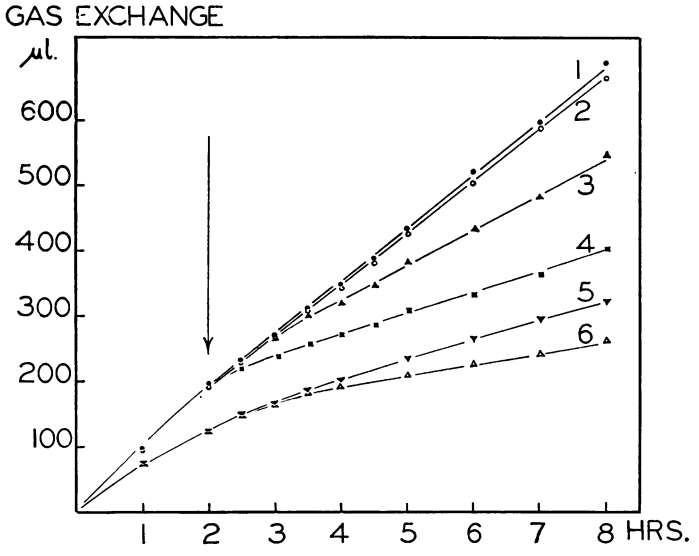

FIG. 4. The progress of aerobic O_2 uptake (curves 1 and 4) and CO_2 output (curves 2 and 3) and anaerobic CO_2 output (curves 5 and 6). Malonate (0.04 M) was added to root samples for curves 3, 4, and 6 after two hours (indicated by arrow).

involved in the latter process. However, malonate in high concentrations (but no higher than have frequently been used to show aerobic inhibition) can bring about extensive inhibition of respiration in nitrogen. The results of an experiment in which 0.04 M malonate was added after two hours to root samples respiring in air and in nitrogen are shown in figure 4. It will be seen that, in air, the control rates of CO_2 output and O_2 uptake continued unchanged with an R.Q. of 0.97, while the rate of O_2 uptake with malonate was reduced by 60% and that of the CO_2 output with malonate was reduced by some 27% (R.Q. 1.8). However, the rate of anaerobic CO_2 production was also reduced considerably by this same concentration of malonate. The rate of CO_2 output in N_2 by the control was about half of the rate of CO_2 production in air, but this rate was reduced by 40% in

the presence of malonate. Figure 5 shows the results of an experiment in which different concentrations of malonate were added to systems in nitrogen and the percentage inhibitions evaluated over controls during the period of two to three hours after addition (curve 1). Curve 3 shows the effects of the same malonate concentrations on aerobic respiration (O_2 uptake) over the same time period. It is clear that those concentrations of malonate which bring about large inhibitions of aerobic respiration are also effective in reducing the rate of respiration in N_2. Curve 2 shows that there was no marked difference in the extent of the effect on aerobic respiration whether or not aerobic respiration was proceeding during the development of the inhibition.

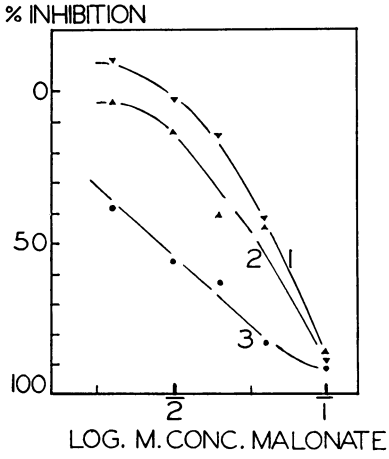

FIG. 5. The effect of different concentrations of malonate on CO_2 output in N_2 with malonate added in N_2 (curve 1), CO_2 output in N_2 with malonate added previously in air (curve 2), and O_2 uptake in air (curve 3).

THE INDUCTION OF AEROBIC PRODUCTION OF ACETALDEHYDE AND ALCOHOL

The fact that R.Q. values were maintained at values considerably greater than unity when roots were treated with concentrations of malonate which reduced O_2 uptake suggested the possibility that the inhibitor was inducing aerobic fermentation. When it was further observed that strong iodoform reactions were obtained after large samples of roots (several hundreds) had been aerated in 0.04 M malonate, the quantitative nature of the production of acetaldehyde and alcohol was investigated. Figure 6 shows the results of experiments in which acetaldehyde trapped from the outgoing air stream and alcohol from a distillate of roots and surrounding liquid were estimated after samples of 150 roots had been aerated in contact with various concentrations of the inhibitor for three and one half hours at pH 4.0. Only trace amounts of acetaldehyde and alcohol were produced in control systems without malonate, and the values refer to the excess of acetaldehyde and

alcohol over such controls, which were used as blanks in the final color analysis. It is clear that as the concentration of malonate is increased beyond 0.003 M there is a striking and regular increase in the amount of acetaldehyde recovered. The corresponding curve for alcohol shows again a sharp rise beginning at a concentration of 0.003 M, but at 0.02 M a peak is reached and thereafter the curve declines. Thus concentrations of malonate which inhibit O_2 uptake and induce high R.Q. values also bring about the aerobic production of acetaldehyde and alcohol in substantial amounts. It will be seen that the total carbon loss due to acetaldehyde and alcohol also reaches a peak at 0.02 M and declines at the highest malonate levels; this fact is in accord with the observation that the greatest excess of CO_2 in manometric experiments was found at this concentration of malonate (fig. 3). When 0.1 M malonate was applied to roots in nitrogen, the alcohol and acetaldehyde accumulations over a period of four hours were reduced by

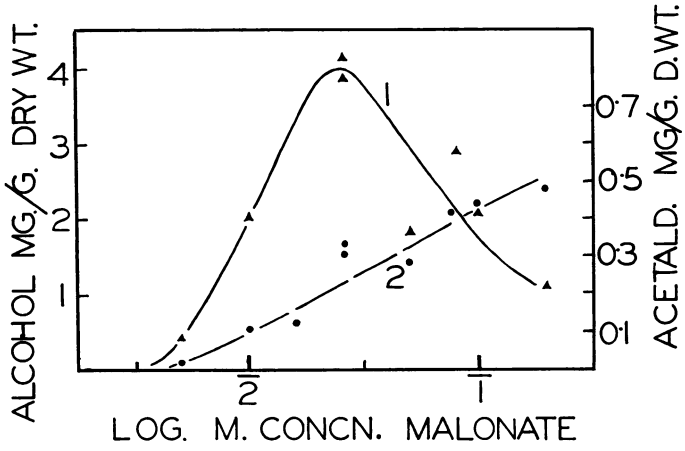

FIG. 6. The accumulation of alcohol (curve 1) and acetaldehyde (curve 2) in a three and one half hour period at different concentrations of malonate.

33 and 37% respectively and the alcohol/acetaldehyde ratio (38) remained near the control level of 43. Malonate, therefore, has no direct effect on the equilibrium between alcohol and acetaldehyde. In air, however, the amounts of acetaldehyde produced are such that the ratio of alcohol/acetaldehyde is lower than 40:1 and, as figure 6 shows, this ratio is drastically reduced as the malonate concentration is increased. Indeed, the rate of acetaldehyde production after the first few hours in contact with the inhibitor may far exceed that of the production of alcohol. Table I shows the results of an experiment in which samples were taken for alcohol analysis after three hours and again after eight hours in contact with the inhibitor. It will be seen that in the case of the lower malonate concentration both alcohol and acetaldehyde increased with time; the alcohol continues to exceed the acetaldehyde but by a ratio much lower than 40:1. When 0.09 M malonate was present, however, there was a large amount of alcohol

TABLE I

THE AEROBIC ACCUMULATION OF ALCOHOL AND ACETALDEHYDE IN THE PRESENCE OF MALONATE (DRY WEIGHT BASIS).

	Accumulation after three hours		Accumulation between three and eight hours	
	Alcohol	Acetaldehyde	Alcohol	Acetaldehyde
	mg./gm.	*mg./gm.*	*mg./gm.*	*mg./gm.*
0.03M malonate	1.23	0.11	1.77	0.16
0.09M malonate	3.2	0.41	0.08	0.63

produced in the first three hours, but only a relatively small amount during the second period, during which the evolution of acetaldehyde was maintained; the ratio during this period was very low (0.13).

Discussion

Malonate, as a competitive inhibitor of succinic dehydrogenase is frequently regarded as one of the most specific inhibitors; and in work with animal tissues, in which the normal content of succinic and other acids is low and strong inhibition (greater than 70%) can be achieved in concentrations as low as 0.001 M, this view is justified (**6**). However in work with plant tissues, in which greater amounts of these acids occur, it is noteworthy that considerably larger amounts of malonate must be applied even at suitable pH levels to induce similarly large responses; and some authors have questioned the specificity of the inhibitor at these high levels (**4, 19**). The results presented here also place doubt on its absolute specificity, in so far as some inhibition of the anaerobic respiration of maize tissue results when malonate is applied in concentrations which have often been used to demonstrate the inhibition of aerobic respiration. The maximum inhibition of aerobic respiration obtainable without, at the same time, inducing some inhibition of anaerobic respiration was 30 to 40%. As the malonate concentration was increased, aerobic respiration continued to decline but anaerobic respiration also was increasingly affected; and it therefore appears that only those inhibitions of aerobic respiration induced by concentrations which do not reduce anaerobic respiration can be wholly ascribable to a specific effect of malonate on the succinic dehydrogenase. In maize roots in air, as in most other plant tissues, it may be presumed that the rate of removal of pyruvate along the aerobic path is sufficiently rapid to prevent its diversion to alcohol *via* acetaldehyde, since these materials cannot ordinarily be detected in any but trace amounts. The fact that alcohol and acetaldehyde do appear in substantial amounts in the presence of malonate might be interpreted as indicating that the breakdown of pyruvate by the cycle is sufficiently reduced to induce its accumulation and consequent diversion towards alcohol. However, when O_2 uptake is strongly inhibited, the CO_2 output, although not so greatly reduced, still falls short of the rate expected if glycolysis was unaffected and all of the unused pyruvate were

being so diverted, and it is in fact clear (fig. 5) that under these conditions one or more of the anaerobic events are also inhibited. As the concentration of malonate is increased from zero, the accumulation of acetaldehyde and alcohol increases but at higher concentrations, as anaerobic events become progressively slower, the curve declines (fig. 6).

In this connection, it is noteworthy that in experiments on the accumulation of succinate in the presence of the inhibitor (**7**), the amounts of accumulant were not related to the amount of added malonate in a way which might have been expected if the sole effect was on the succinic dehydrogenase; at the highest malonate levels, there was only a small increase over the control level of succinate. It is clear then that there are additional points in the respiration sequence which are malonate sensitive; and although these can only be surmised at present, it seems likely that at least one point is a glycolytic step common both to anaerobic and aerobic respiration.

While malonate inhibits anaerobic respiration, it does not change the relative amounts of acetaldehyde and alcohol accumulated. The much lower ratios of alcohol/acetaldehyde, which may approach zero, when the tissue is inhibited in air are therefore not due to a direct influence on the alcohol dehydrogenase. It is clear that the reduction of acetaldehyde to alcohol would require a continuous supply of diphosphopyridine nucleotide, and it seems likely that the reaction is slowed down because the acetaldehyde-alcohol system cannot compete successfully for the reduced coenzyme with the aerobic oxidation mechanism. A similar explanation would account for the low alcohol/acetaldehyde ratios which have been observed in unpublished experiments by the author when the level of pyruvate was artificially increased in air by adding it to the medium or when dinitrophenol was added in suitable amounts.

For root tissues of maize, malonate may be added to the list of respiratory inhibitors including terminal oxidase inhibitors such as HCN, azide, CO, and H_2S (**10, 17**) and also high concentrations of CO_2 (**15**) which induce aerobic fermentation or zymasis (**15, 16**). It is not known to what extent such production of acetaldehyde and alcohol has been a feature when malonate has been applied to other plant tissues in air, but some recorded high R.Q. values suggest that the phenomenon may be of general occurrence. When the possibility of inhibition of reactions other than that controlled by the succinic dehydrogenase is also considered, it will be apparent that there are good reasons for supposing that strong inhibitions by malonate will not readily be reversed by supplying succinate. It has not been found possible to induce such reversal in maize tissue (*i.e.*, a recovery when succinate was added after the development of the inhibition) which is in accord with the experiences of some authors (**9, 18**), although some protective action of succinate against malonate added subsequently has been demonstrated.

Summary

The relationship between the pH of the medium and the efficiency of malonate as a respiratory inhibitor has been re-examined. The results show that in this respect the behavior of malonate is similar to that of other inhibitors which are weak acids; there is no evidence that it penetrates predominantly as the monobasic ion.

As the concentration of malonate was raised, both CO_2 output and O_2 uptake were increasingly inhibited, but the former was relatively less strongly affected so that the R.Q. rose steadily from the control value. As the malonate concentration was raised to levels beyond 0.01 M, anaerobic as well as aerobic respiration was increasingly inhibited.

Malonate in high concentrations in nitrogen did not alter the alcohol/acetaldehyde ratio, although the rate of anaerobic respiration of the roots was diminished. Roots whose aerobic respiration was inhibited by malonate produced acetaldehyde and alcohol in amounts and in a proportion determined by the concentration of the inhibitor. At high levels of malonate, the rate of alcohol production was greatly reduced while acetaldehyde production continued.

The results are interpreted as indicating that in maize root tissue there are at least two distinct effects of malonate; those concentrations which strongly reduce O_2 uptake in air also slow down certain of the anaerobic events. The origin of the acetaldehyde and alcohol is considered to be some of the pyruvate which is not used when the cyclic break-down system is inhibited by malonate. Some implications are discussed.

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