THE PRODUCTION OF CARBON DIOXIDE BY ISOLATED FRUIT TISSUES

by

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INTRODUCTION

The importance of respiration in fruit metabolism is reflected in the extent to which it has been studied and the extensive knowledge that has been accumulated. Of the respiratory mechanism, however, relatively little is known. For example, the phenomenon of the climacteric rise in the respiration of such fruits as apples, pears, and bananas has not been explained. The reactions involved in ethylene production by ripening fruit and the nature of the stimulatory effect of ethylene on respiration and ripening of fruit have not been elucidated. Scald as it occurs on apples and pears in storage appears to be associated with volatiles, often referred to as scald gases, but he exact nature of these volatiles and their source is still obscure. These examples reflect the importance of an understanding of fruit respiratory mechanisms in dealing with practical storage problems. This investigation concerns the phase of respiration in apple tissue involving liberation of carbon dioxide.

In respiratory systems of most of the plant and animal materials so far studied, carbon dioxide liberation is a consequence of decarboxylation of organic acids which function as intermediaries in an oxidative cycle referred to as the organic acid cycle or the Krebs cycle. At the

present time however, there is little information which indicates the nature of the mechanism functioning in carbon dioxide production by apples. This is an exploratory study employing techniques which yield indirect evidence of the nature of the respiratory processes. The information thus obtained may be used to orient a more direct and fundamental approach to the problem.

REVIEW OF LITERATURE

Considerable evidence supports the contention that plants may have respiratory mechanisms which are similar, at least in part, to yeasts, some bacteria, and animal tissues, (1, pp. 156-161; 22, pp. 417-434; 7, pp. 497-518; 47, pp. 529-544; 16, pp. 207-232).

Respiration has been described by Lardy (28, p. 1) as, "the aggregate of those processes by which oxygen is introduced into a system and carbon dioxide is removed." Oxidation of organic materials in living cells occurs in steps involving many enzymes and intermediate compounds between the various substrates (carbohydrate or derivatives) and molecular oxygen. Enzymes control the rate of formation and degradation of each substance resulting in a gradual release of energy. There appear to be few enzymes which bring about reaction of the substrate with molecular oxygen, but cellular oxidation-reduction may proceed through a coordinated series of enzymes and carriers which effect the transfer of hydrogen or electrons and eventual reaction with molecular oxygen.

The present understanding of carbohydrate breakdown is associated with such names as Embden, Meyerhoff, Parnas, Cori, Warburg, Szent-Gyorgyi, and Krebs from whose work has evolved the concepts of the glycolysis and tricarboxylic acid cycle.

Barron (1, p. 184) considers pyruvate to be the focal point or hub of synthesis and degradation of metabolic compounds. It is convenient to examine the process of respiration as consisting of two major phases: the degradation of carbohydrate to pyruvic acid by the Embden-Meyerhoff glycolytic scheme, (1, pp. 151-156) and the oxidation of pyruvic acid in the tricarboxylic acid cycle, (27. p. 235). These schemes were elaborated from work on animal tissues and yeasts and are probably applicable to plants. All details, however, may not be valid nor is it likely that all enzymes and intermediates have been found, or that this is necessarily the only pathway of carbohydrate breakdown.

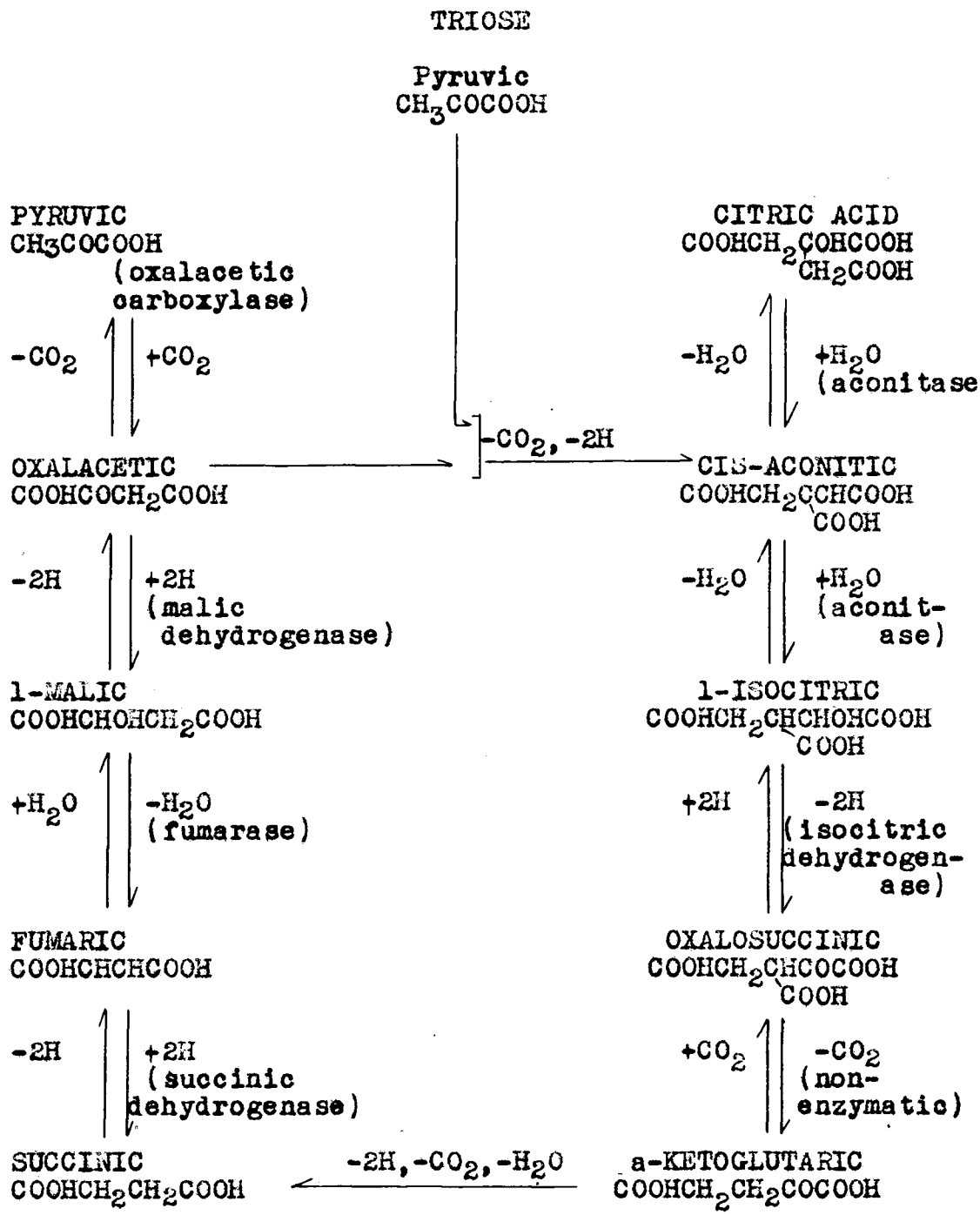
Since this investigation is primarily concerned with reactions involving liberation of carbon dioxide, the acid cycle will be discussed in some detail. In either aerobic or anaerobic conditions of plant respiration, pyruvate is the common intermediate, but oxygen tension has considerable influence on the further reactions of pyruvate. Thus in plant tissues such as fruit with limited oxygen supply, carbon dioxide and ethyl alcohol are produced as a result of decarboxylation of pyruvic acid and the reduction of the resulting acetaldehyde to ethyl alcohol.

$$\text{Pyruvic acid} \xrightleftharpoons[\text{(carboxylase)}]{} \text{acetaldehyde} + CO_2$$

$$\text{acetaldehyde} + H_2.CO\text{-}I \underset{\text{(alcohol dehydrogenase)}}{\rightleftharpoons} \text{ethyl alcohol} + CO\text{-}I$$

The enzymes carboxylase and alcohol dehydrogenase occur widely in plant materials, (23, p. 472; 4, pp. 290-297; 10, pp. 685-689; 46, pp. 445-446; 45, pp. 301-314; 32, pp. 62-72) and acetaldehyde and alcohol (37, pp. 1629-1642; 38, pp.215-219; 14, pp. 421-426; 15, pp. 41-64) are readily observable, particularily in fruits.

Under normal aerobic conditions plants can oxidize carbohydrates to carbon dioxide and water with a consequent uptake of oxygen in the same molecular ratio as the carbon dioxide evolved. The oxidation of pyruvic acid by its participation in an acid cycle similar to that described by Krebs (26, pp. 148-156; 25, pp. 223-238) is represented schematically as follows.


TRIOSE
Pyruvic
CH3COCOOH
PYRUVIC
CH3COCOOH
(oxalacetic carboxylase)
-CO2
+CO2
CITRIC ACID
COOHCH2COHCOOH
CH2COOH
-H2O
+H2O
(aconitase)
-CO2,-2H
OXALACETIC
COOHCOCH2COOH
CIS-ACONITIC
COOHCH2CCHCOOH
COOH
-2H
+2H
(malic dehydrogenase)
-H2O
+H2O
(aconitase)
1-MALIC
COOHCHOHCH2COOH
1-ISOCITRIC
COOHCH2CHCHOHCOOH
COOH
+H2O
-H2O
(fumarase)
+2H
-2H
(isocitric dehydrogenase)
FUMARIC
COOHCHCHCOOH
OXALOSUCCINIC
COOHCH2CHCOCOOH
COOH
-2H
+2H
(succinic dehydrogenase)
+CO2
-CO2
(non-enzymatic)
SUCCINIC
COOHCH2CH2COOH
-2H,-CO2,-H2O
a-KETOGLUTARIC
COOHCH2CH2COCOOH

Pyruvic acid, or a C_2 compound arising from pyruvic (49, pp. 198-246), condenses with oxalacetic to form cis-aconitic, the oxidative process proceeding around the cycle as indicated releasing carbon dioxide and eventually returning oxalacetic to the cycle. Since oxalacetic acts catalytically, only a low concentration is required. The formation of organic acids in plants by a process of carbon dioxide fixation with pyruvate has been suggested (46, pp. 445-446; 45, pp. 301-314; 9, pp. 133-143)

pyruvate + CO_2 → oxalacetic → malate → fumarate → succinate
+
pyruvate
|
citrate

Stauffer (12, p. 385) states that all acids of the tricarboxylic cycle, excepting oxalosuccinic which decomposes spontaneously, have been found in plants. These acids have been adequately reviewed by Vickery (47, pp. 529-544), Bennet-Clark (3, pp. 65-92), and Thimann and Bonner (36, pp. 75-108). Malic, citric, succinic, and lactic acids have been identified in apples, (35, pp. 1373-1374; 41, pp. 138-153). The enzyme aconitase which catalyzes the reversible hydration of cis-aconitic to citric and isocitric (5, pp. 11-19), isocitric dehydrogenase (7, pp. 497-518; 30, pp. 284-299), fumarase (7, pp. 497-518; 4, pp. 290-297) have also been shown to occur in plants. According to James (22, pp. 417-434), three terminal oxidases occur in plants: cytochrome

(indophenol) oxidase, tyrosinase (catechol, polyphenol oxidase), and ascorbic acid oxidase. These three oxidases are cyanide inhibited. Cytochrome and polyphenol oxidases are inhibited by carbon monoxide. Cytochrome oxidase inhibition by CO is reversible by light while ascorbic acid oxidase is not affected by carbon monoxide. Polyphenol and ascorbic acid oxidases were shown by Hackney (19, pp. 439-454; 20, pp. 455-465) to function in apple tissue, but Goddard (16, p. 212) questions the evidence upon which Hackney based her conclusions.

The actual mechanism of oxidation in a repiratory system utilizing the glycolytic and acid cycles consists of removing pairs of hydrogen atoms in six intermediate reactions catalysed by dehydrogenases, the ones functioning in the acid cycle being isocitric, succinic and malic. Various coenzymes and carriers are also involved in certain steps. The general aeobic system could be represented thus: substrate --dehydrogenase-- carrier -- reductase -- carrier -- oxidase --O_2 (12, p. 192).

The occurrence in plants of the postulated enzymes and intermediates of the acid cycle provides only indirect evidence of the operation of the cycle. Goddard (16, pp. 207-232) suggests methods by which the existence of the tricarboxylic acid cycle may be demonstrated:
(1) demonstration of the presence of substances and enzymes connected with the cycle,

(2) proof that pyruvic acid may be oxidized in cell preparations where normal pyruvic supply has been blocked by poisons,

(3) demonstration that interruption of the cycle at any point leads to accumulation of the substance postulated as occurring before the block.

The first consideration has been met in the case of many of the higher plants. The second point requires the blocking of pyruvate formation by an inhibitor such as sodium fluoride which, by forming a magnesiumfluorophosphate complex, removes magnesium from the enzyme enolase, thus inhibiting the conversion of 2-phosphoglycerate to pyruvate. If addition of pyruvate restores the respiration rate to a fluoride inhibited system, it can be assumed that pyruvate is a necessary intermediate. Compliance with Goddard's third point is considered strong evidence for the existence of the cycle. The ideal inhibitor affects one enzyme only, when introduced into a system. Malonate is in this category (25) due to its specific inhibition of succinic dehydrogenase. Krebs and Eggleston (26, pp. 442-459) showed that malonate inhibition resulted in the accumulation of succinate and that the addition of fumarate and, or pyruvate caused further increase of succinate. Bonner (6, pp. 311-326), working with avena coleoptile, obtained reversal with fumarate,

isocitrate, alpha-ketoglutarate and succinate. Since the reduction of fumarate to succinate is blocked by malonate an oxidative mechanism is indicated:

fumarate $\rightarrow$ oxalacetate $\rightarrow$ pyruvate $\rightarrow$ citrate $+ CO_2 \rightarrow$ alpha-ketoglutarate $+ CO_2 \rightarrow$ succinate $+ CO_2$.

Bonner (7, pp. 497-518), working with spinach leaves, was probably first to demonstrate, via the above approach, the operation of the tricarboxylic acid cycle in plants. While the occurrence of the acid cycle has been demonstrated in such plants as spinach (7) and Oats (6) no conclusive evidence has been presented for its occurrence in fruits. Turner (41, pp. 138-153) studied various metabolic and respiratory trends of Australian Granny Smith apple. During storage, total organic acids showed a tendency to decrease with fluctuations paralleling those of respiration intensity, while citric acid tended to increase after the 160'th day, again with fluctuations correlated with respiration. Turner concluded that the data were compatible with, but did not prove participation of an organic acid cycle.

MATERIALS AND METHODS

Measurement of gaseous exchange

Oxygen and carbon dioxide exchange was determined with a Warburg constant volume respirometer using the direct method as described by Umbreit (44, pp. 17-20). The temperature of the water bath was 24.8 deg. C and the shaker operated at 90 oscillations per minute. The respirometer flasks were the double sidearm type with a capacity of 18-22 ml. The control tissue samples were suspended in 3 ml. of .02M KH_2PO_4 buffer, pH 4.5. Acid reagents used in treatments were made up as stock solutions and adjusted to pH 4.0 - 4.5 with sodium hydroxide. The desired concentration of reagent was obtained by adding to the flask a calculated amount of reagent solution together with sufficient buffer to total 3 ml. In flasks used for determination of oxygen uptake, carbon dioxide was absorbed in .4 ml. of 20% KOH placed in one sidearm together with a piece of filter paper to increase the active surface. To the sidearm of flasks not containing KOH were added .4 ml. of water to maintain the volume of fluid per flask at 3.4 ml.

Preparation of tissue samples

The mature fruit tissue used in this study was taken from Yellow Newtown apples that had been stored four to five months. Sample material was obtained by cutting

cylinders of tissue frome the cortical region of a 15mm. thick median section of the apple with a 10 mm. cork borer and sectioning these cylinders into disks 1 mm. thick with a hand microtome. Except where otherwise indicated, tissue disks were rinsed briefly with distilled water and blotted with filter paper to remove excess moisture. Random samples of fifteen disks, approximately 0.8 gm., were weighed and placed in the flasks without further delay.

Respiration of whole mature fruits

In view of the consistently high respiratory quotients obtained with isolated tissue, the respiratory behaviour of mature, whole apple was examined. The fruit was enclosed in a vacuum desiccator with KOH to absorb carbon dioxide and maintained at 25 deg. C. in a constant temperature water bath for 24-36 hours. A sample of the atmosphere was then withdrawn and the oxygen content determined in an Orsat apparatus. The carbon dioxide absorbed by the KOH was determined by HCL titration using thymolphthalein indicator. The respiratory quotient calculated from these values was less than unity, possibly due to the retention of carbon dioxide by the tissue. Accordingly, the carbon dioxide content of the tissue was determined using a method described by Denny (11, pp. 273-298) in which the tissue was ground up in a Waring

blender in the presence of alkali, then transferred to a reaction cylinder where acid was introduced releasing carbon dioxide which was removed by aeration and trapped in KOH solution.

Washing treatments

Sectioning plant material affects the respiratory intensity. Sectioning carrot tissue, for example, resulted in an abnormally high respiration rate, termed wound respiration by Turner (40, pp. 273-298), which upon washing about 200 hours with water dropped to a lower constant rate. According to Bennet-Clark (3, pp. 65-92) the respiration rate of beet tissue is increased by cutting, attaining a steady value after 300 hours of washing. Boswell (8, pp. 847-862) observed that the respiration rate of potato tissue increased with washing for a period up to two days, after which it remained fairly constant. While it is desirable to use material having a steady respiration rate, it is not entirely necessary where controls are used with all treatments and the duration of the experiment is relatively short. It seemed possible, however, that washing apple tissue might restore the balance between oxygen and carbon dioxide exchange and, in addition, might reduce the concentration of certain substrates so that definite response would be obtained upon the addition of these substrates in the respirometer flasks. Accordingly, tissue

disks were washed for various periods in the following solutions and their respiratory activity, as it responded to various treatments, determined: water, 10 percent sucrose, 0.25 M. calcium chloride.

Treatment of tissue with organic acids

If acids of the Krebs cycle are active in the metabolism of apples it might be expected that intermediates such as, pyruvate, citrate, alpha-ketoglutarate, succinate, fumarate and malate could be utilized in tissue respiration with a resulting change in respiration rate and possibly the respiratory quotient. The effect on respiration of various concentrations of the above acids, glutamic acid, aspartic acid, glucose, and apple juice was determined.

The use of respiratory inhibitors

Depending on the specificity, respiratory inhibitors provide some information about the respiratory mechanism. Iodoacetate, fluoride, malonate and p-nitrophenol inhibitions were investigated. Here, as in the case of acid intermediates, optimum concentrations were determined by trial.

The use of substrates to reverse inhibition

Reestablishment of respiration by the addition of a substrate to an inhibited system is evidence that this substrate may participate in respiration. Pyruvate, citrate, alpha-ketoglutarate, succinate, and malate were used in an effort to demonstrate reversal of iodoacetate, fluoride and malonate inhibition.

Experiments with immature fruit

Young, actively metabolizing material which would be expected to possess a complete, vigorous enzyme system might respond more uniformly to treatment than older mature fruit. A series of determinations similar to those outlined for mature fruit was carried out on very small Newtown apples in the early stages of growth following fertilization. The disadvantage of using a variety of tissue types was recognized, but there was distinct advantage in being able to determine accurately the respiration characteristics of an intact fruit. Depending on the stage of growth, a suitable sample consisted of one to three fruits per flask. It was therefor possible to determine the respiratory activity of a whole fruit and the effect of subsequent sectioning.

Demonstration of dehydrogenase activity

The reduction of triphenyltetrazolium chloride to formozan in the presence of dehydrogenase and a suitable substrate has been used effectively in colorimetric estimation of dehydrogenase activity in animal tissues (28, pp. 144-146) and certain plant materials (24, pp. 65-66; 13, pp. 169-170). This test was used in an effort to demonstrate the presence of dehydrogenase enzymes in tissue slices and homogenates.

RESULTS

The effect of cutting on respiration

Data obtained from respiration studies of whole apples and tissue samples demonstrate the pronounced increase in respiration rate accompanying sectioning.

TABLE 1

Respiration rates of tissue and whole fruit

Experiment	Whole fruit (mature)			1x10 mm. tissue disks		
	O_2	CO_2	R.Q.	O_2	CO_2	R.Q.
1	11.7	7.1	.606	29	58	2.0
2	15.3	10.8	.705	49	105	2.14
3a	12.6	11.6	.92			
3b	12.6	13.5	1.07			

The results tabulated in table 1 show respiration rates in microlitres per hour per gm. fresh tissue for the whole fruit and tissue samples taken from the same fruit. The value for carbon dioxide in 3b includes that retained in the tissue and extracted by the method described previously. This value is probably high since carbon dioxide content of the tissue was not determined when the fruit was taken from storage. The values for oxygen utilization obtained by sampling the external

atmosphere may be reasonably accurate according to Denny (11, p. 274), who found that oxygen content of potato tissue changed only slightly compared to the carbon dioxide. It is apparent that cutting the fruit resulted, not only in increased respiration rate, but also in considerable increase in the respiratory quotient.

That the rates of diffusion of oxygen and carbon dioxide have a bearing on the respiration rate is shown by experiments with tissue slices of different thicknesses. The data given in table 2 show decreasing respiration rate with increasing thickness. The apparently anomalous value for oxygen uptake by the 10x10 tissue sections was obtained in duplicate.

TABLE 2

Respiration rate of tissue disks of various thicknesses

Size of tissue (mm)	CO_2	O_2	R.Q.
1x10 disks	117.2	32.6	3.6
3x10 disks	95.5	23.9	4.0
5x10 disks	92	20.4	4.5
10x10 disks	61	30.7	2
1x10 disks	147	46	3.2
15x10 cylinders	72	32	2.2

Turner (39, pp. 232-253) considered adequate aeration occurred at the centre of carrot tissue slices 1 mm. thick. Goddard (16, p. 223) considers high carbon dioxide content of tissue as a limiting factor in respiration and cites thesis work of Scott, Cambridge University, in which respiration rate of beet, carrot and potato was increased by flushing the internal tissue with nitrogen. Increasing the oxygen content had no effect. Increased facility for gaseous diffusion, however, cannot account for the high respiratory quotients obtained with apple tissue.

The effect of washing on tissue respiration

In general, washing resulted in reduced respiration rate and reduced respiratory quotient. The respiration rate of tissue washed with water (Figure 1) dropped rapidly to zero in approximately two hours. The flaccid, shrunken appearance of the tissue indicated cell damage from unfavourable osmotic conditions. Washing with 10 per cent sucrose (Figure 2) maintained respiration at a high level for over 135 hours. The increased carbon dioxide output and decreased oxygen uptake occurring towards the termination of the experiment probably resulted from anaerobic respiration. Tissue washed with a .25 M calcium chloride retained firmness and active respiration over a considerable period (Figure 3). Since

calcium chloride would not serve as a respiratory substrate and would not become contaminated as readily as sucrose, it appeared to form a suitable washing agent.

Respiratory responses to acid intermediates

Samples of tissue from different fruits showed variation in response to treatments; for example, a given concentration of reagent did not always give the same effects. Certain trends, however, were evident when sufficient replications were made. Except where calcium chloride treatment is indicated, the following results are those obtained with mature tissue receiving only a brief water rinse.

Pyruvate. Pyruvate (0.05M) increased carbon dioxide production and invariably increased the respiratory quotient. In some cases oxygen uptake was increased (Figure 4) and in other experiments it was decreased. Tissue washed for 56 hours with calcium chloride responded to 0.05M pyruvate with increased respiration rate and respiratory quotient. Tissue treated with pyruvate possessed a distinct odor of acetaldehyde which was identified qualitatively with Schiff's reagent. Acetaldehyde could be detected only with pyruvate treated tissue in mature and immature fruit.

Citrate. All 0.02M citrate treatments decreased carbon dioxide output of fresh tissue, but appeared to

Figure 1. EFFECT OF WASHING WITH WATER.

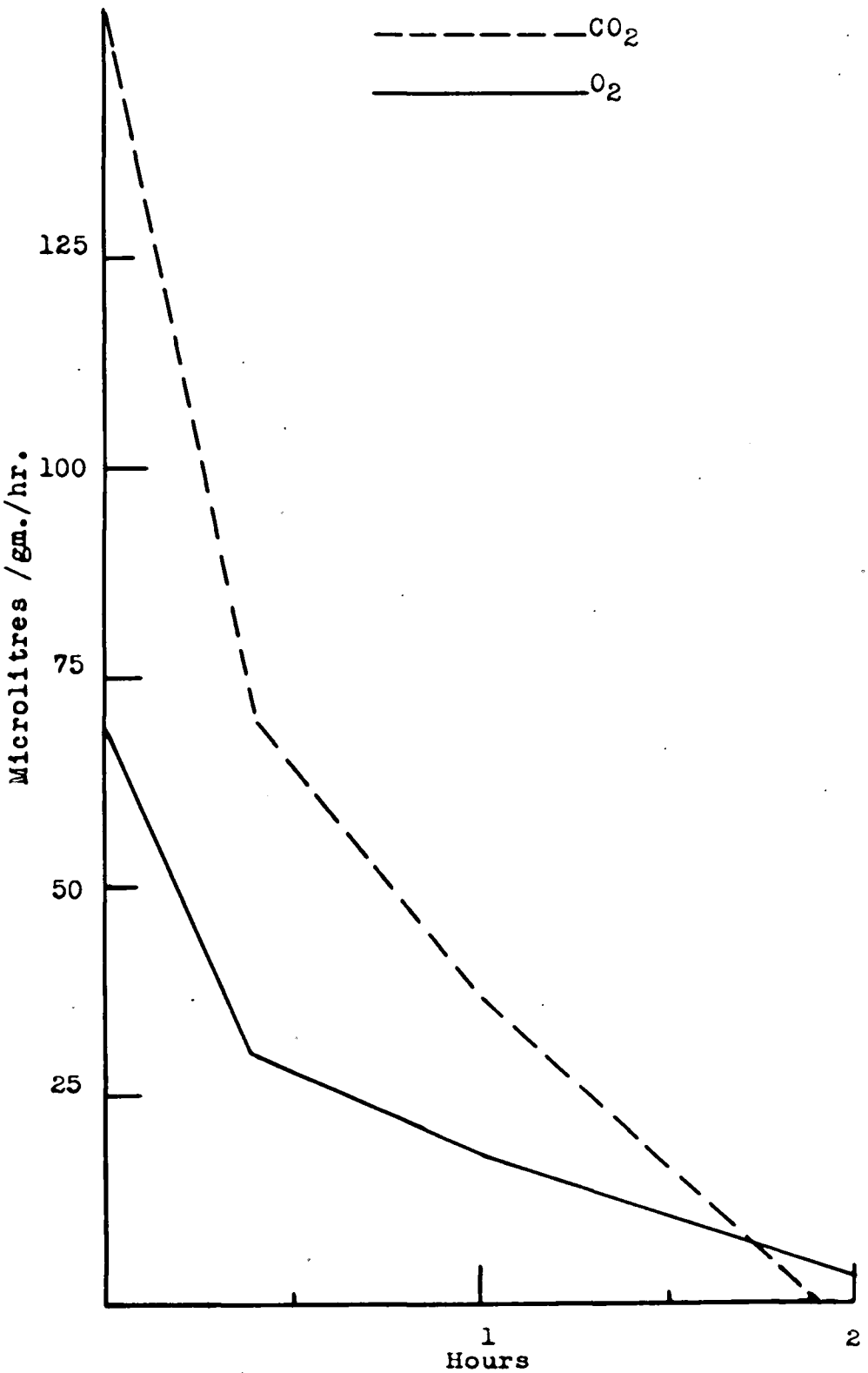


Figure 2. EFFECT OF WASHING WITH 10% SUCROSE

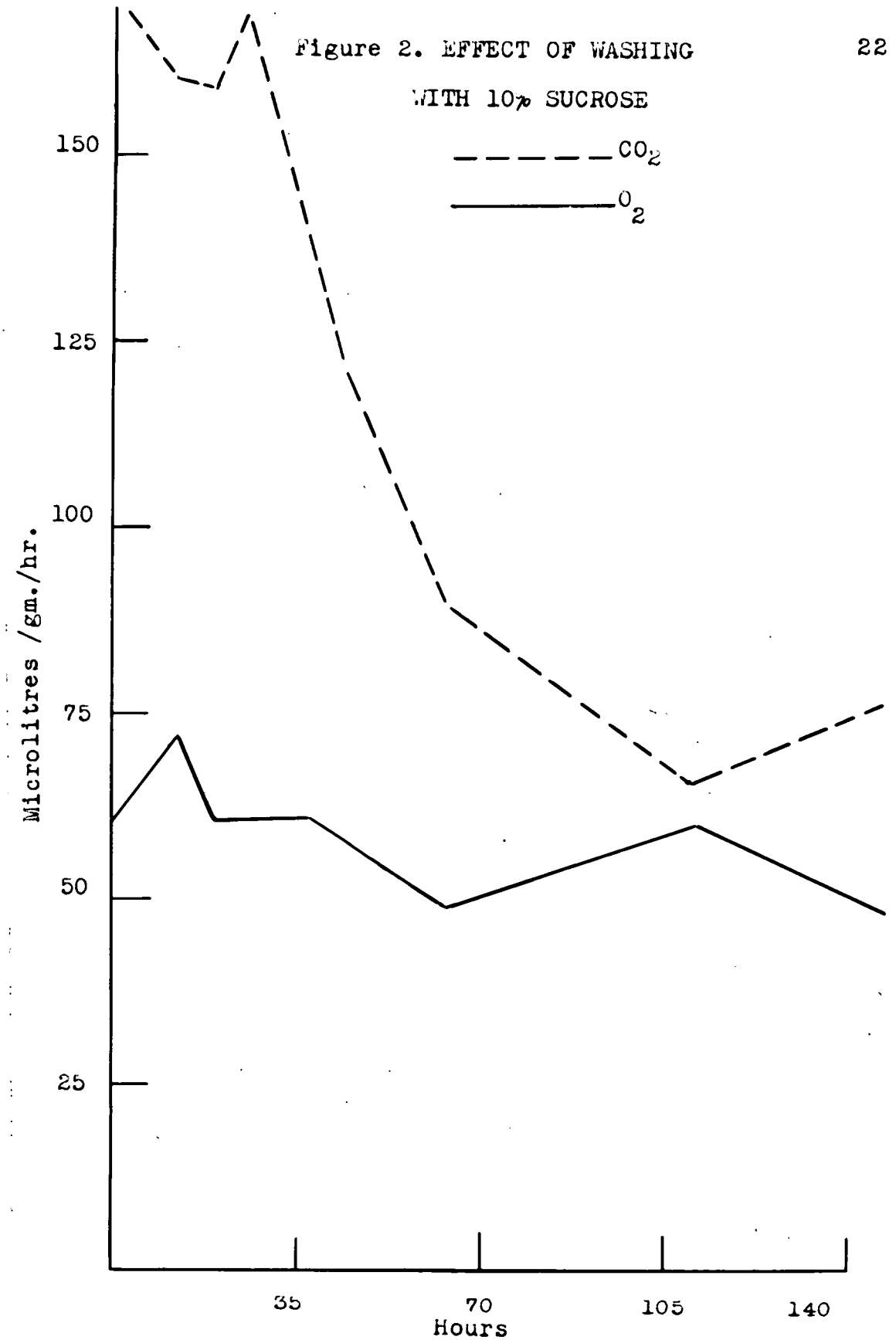


Figure 3. EFFECT OF WASHING WITH $CaCl_2$ .25M

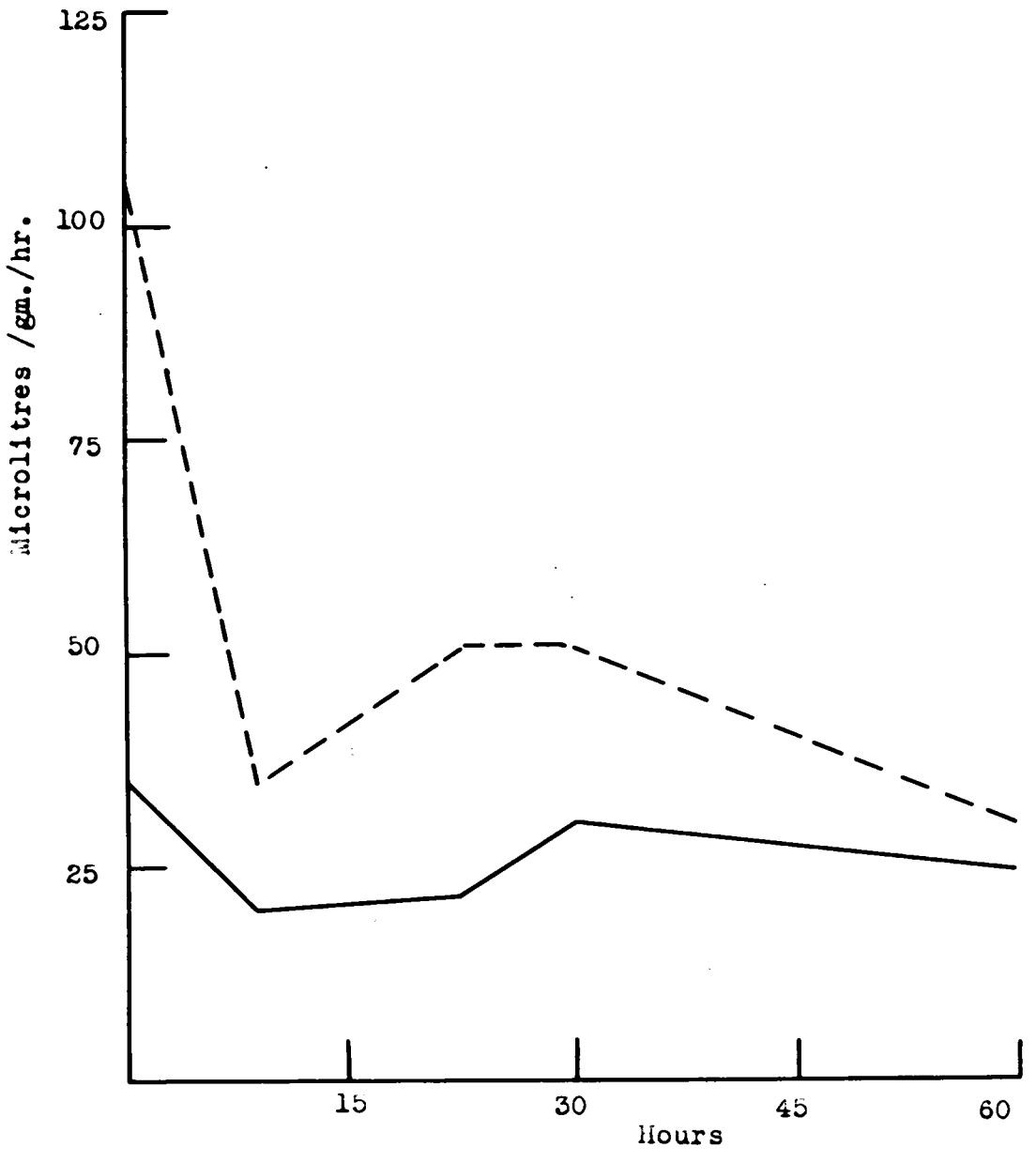
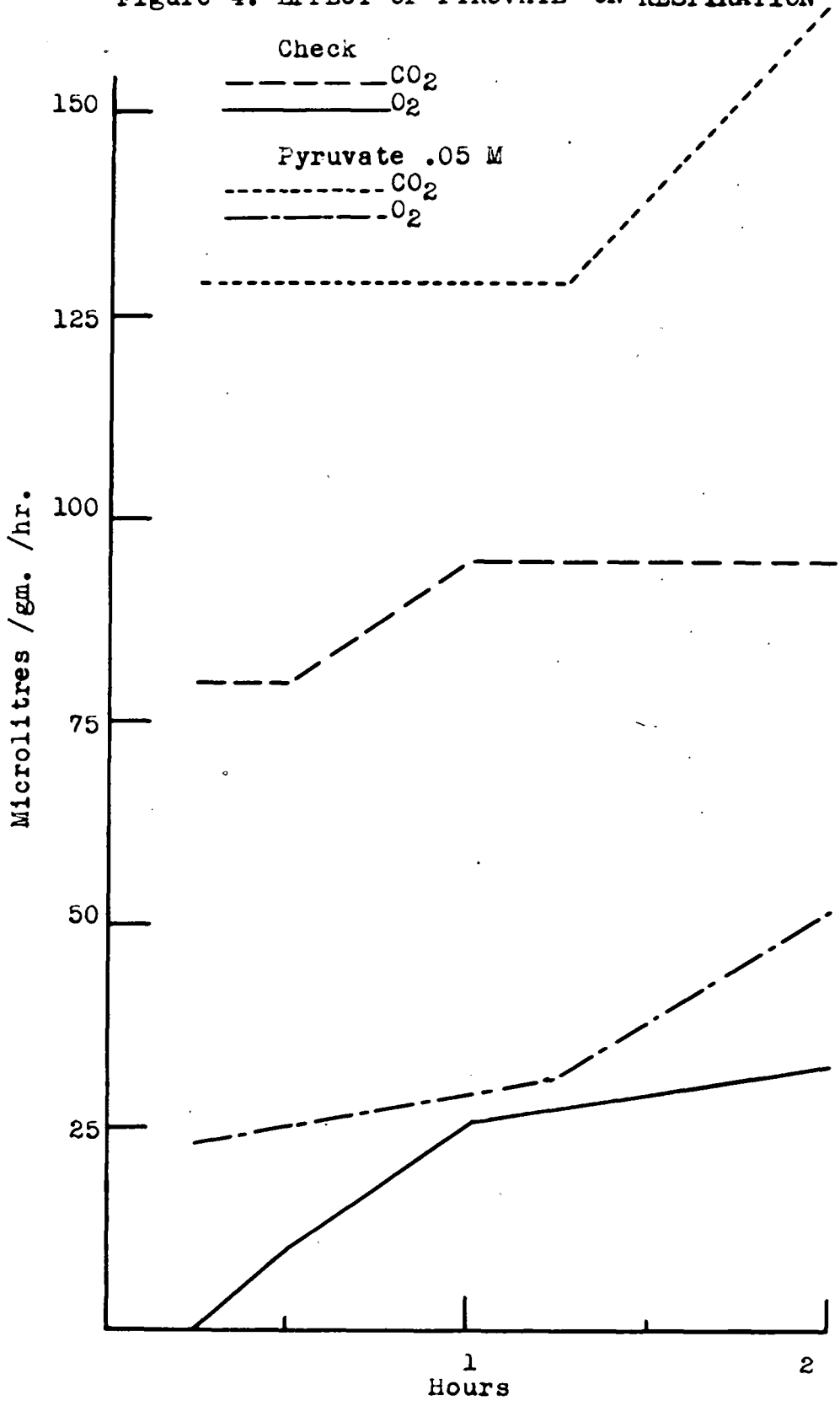


Figure 4. EFFECT OF PYRUVATE ON RESPIRATION 24



increase carbon dioxide production from calcium chloride-washed tissue. High concentrations of citrate (0.06 M) depressed respiration. In the series shown in Figure 5, 0.04 M and 0.02 M citrate treatments decreased the respiratory quotient and increased oxygen utilization.

Alpha-ketoglutarate. Alpha-ketoglutarate at 0.033 M and 0.016 M depressed respiration of fresh tissue; had little effect on oxygen consumption, but caused a distinct increase in carbon dioxide production of calcium chloride washed tissue (Figure 6).

Succinate. The effect of succinate on respiration was governed by concentration. Stimulation of both oxygen uptake and carbon dioxide output occurred with 0.02 M succinate for both fresh (Figure 7) and washed tissue. In the series shown in Figure 8, 0.025 M and 0.01 M caused some stimulation, but definite inhibition of respiration occurred with concentrations of 0.05 and 0.1 M succinate (Figures 9, 8). High concentrations of succinate inhibited carbon dioxide production more than oxygen consumption with a consequent lowering of the respiratory quotient.

Fumarate. Fumarate at 0.03 M concentration depressed respiration in all cases. Lower concentrations, 0.01 and 0.005 (Figure 10), reduced carbon dioxide output to a considerable extent, but maintained oxygen uptake at the level of the control.

Figure 5. EFFECT OF VARIOUS CONCENTRATIONS OF CITRATE ON RESPIRATION

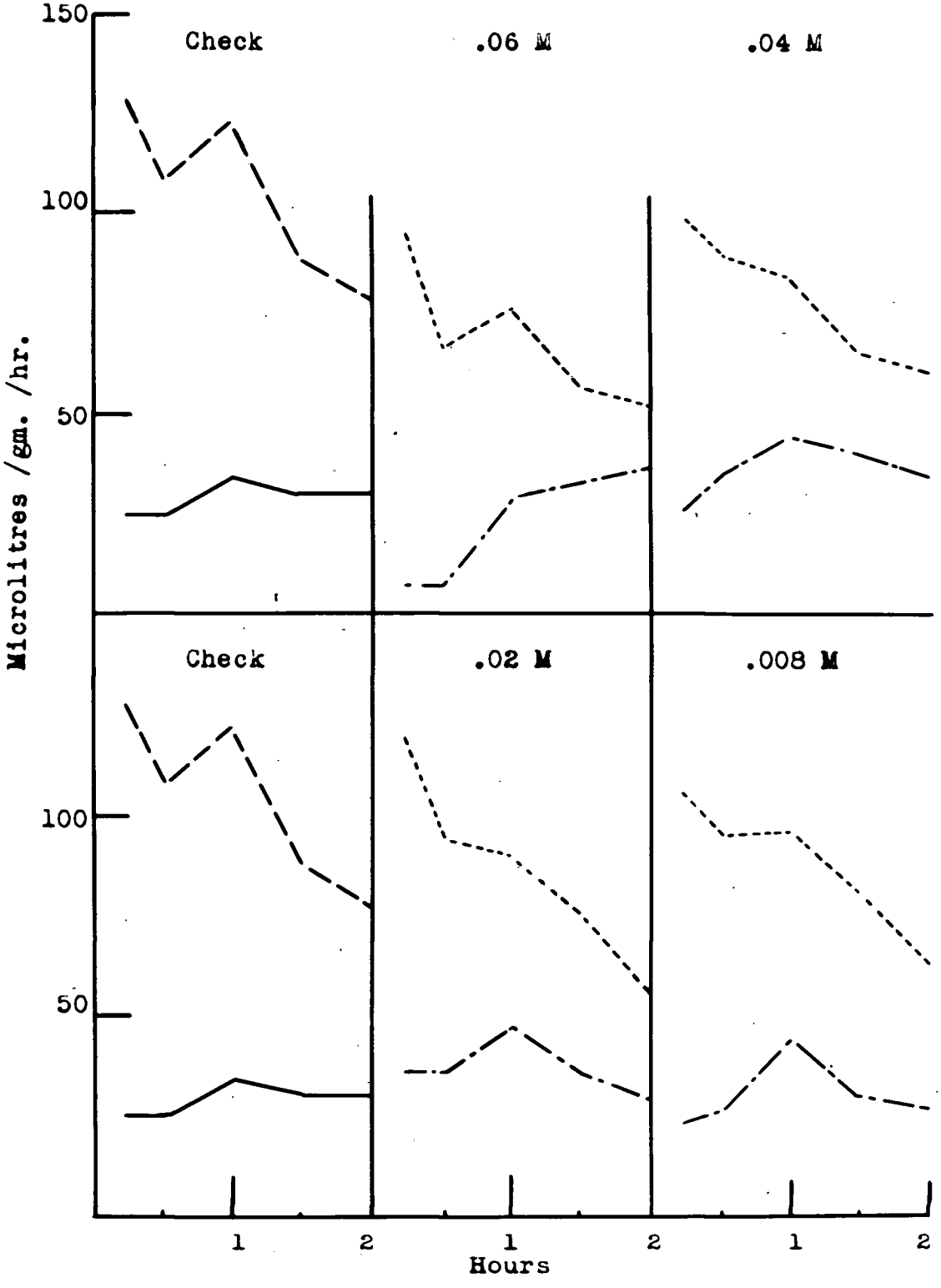


Figure 6. EFFECT OF ALPHA-KETOGLUTARATE ON TISSUE WASHED 56 HOURS WITH $CaCl_2$

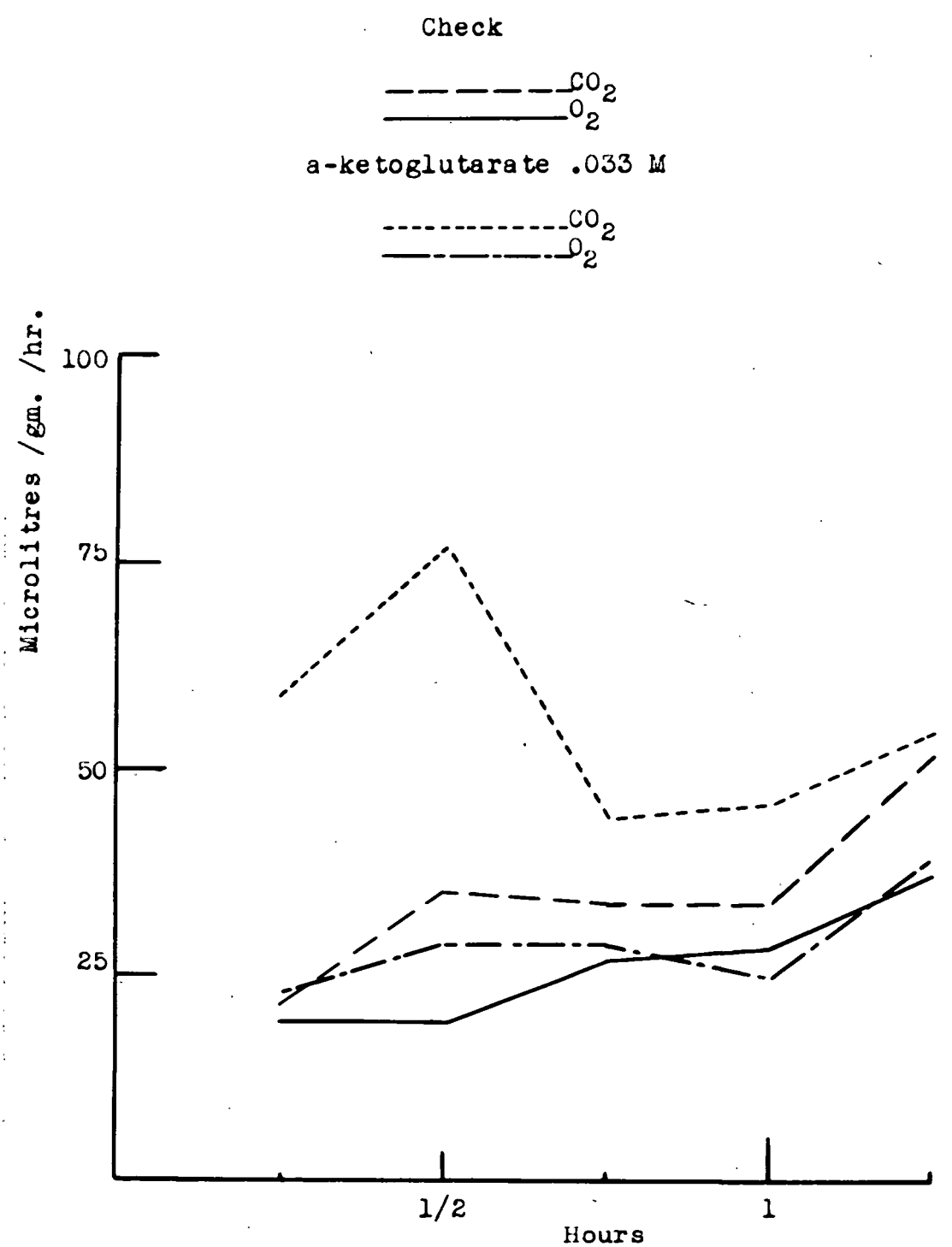


Figure 7. EFFECT OF SUCCINATE ON RESPIRATION.

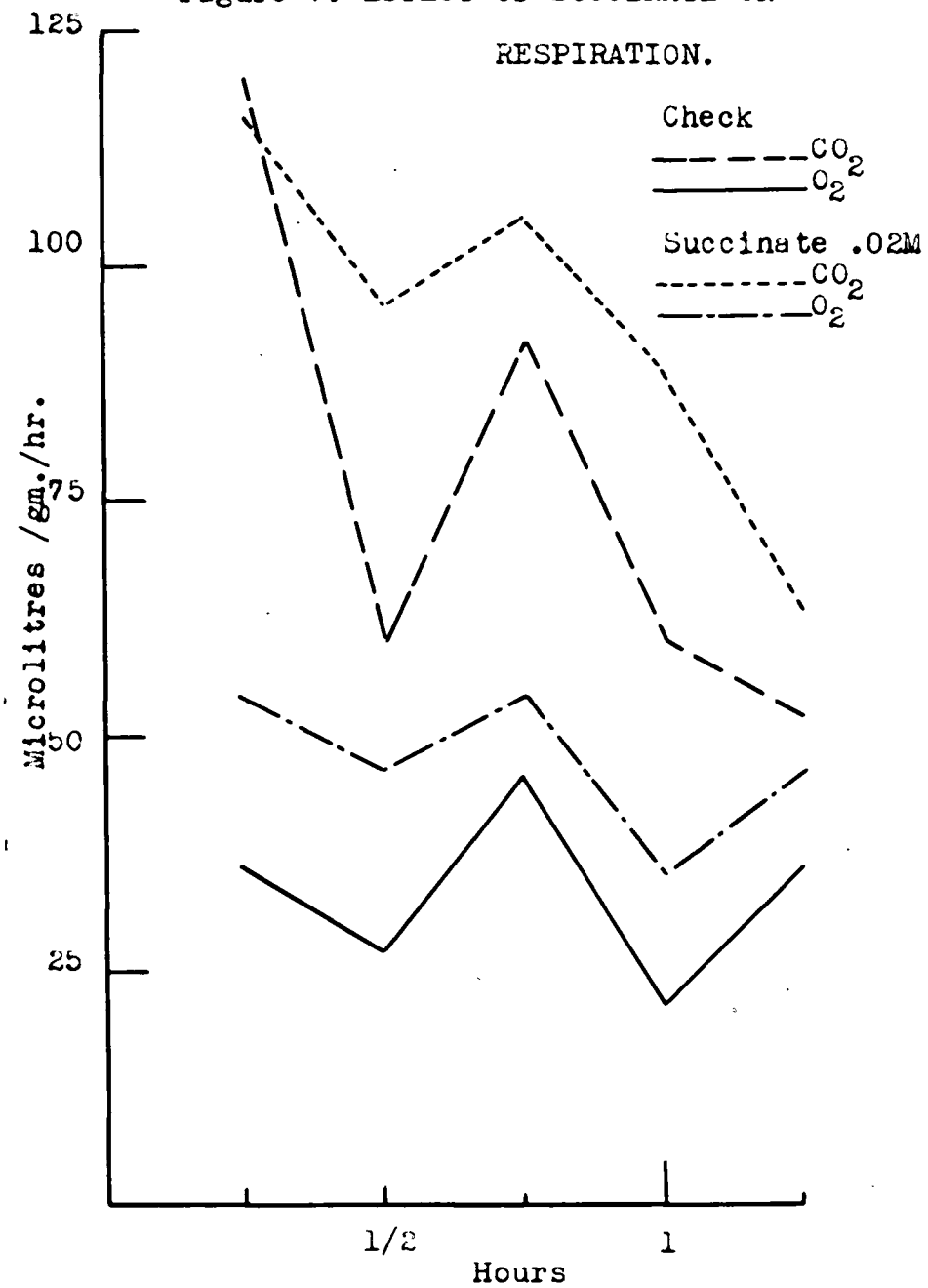


Figure 8. EFFECT ON RESPIRATION OF VARIOUS CONCENTRATIONS OF SUCCINATE

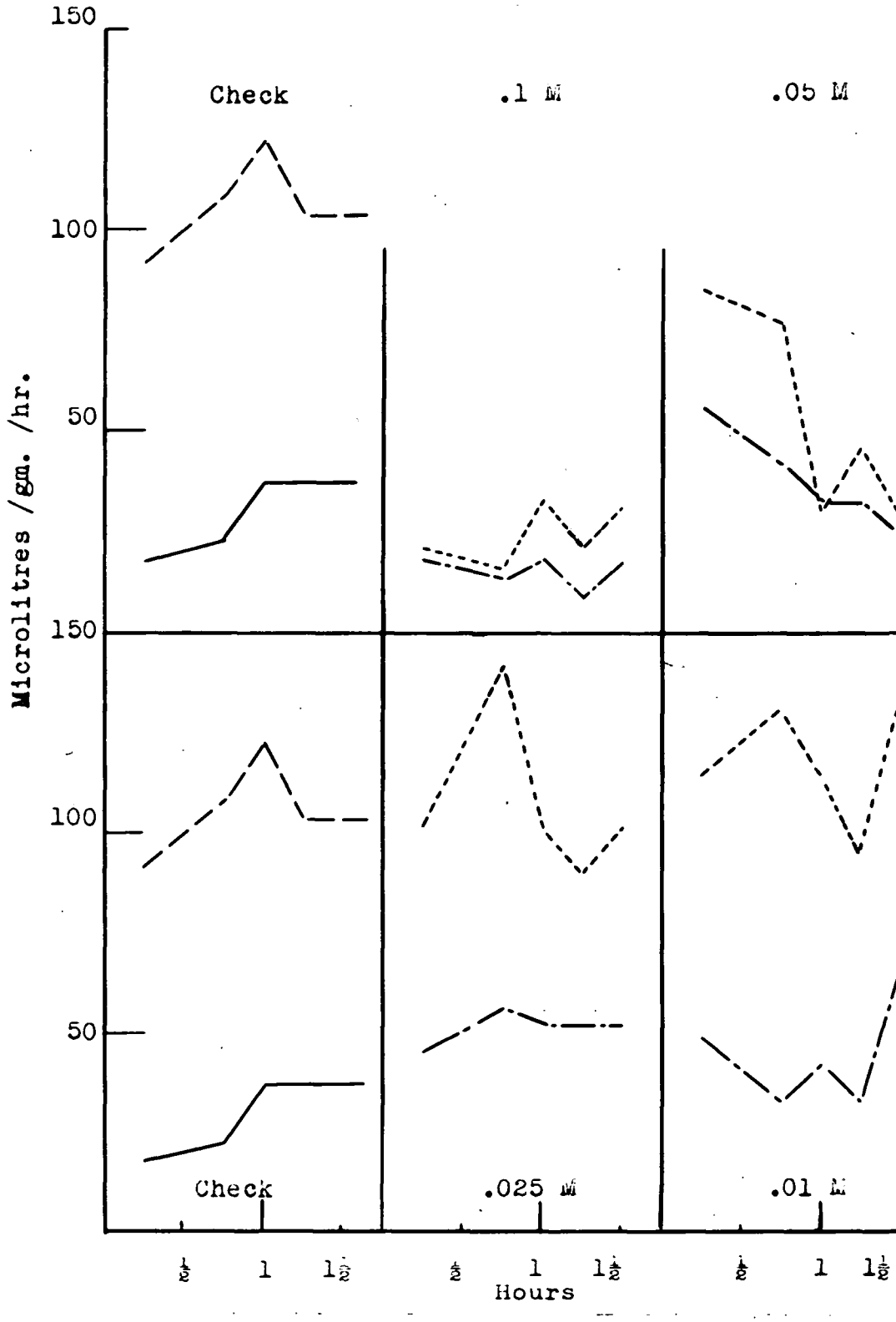


Figure 9. INHIBITION WITH SUCCINATE

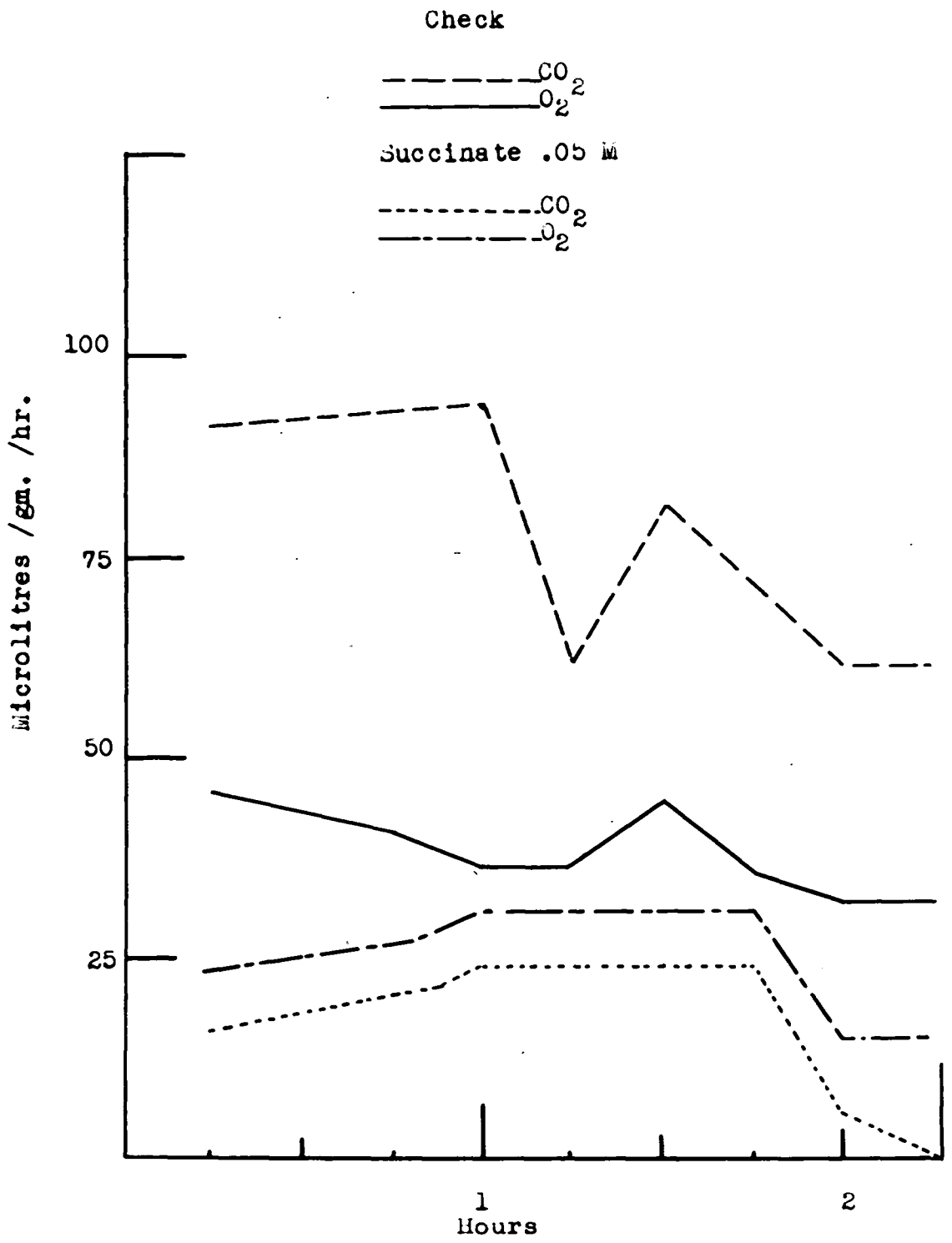
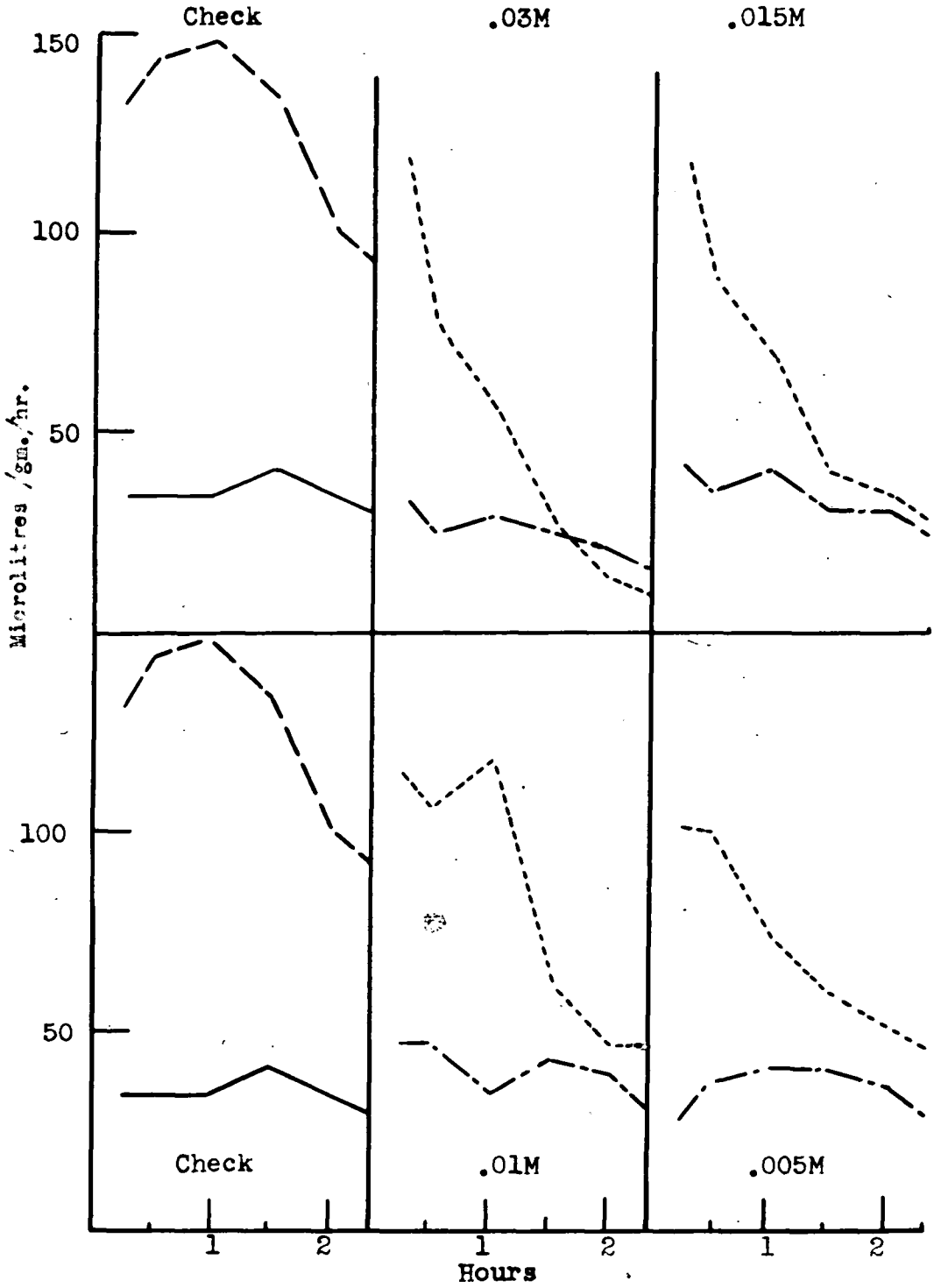


Figure 16. EFFECT OF VARIOUS CONCENTRATIONS OF FUMARATE ON RESPIRATION



Malate. Malate concentration appeared not to be a critical factor. A range of concentrations 0.025, 0.05 and 0.075 M (Figure 11) produced comparable responses, i.e. stimulation of carbon dioxide production, increase of respiratory quotient, and in the case of 0.05 and 0.075 M, some increased oxygen uptake.

Respiratory response with addition of amino acids

Both dl-asparagine and d-glutamate, (0.05 M) increased respiration rate and respiratory quotient of mature tissue (Figures 12, 13).

The effect on tissue respiration of added juice

Apple juice, boiled and diluted 1:3 with buffer solution, increased carbon dioxide production of mature tissue by as much as 35 per cent in a three-hour period. Total oxygen uptake increased only slightly (Figure 14).

The effect of inhibitors on tissue respiration

Malonate. Malonate in concentrations of 0.005, 0.01, 0.025 or 0.05 M produced respiratory inhibition ranging from less than 10 per cent to over 75 per cent. While there appeared to be some variation in response of different samples, 0.05 M malonate produced consistently greater inhibition than lower concentrations (Figure 15).

Figure 11. EFFECT OF MALATE ON RESPIRATION

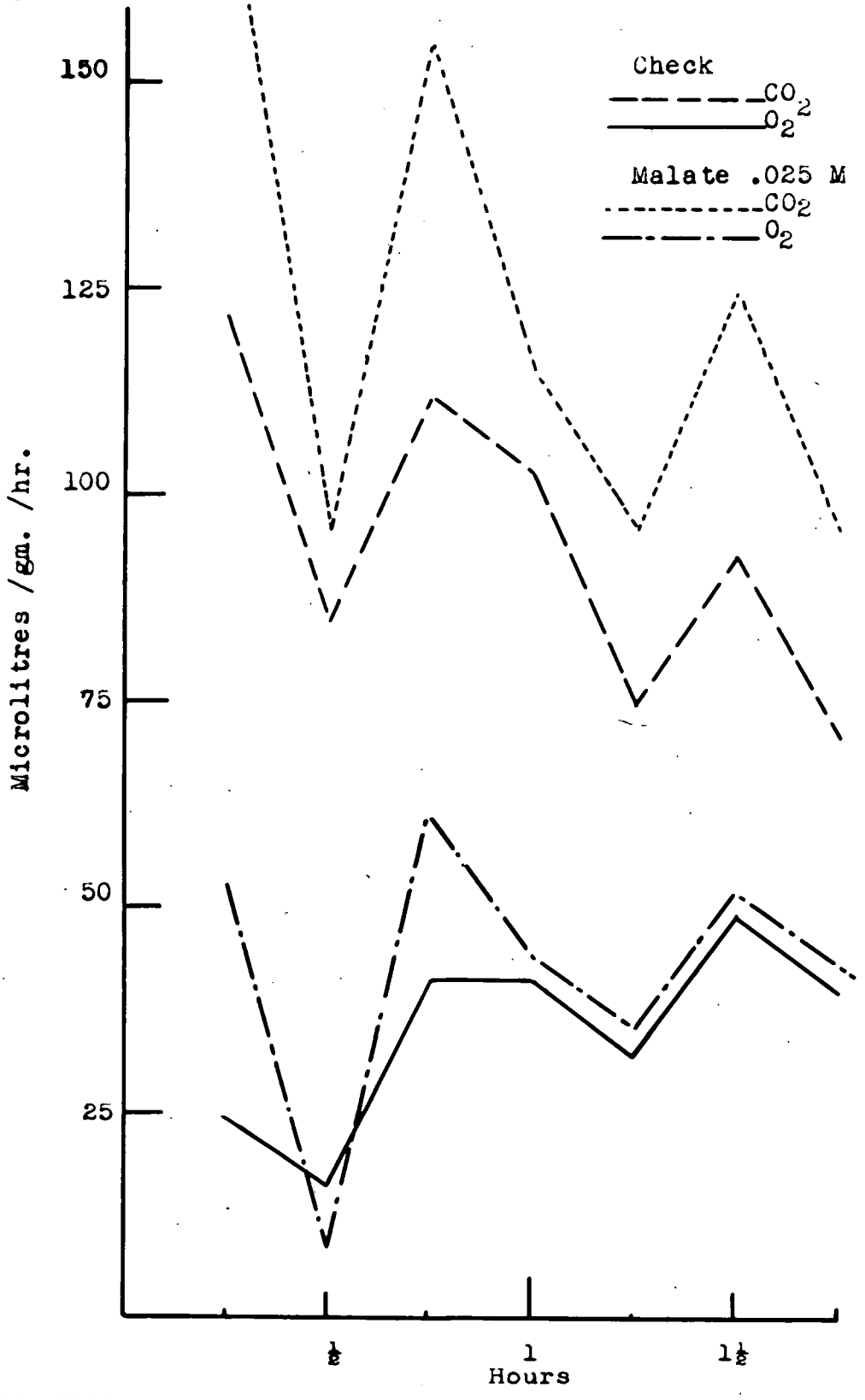


Figure 12. dl-ASPARAGINE

EFFECT ON RESPIRATION

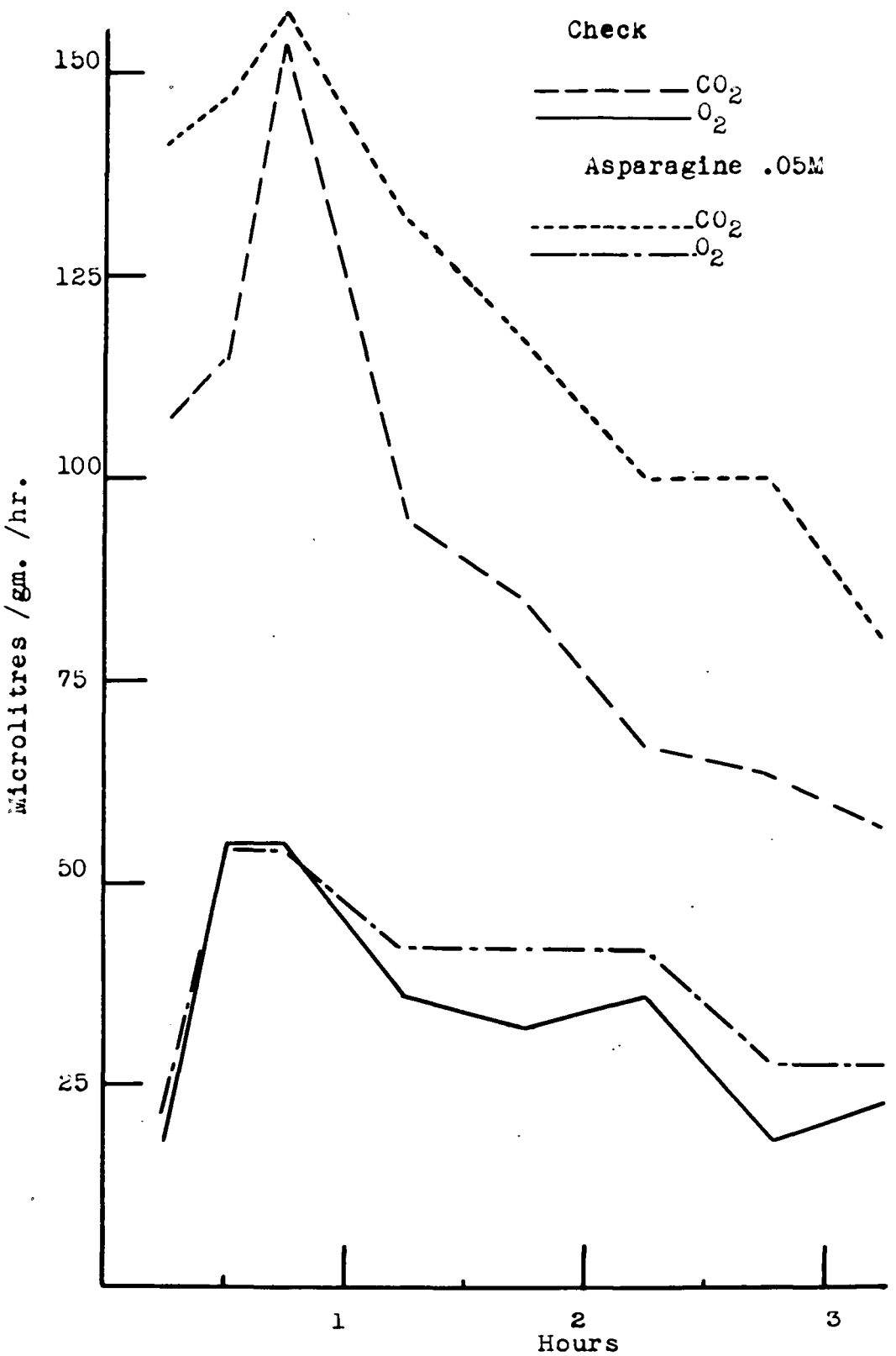


Figure 13. EFFECT OF d-GLUTAMATE ON RESPIRATION 35

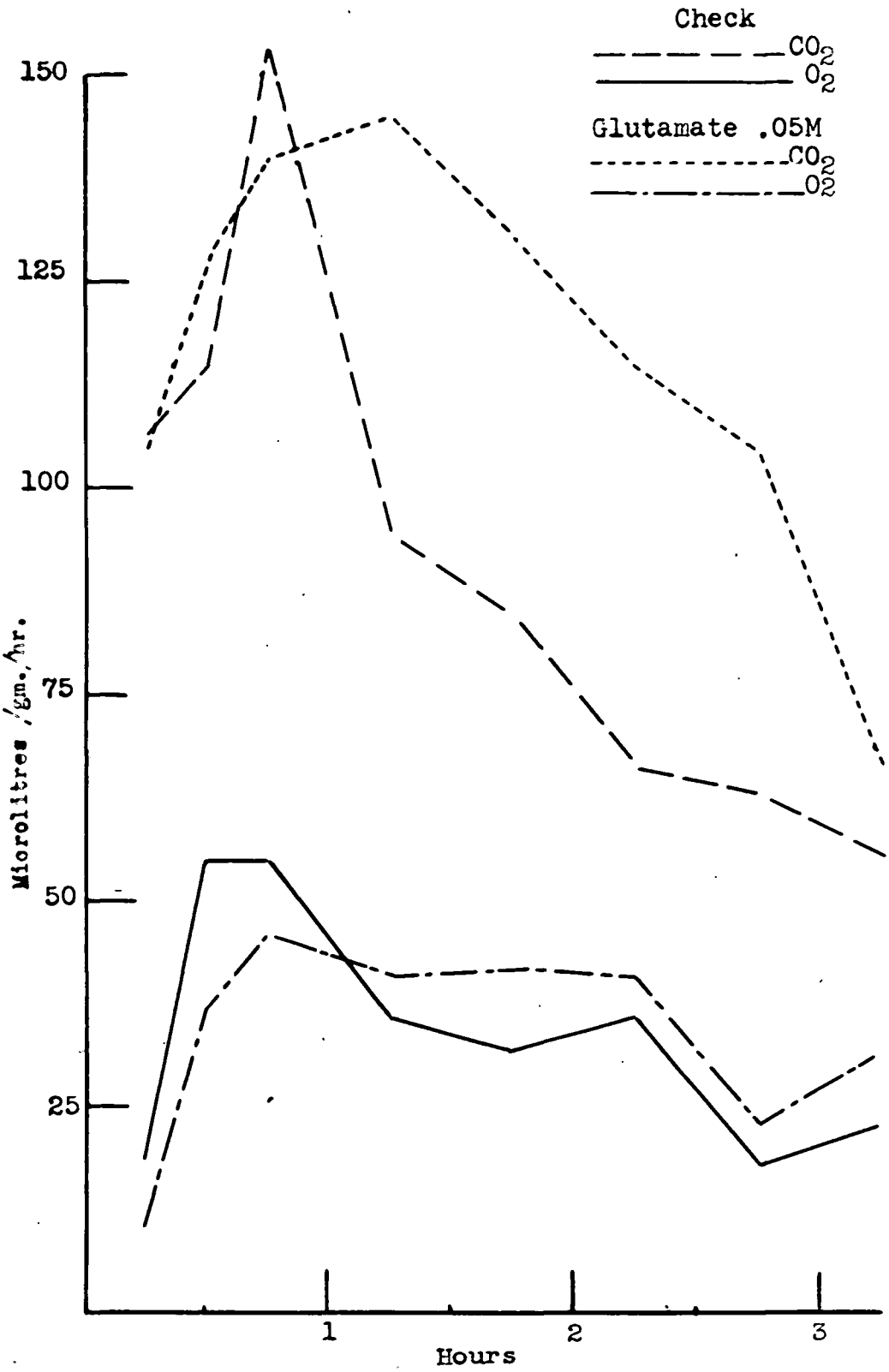


Figure 14. EFFECT OF APPLE JUICE 1:3 DILUTION

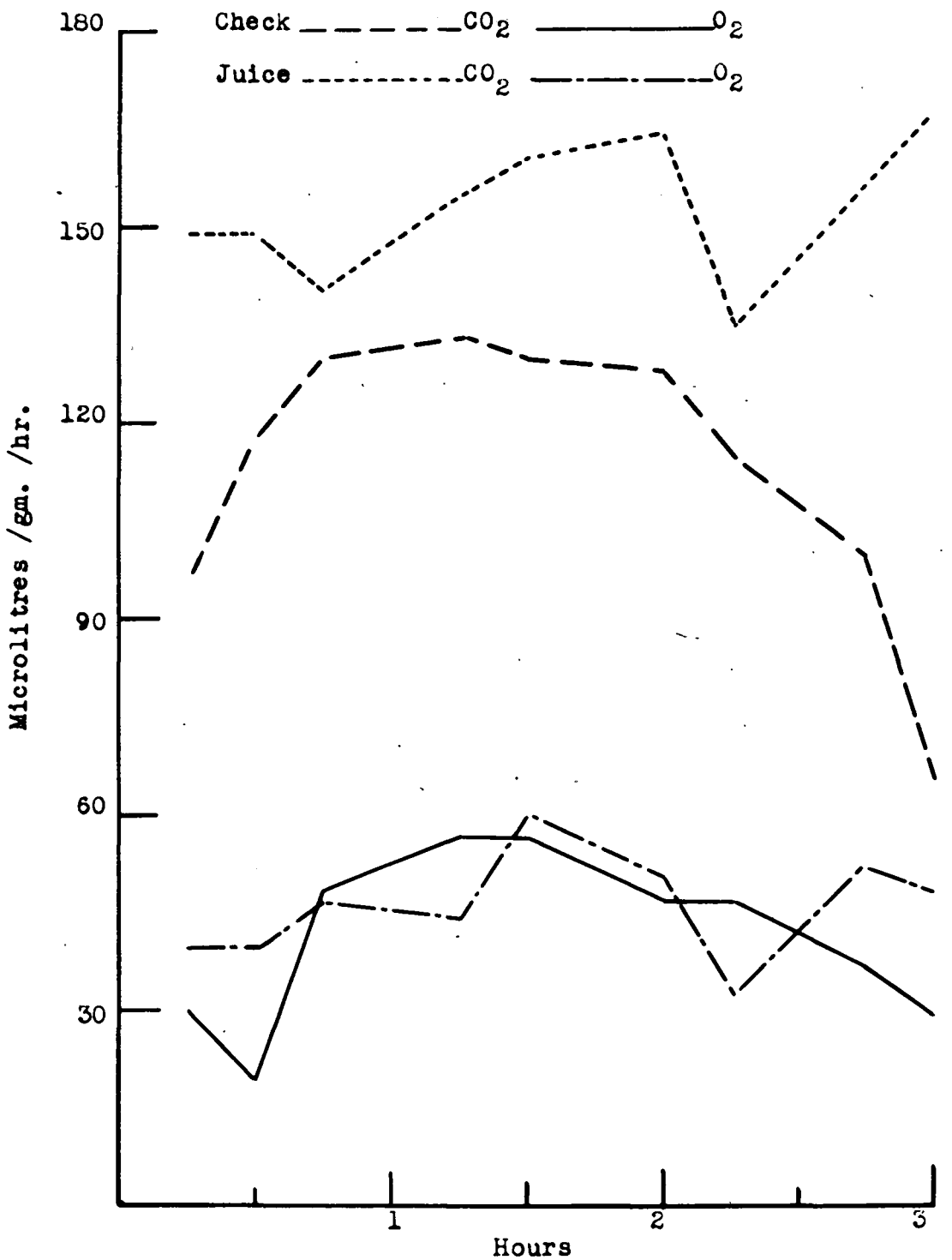
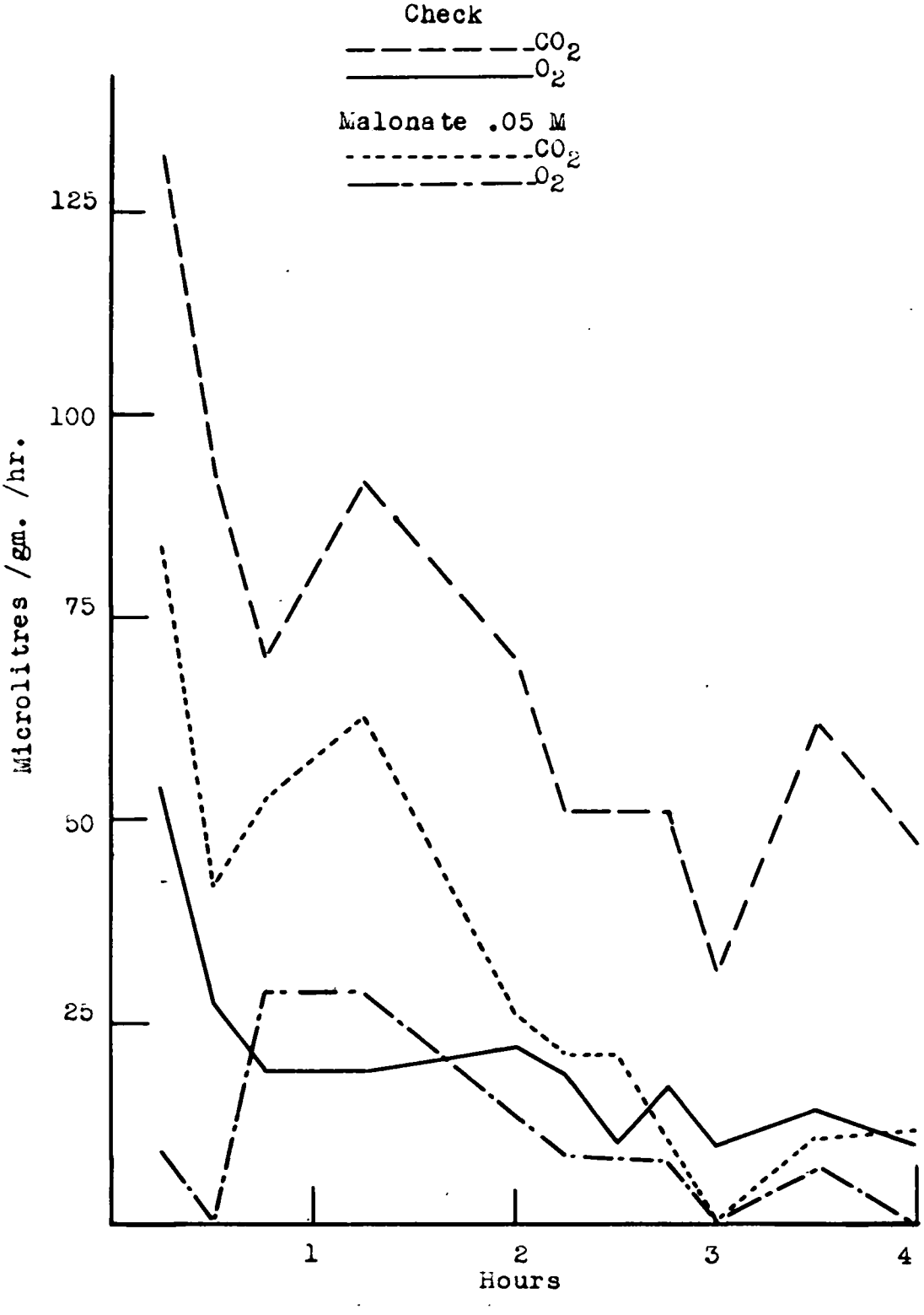


Figure 15. MALONATE INHIBITION



Iodoacetate. Iodoacetate caused pronounced inhibition of tissue respiration (Figure 16). Concentrations of 0.0001 and 0.001 M reduced respiration by as much as 50 and 90 per cent respectively. In every instance of iodoacetate inhibition, the respiratory quotient was markedly reduced.

Fluoride. Almost complete inhibition of respiration could be obtained with 0.025 M fluoride as shown in (Figure 17), while as low as 0.004 M reduced respiration by more than 50 per cent.

Para-nitrophenol. Both oxygen consumption and carbon dioxide production were inhibited 90 per cent or more by 0.01 M p-nitrophenol (Figure 18), a fairly specific test for polyphenoloxidase according to Bonner and Wildman (7, pp. 497-518). The specificity of p-nitrophenol is, however, contested by Stenlid (34, pp. 61-69) who considers it to be a general respiratory inhibitor.

The effect of added substrates on an inhibited system

Malonate inhibition was not reversed by succinate. Since malonate is considered to be a competitive type of inhibitor, the concentration of the added substrate should at least equal that of the inhibitor. Malonate inhibition was most effective at 0.05 M, but at this concentration succinate may also cause inhibition. Malate at 0.05 or 0.075 M normally stimulated respiration, but had no

Figure 16. IODOACETATE INHIBITION

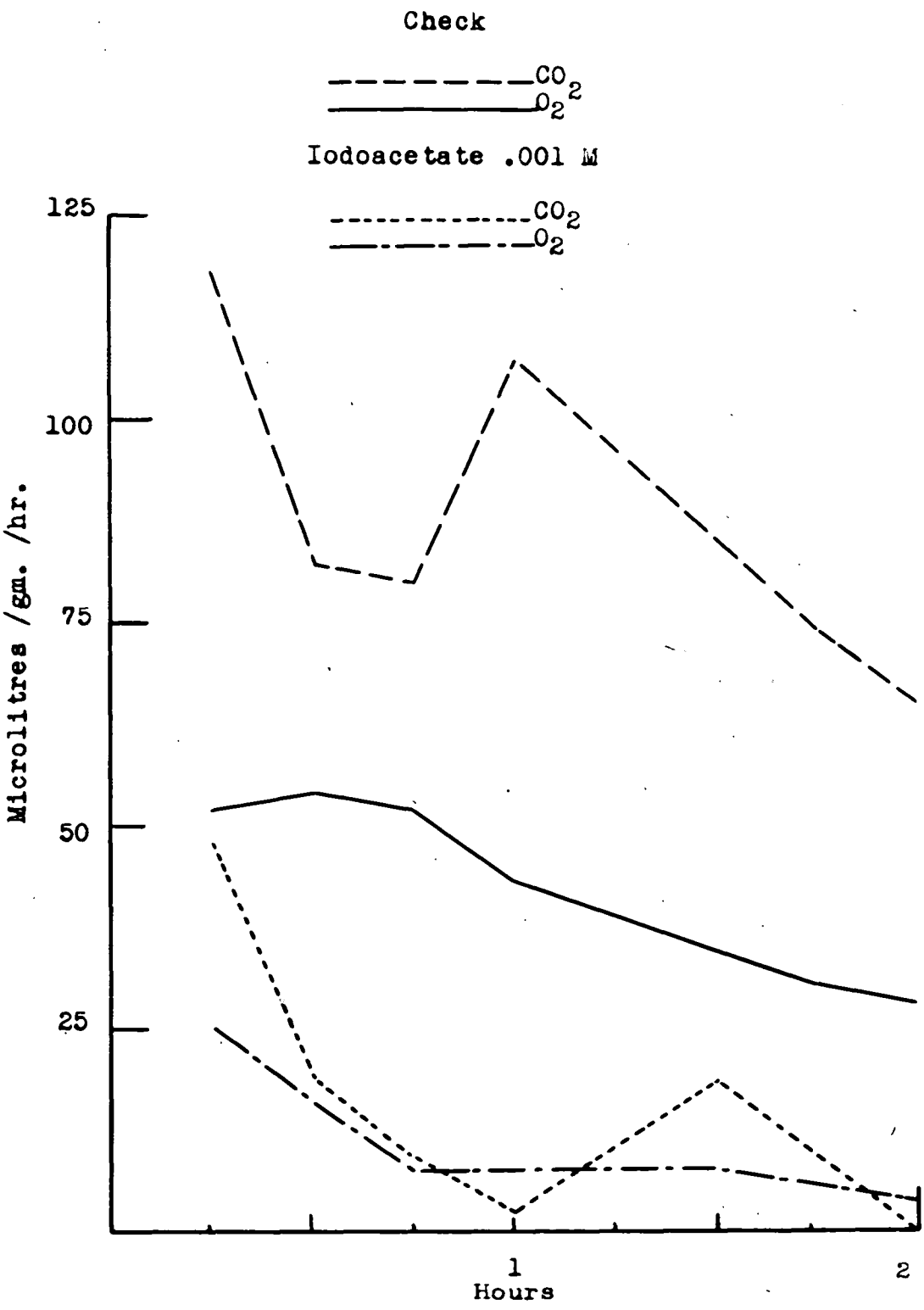


Figure 17. FLUORIDE INHIBITION

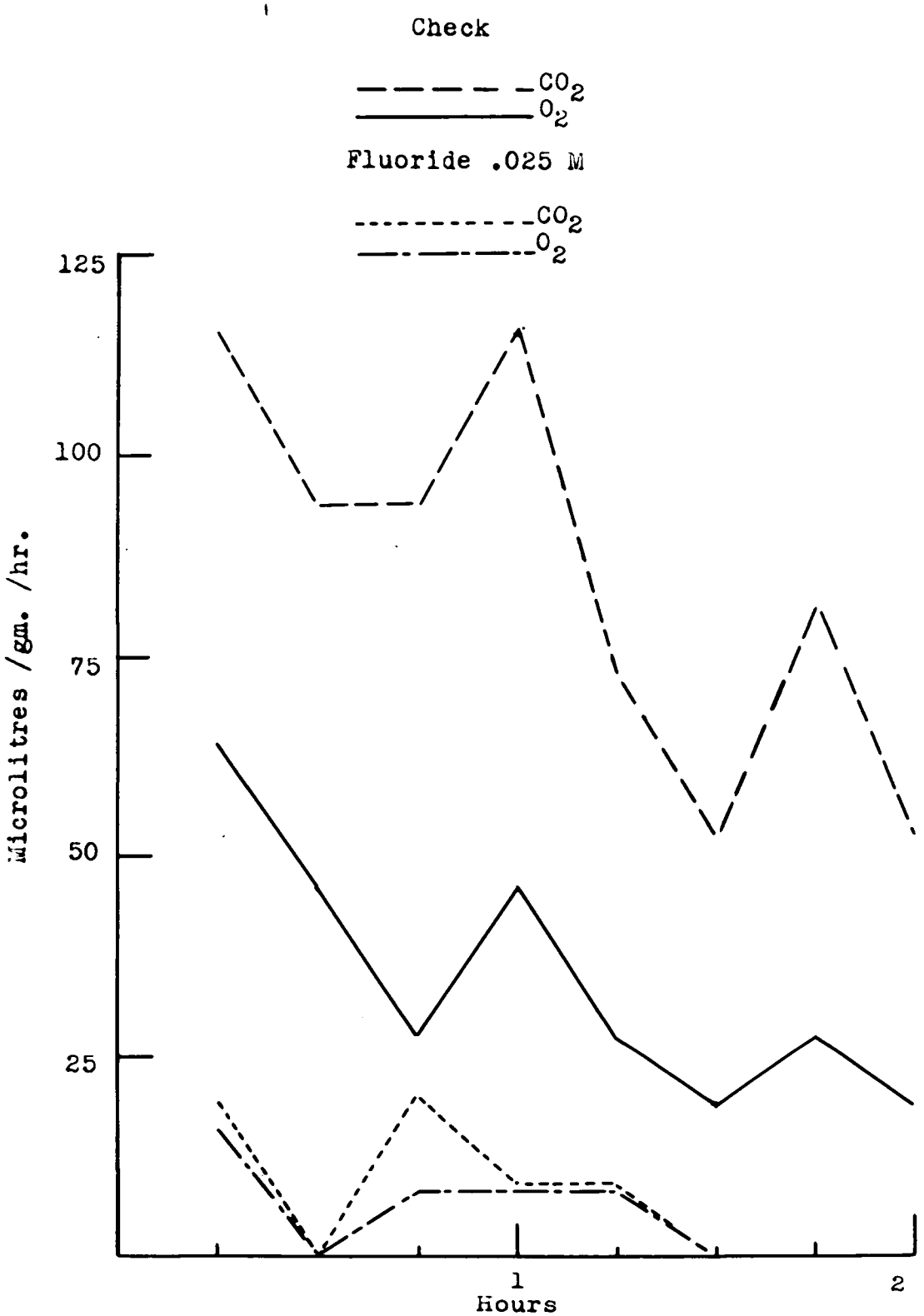


Figure 18. p-NITROPHENOL INHIBITION

effect on malonate inhibited respiration. Neither fluoride nor iodoacetate inhibited respiration was restored with pyruvate.

Respiratory behaviour of immature fruit

Respiration rate of young Newtown apples, approximately three weeks after bloom, was almost 10 times that of mature tissue. The respiratory quotient of whole, intact fruits was close to unity, but often increased considerably when fruit was disturbed by removing epidermis and calyx, or when sectioned. Respiratory quotients of fruit with epidermis, stem and calyx removed was as high as 3.5. Table 3 shows respiration rates of whole apple with only the stem cut and of the same apple after being cut in half or in thin slices.

The pronounced increase in respiration rate which accompanied sectioning of mature fruit did not occur with small, immature fruit. This might be explained by the fact that young tissue, having smaller, denser cells with much less intercellular space than mature tissue, would not be as effectively aerated by sectioning as mature tissue. Furthermore, a greater number of cells would be damaged in the young tissue than in mature tissue. This fact might account for the lowered respiration rate that sometimes followed sectioning of immature fruits.

TABLE 3

Respiration of immature fruit

Time (hours)	Sample	CO_2 Microlitres	O_2 /gm./hr.	R.Q.
1	whole fruit	515	545	.95
2	slices	615	385	1.60
3	slices	1005	668	1.57
1	whole fruit	770	778	.98
2	slices	514	268	1.9
3	slices	838	468	1.8
1	whole fruit	233	267	.87
2	slices	413	308	1.34
3	slices	300	261	1.15
1	whole fruit	362	310	1.17
2	halves	347	181	1.92
3	slices	465	365	1.27
1	whole fruit	422	407	1.03
2	halves	289	212	1.36
1	whole fruit	224	194	1.16
2	halves	375	212	1.77
3	halves	235	209	1.37

Respiratory inhibitors, in concentrations used on mature tissue, had little effect on immature tissue. Iodoacetate at 0.001 M had no effect, but 0.017 M reduced oxygen consumption about 20 per cent and carbon dioxide production 50-70 per cent. Fluoride, 0.1 M produced over 90 per cent inhibition. No reduction of respiration occurred with 0.05 M malonate, but 0.2 M reduced oxygen and carbon dioxide exchange by 30 and 60 per cent respectively.

Carbon dioxide output increased and oxygen consumption usually decreased somewhat upon addition of 0.1 M pyruvate, 0.1 malate or 0.04 M citrate. Depression of oxygen by malate was sometimes followed by an increase after 1-2 hours. Fumarate, 0.03 M and 0.066 M alpha-ketoglutarate had no effect on respiration. Pyruvate failed to restore respiration to fluoride or iodoacetate inhibited tissue.

Identification of dehydrogenase activity with triphyltetrazolium chloride

Triphenyltetrazolium chloride at pH 7.4 is aerobically reduced to red formozan by dehydrogenases in presence of suitable substrates. The tetrazolium chloride test is adaptable to sensitive colorimetric methods when used with extracts or homogenates. Apple homogenates, however, were subject to severe browning which entirely masked the formozan color. Attempts to limit polyphenoloxidase activity

with 8-hydroxyquinoline, which reacts with the copper prosthetic group, were unsuccessful.

Since formozan formation proceeds in air it is possible to observe the progress of the reaction on tissue slices which do not brown as rapidly as homogenates.

Mature tissue. The seed was the only portion of mature tissue to show visible color change in the presence of succinate and tetrazolium chloride.

Immature tissue. Color developed rapidly in thin slices of young apple tissue with or without the addition of substrates. While this test indicated vigorous dehydrogenase activity, it was not suitable for identifying specific enzymes. Observed under a microscope, transverse tissue sections showed color developing first in cells of the seed, then in the area of the vascular bundles and later in the cortical region.

DISCUSSION

This investigation has attempted to reveal the mechanism of carbon dioxide production in apple tissue. The methods were those which have been used with other plant and animal tissues to demonstrate participation of organic acids in respiration. The results have not provided satisfactory evidence of an organic acid cycle in apple fruits.

In experiments of this type, tissue is moved from a natural, physiological environment in which intermediates may be transitory and in minute amounts, to an unnatural site where concentrations of intermediates are changed and respiratory poisons are used. Demonstration of an intermediary reaction under these circumstances could be accepted as evidence, not proof of its occurrence under natural conditions. On the other hand, failure to show a certain reaction does not preclude its occurrence in the natural state.

Many of the techniques used in studying plant respiration are applications of methods developed for study of animal tissue. Optimum experimental conditions differ for plant and animal and may even vary among different plants. Unsuitable conditions may lead to erroneous conclusions. For example, Laites (30, pp. 284-299) and Turner (42, pp. 296-297) point out the necessity of low pH in obtaining malonate inhibition. Stenlid (34, p. 67)

shows that use of too high a pH resulted in failure by Marsh and Goddard (31, pp. 724-728) to obtain azide inhibition of oxygen uptake in old carrot leaves and led to the false assumption that the terminal oxidase was different from that in young leaves.

Krebs (25, p. 196), considering animal metabolism, points out several facts which have considerable bearing on the interpretation of results: malonate in low concentration (0.001-0.005 M) probably acts exclusively on succinic dehydrogenase, but at higher concentration may interfere with other enzymes. An added substrate, although a normal intermediate, may inhibit the oxidation of other substrates by combining with ions and enzymes needed elsewhere. For example, 0.05 M citrate may inhibit the oxidation of alpha-ketoglutarate which accumulates, excess succinate may cause accumulation of fumarate and malate, and excess oxalacetic may cause accumulation of citrate, alpha-ketoglutarate and succinate.

A French worker, Jacobsohn, (21, p. 116) observed iodoacetate inhibition of fumarase. Fluoride, which is known to inhibit enolase, may not be entirely specific but will inhibit other enzymes particularily those requiring magnesium ion (17, p. 690). Para-nitrophenol inhibition, used by Bonner (7, p. 497-518) to indicate polyphenol oxidase in spinach, is likewise non-specific

according to Stenlid (34, p. 67) who showed it to be a general inhibitor even for carrot in which cytochrome is considered to be the terminal oxidase.

In view of the foregoing, some of the results of this study can be accounted for without departing from the basic scheme of respiration that has been shown to function in many plants.

Krebs (25, p. 192) contends that a tissue which metabolizes carbohydrate or carbohydrate derivitives under physiological conditions will be able to deal only with carbohydrate or related substances. If substances, therefore, are added and found metabolized they must be associated with carbohydrate metabolism. It is difficult, however, to determine, without chemical analysis, if a material acts as a metabolite or merely a stimulant of respiration. It has been suggested by Stare (33, pp. 338-357) that stimulation alone would not change the respiratory quotient, but oxidation of an acid intermediate would increase the respiratory quotient. On the basis of this concept, pyruvate, citrate, alpha-ketoglutarate, succinate, malate, glutamate and asparagine have been shown by the present experiments to enter into the metabolism of apple tissue. Pyruvate carboxylase was shown to be active in apple tissue. Presence of dehydrogenase enzymes was also demonstrated but specific types have yet

to be identified.

Reason for the high respiratory quotient of cut tissue is obscure. Hackney (18, pp. 333-345) appears not to have encountered this problem in her study of apple tissue respiration. High respiratory quotients were obtained by Bennet-Clark (3, pp. 65-92), when beet root was exposed to expressed sap or when suspended in succinate or malate in concentrations similar to those found in the sap. The respiratory quotient (1.5-2.3) was higher than would be produced by "burning" the acid: $C_4H_6O_5 + 3O_2 \rightarrow 4CO_2 + 3H_2O$, R.Q. 1.33. An oxidative anabolism theory was therefore proposed which involves decarboxylation of the acid and conversion of the C_3 residue into carbohydrate, thus: $C_4H_6O_5 \rightarrow \frac{1}{2} C_6H_{12}O_6 + CO_2$. One mole of CO_2 produced per mole of malic acid used was demonstrated.

Turner (43, pp. 149-171) studied the effect of succinate on carrot tissue respiration. He concluded that a gross respiratory quotient of about 2, obtained with succinate stimulation, was the result of the following:
(1) normal respiration with an R.Q. of 1.0,
(2) salt stimulated respiration, R.Q. of 1.0,
(3) succinate stimulated gas exchange with a net R.Q. of 4.0. Turner considered a respiratory quotient of 4 too high for oxidative anabolism and proposed the following: conversion of excess acid to pyruvate, decarboxylation of

pyruvate and reduction of acetaldehyde formed to alcohol. Alcohol, which is difficult to detect, was not found.

Loss of malic acid by apples was shown by Fidler (15, pp. 41-64) to proceed at the same rate under aerobic or anaerobic conditions. He suggests that there may be interconversion of acid and carbohydrate by carbon dioxide transference which is essentially the oxidative anabolism theory.

Exposure of apple tissue to juice and some of the organic acids known to be present in apple, resulted in a high respiratory quotient. Sectioning the tissue undoubtedly spreads the contents of damaged cells over the tissue surface, but it is difficult to account for the long period of washing required to lower the respiratory quotient.

While the data obtained indicate that organic acid metabolism is related to the formation of carbon dioxide in apple tissue, conclusive evidence will require considerably more fundamental investigation. Chemical analysis is needed to identify the intermediates, to determine changes that occur during normal respiration, upon treatment with inhibitors, and as a result of adding intermediates. More knowledge is also required of the enzyme system particularily the terminal oxidase.

SUMMARY

A study was made of the respiratory behaviour of Newtown apples to determine the mechanism of carbon dioxide production.

Since, in many living organisms carbon dioxide is released from intermediates in an organic acid cycle, participation in apples of this cycle was investigated by using methods which have revealed its operation in a variety of animal and plant materials.

Results indicated that a number of acid intermediates could be metabolized in apple, and respiration could be checked with inhibitors known to block the acid cycle at certain points, but reversal of fluoride, iodoacetate or malonate inhibition could not be accomplished.

Operation of the organic acid cycle in apple was indicated but not conclusively demonstrated by the techniques employed.

No satisfactory explanation was found for the high respiratory quotient which became evident after sectioning and which decreased only after long periods of washing.

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