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THE OXIDATION OF MALATE BY MITOCHONDRIA FROM NORMAL AND ABNORMAL TOMATO FRUIT

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Abstract—An improved method for the isolation of mitochondria from the outer walls of tomato fruit during maturation and ripening has been developed and the oxidation of malate by the particles so obtained has been studied. When particles from fruit showing some external red colour were tested, especially in the presence of thiamine pyrophosphate or sodium glutamate (plus pyridoxal phosphate), either of which increased the rate of malate oxidation considerably, a short period of inhibited oxidation interposed between the ADP-stimulated rate (State 3) and the true ADP-limited rate (State 4) was found. Mitochondria from both the green and the red areas of non-uniformly ripened ("blotchy") fruit behaved similarly. In any set of conditions the extent of the inhibition was proportional to the amount of ADP available to the mitochondria. It is suggested that the inhibition is due to an accumulation of oxaloacetate and that the addition of any substance capable of lowering its concentration increases the rate of malate oxidation.

INTRODUCTION

SEVERAL techniques are available for obtaining mitochondria from tomato fruit so that the particles show a high degree of coupling between oxidation and phosphorylation and also display the phenomenon of respiratory control.[1-4] Respiratory control is defined as the ratio of the rate of oxidation of a substrate such as succinate or malate when adenosine diphosphate (ADP) is present in non-limiting amounts (State 3 according to Chance and Williams)[5] to that when supplies of the phosphate acceptor are exhausted (State 4). In an attempt to improve further the integrity of the particles, modifications to a previous method[3] have been introduced which result in higher rates of substrate oxidation/mg of mitochondrial nitrogen.

During an investigation of the metabolism of malate by mitochondria from ripening tomato fruit using a Clark oxygen electrode, it was noticed that there was often a short period of a slow oxidation rate immediately following the termination of the State 3 rate and prior to the assumption of a true State 4; this will be referred to as the "inhibited State 4" rate. Some factors affecting the extent of the inhibition during malate oxidation by particles from evenly ripening tomatoes have been studied and the investigation then widened to include mitochondria from fruit that do not ripen uniformly ("blotchy" ripened).[6,7]

RESULTS

The relative rates of oxidation of malate by mitochondria drawn from tomato fruit at selected stages during maturation and ripening have already been published.[3] The use of modified preparatory methods given below led to substantially similar results which are

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[1] D. B. DICKINSON and J. B. HANSON, *Plant Physiol.* **40**, 161 (1965).

[2] H. S. KU, H. K. PRATT, A. R. SPURR and W. M. HARRIS, *Plant Physiol.* **43**, 883 (1968).

[3] G. E. HOBSON, *Qualitas Plant Mater. Vegetabiles* **19**, 155 (1969).

[4] S. A. GARRISON, Ph.D. Thesis, University of Illinois. [*Diss. Abstr.* **28B**, 4868] (1967).

[5] B. CHANCE and G. R. WILLIAMS, *J. Biol. Chem.* **217**, 383 (1955).

[6] W. F. BEWLEY and H. L. WHITE, *Ann. Appl. Biol.* **13**, 323 (1926).

[7] A. J. COOPER, *Rep. Glasshouse Crops Res. Inst.* 1956, p. 39 (1957).

summarized in Fig. 1. The curves indicate that maximum efficiency (in terms of ADP to oxygen uptake ratios) and integrity (measured by the respiratory control ratios) coincide with the period between the mature green and the orange-green stage when the climacteric peak in respiration seems most likely to occur (see Winsor *et al.*).[8] The addition of thiamine pyrophosphate (TPP) invariably stimulated the rate of malate oxidation; this cofactor had little effect on the respiratory control ratios but it did depress the ADP to oxygen ratios. As previously explained,[3] the different method by which the latter figures were obtained may account for this.

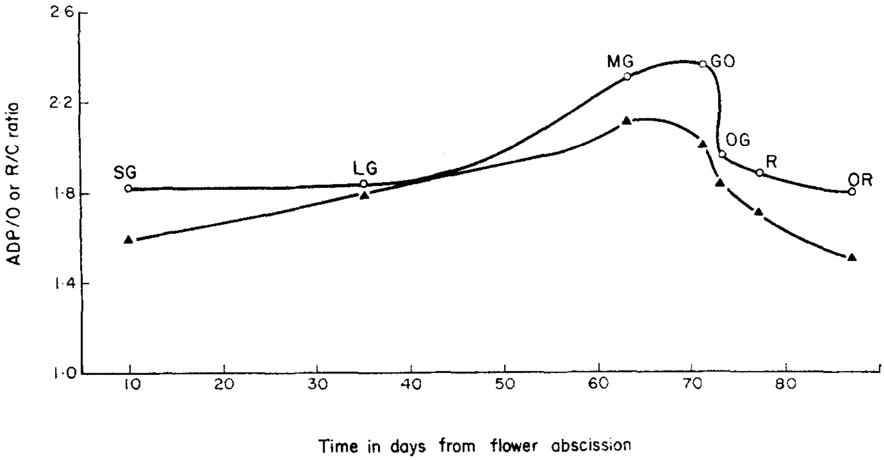

FIG. 1. VARIATION IN THE ADP TO OXYGEN UPTAKE AND RESPIRATORY CONTROL RATIOS DURING THE OXIDATION OF MALATE BY MITOCHONDRIA FROM TOMATO FRUIT AT VARIOUS STAGES OF DEVELOPMENT. ADP to oxygen uptake ratios (○—○); respiratory control ratios (▲—▲). SG—small green tomatoes; LG—large green; MG—mature green; GO—green-orange; OG—orange-green; R—red; OR—overripe.

Oxygen traces given by mitochondria extracted from small green fruit in the presence of

TABLE 1. RATES OF OXIDATION OF MALATE BY MITOCHONDRIA FROM GREEN-ORANGE TOMATOES IN THE PRESENCE OF VARIOUS COFACTORS

<table>
<tr><th rowspan="2">Cofactors added with malate</th><th colspan="2">Rate of oxidation [Q_{O_2} (N)]*</th></tr>
<tr><th>Initial</th><th>Final</th></tr>
<tr><td>None</td><td colspan="2">160†</td></tr>
<tr><td>Coenzyme A</td><td>149</td><td>188</td></tr>
<tr><td>NAD</td><td>218</td><td>247</td></tr>
<tr><td>Glutamate + pyridoxal phosphate</td><td colspan="2">257</td></tr>
<tr><td>TPP</td><td>246</td><td>289</td></tr>
<tr><td>All the above</td><td>363</td><td>417</td></tr>
</table>

* Rate after the initial addition of ADP. The other figures are computed from the rates following the second addition of ADP. Where only one figure is shown on a line there was no difference between the initial and the final rate.

† The rate of oxidation is expressed as μl O_2/h/mg mitochondrial nitrogen in the presence of non-limiting amounts of ADP (State 3).

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[8] G. W. WINSOR, J. N. DAVIES and D. M. MASSEY, *J. Sci. Food Agri.* **13**, 108 (1962).

serum albumin are shown in Fig. 2. A number of substances whose addition increases the rate of malate oxidation are listed in Table 1 in order of effectiveness. Since most of them take a short time to become fully effective (e.g. Fig. 2C), initial and final values for the State 3 oxidation rates are given in the table. In these circumstances both the ADP to oxygen and the respiratory control ratios improve with successive additions of ADP. Glutamate is an exception in these respects, showing linear rates of oxidation and constant ADP/O and respiratory control ratios (Fig. 2B).

A further point of interest was noted during a study of the rapid oxidation of malate which occurs with mitochondria from ripening fruit especially in the presence of TPP or glutamate. The transition from State 3 to the linear State 4 rate was separated by a short period of a rather slower rate, the "inhibited State 4" (broken line in Fig. 3A). The length of the plateau immediately following the termination of State 3 is, in general, proportional to the amount of ADP added, hence the first addition of ADP to give 100 μM shows no detectable inhibition in Fig. 3A but twice this concentration of the nucleotide does. The inhibited rate is of the same order as that of the basic respiration rate, i.e. the oxidation observed in the presence of substrate but prior to the addition of any ADP.

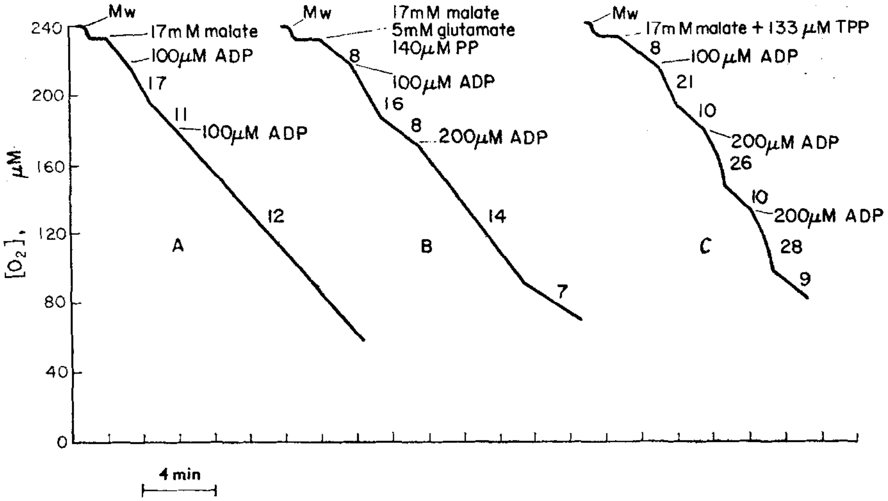

FIG. 2. POLAROGRAPHIC TRACES SHOWING THE OXIDATION OF MALATE BY MITOCHONDRIA FROM SMALL GREEN TOMATOES.

Trace A—no cofactor addition; trace B—glutamate and pyridoxal phosphate (PP) present; trace C—thiamine pyrophosphate (TPP) present. Mitochondrial nitrogen, expressed as μg N/ml, was 124 μg for trace A, 134 μg for trace B and 79 μg for trace C.

Particles were also extracted from the green and the red areas of blotchy ripened fruit. In the presence of TPP the rates of malate oxidation in States 3 and 4 were increased,[3] and the particles from both these tissues also displayed a temporary inhibition at the beginning of State 4 (Figs. 3B and 3C). It is, however, unusual for mitochondria from normally ripening fruit to show this phenomenon before the tomatoes reach the green-orange colour stage. It must be concluded that, in this respect at least, green blotchy tissue has reached a point in development equivalent to the green-orange stage of evenly ripening fruit.

The inclusion with TPP of a number of the other substances listed in Table 1 resulted in the progressive elimination of the "inhibited State 4". Thus TPP with Co-A or TPP with

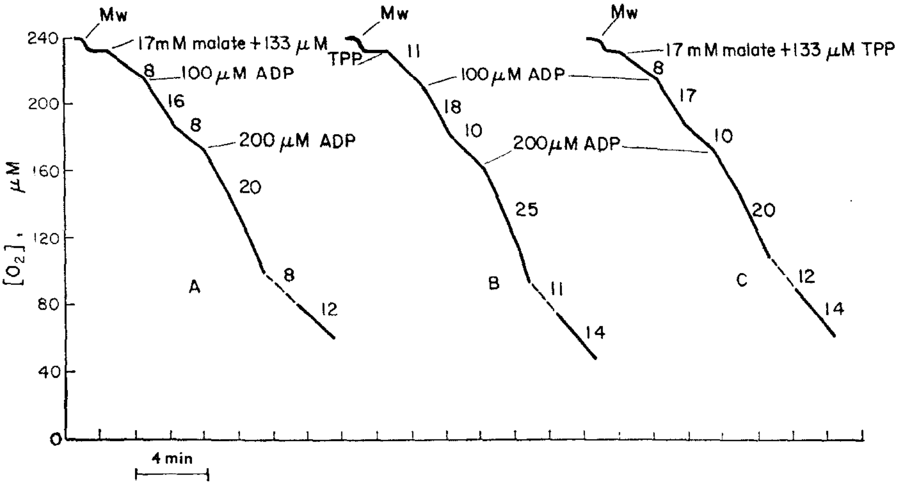

FIG. 3. INHIBITION OF THE STATE 4 RATE DURING MALATE OXIDATION.

Trace A illustrates an "inhibited State 4" rate (shown as a broken line) between a typical State 3 and a linear State 4 rate; the trace was given by mitochondria from orange-green fruit (110 µg N). Traces B and C show similar inhibitions which are given by mitochondria derived from the green (114 µg N) and the red (148 µg N) areas of blotchy fruit respectively.

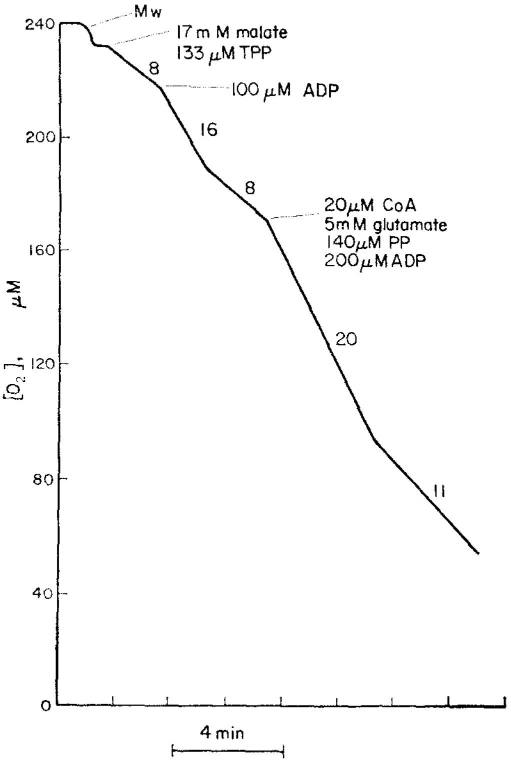

FIG. 4. RELIEF OF THE "INHIBITED STATE 4".

The State 4 inhibition given by evenly ripening orange-green fruit particles (Fig. 3A) is avoided by the addition of Co-A and a transamination substrate.

glutamate are able to overcome mild inhibitions. Particles that show signs of more complete inhibition at the beginning of State 4, illustrated in Fig. 3A, respond only to the inclusion of Co-A and glutamate with the TPP already present before a completely linear State 4 trace is produced (Fig. 4).

DISCUSSION

Serum albumin is now widely accepted as being a desirable constituent of media employed for the isolation of active mitochondria (see Stinson and Spencer),[9] although its function is not known with any certainty. It is thought to counteract the damaging effects of fatty acids arising from the mitochondria when Tris (2-amino-2-(hydroxymethyl)propane-1,3-diol) is used as the buffer. The substitution of *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid ("TES") (Good *et al.*)[10] for Tris has been found to diminish the advantages of adding albumin[3,10] but the present results indicate that its retention does aid the integrity of the particles. Thus the inclusion of albumin in the medium, reduction in the sucrose concentration from 0·4 to 0·3 M (see Baker *et al.*),[11] together with gentle agitation of the disintegrated fruit tissue to help extract undamaged particles, all appear to improve the quality and yield of mitochondria.

Tomato mitochondria are by no means unique in exhibiting an inhibition during malate oxidation.[12] Wiskich and Bonner[13] suggested that the progressive decrease in the rate of malate oxidation by sweet potato mitochondria was due to the accumulation of oxaloacetate. In further work Wiskich *et al.*[14] showed that ripening avocado fruit gave particles which displayed product inhibition during malate oxidation but this could be circumvented by the addition of a transamination agent such as glutamate. An analysis of malate oxidation throughout the respiratory cycle of the avocado[15] indicated that there was a dependence by the mitochondria on exogenous TPP prior to the climacteric rise, but the final stages of ripening were accompanied by a rising rate of malate oxidation during which product inhibition became increasingly obvious. These experiments led to the conclusion that oxaloacetate was closely involved in malate oxidation, and subsequent work[16] making use of ^{14}C-labelled malate provided further evidence that oxaloacetate was acting as an intermediary in the formation of pyruvate from malate. Pyruvate would then be available for decarboxylation to yield acetyl coenzyme A, which could then condense with a further molecule of oxaloacetate to form citrate. Alternatively, the scheme allowed for the rapid transamination of oxaloacetate by glutamate. These and other possible reactions of oxaloacetate have been brought together by Slater[17] and their relevance to fruit ripening in general were considered by Hulme and Rhodes.[18] Soybean mitochondria subjected to certain conditions probably also show an inhibited State 4 during malate oxidation.[19]

[9] R. A. Stinson and M. Spencer, *Can. J. Biochem.* **46**, 43 (1968).

[10] N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa and R. M. M. Singh, *Biochem.* **5**, 467 (1966).

[11] J. E. Baker, L. G. Elfvin, J. B. Biale and S. I. Honda, *Plant Physiol.* **43**, 2001 (1968).

[12] A. C. Hulme, M. J. C. Rhodes and L. S. C. Wooltorton, *J. Exptl. Botany* **18**, 277 (1967).

[13] J. T. Wiskich and W. D. Bonner, *Plant Physiol.* **38**, 594 (1963).

[14] J. T. Wiskich, R. E. Young and J. B. Biale, *Plant Physiol.* **39**, 312 (1964).

[15] C. Lance, G. E. Hobson, R. E. Young and J. B. Biale, *Plant Physiol.* **40**, 1116 (1965).

[16] C. Lance, G. E. Hobson, R. E. Young and J. B. Biale, *Plant Physiol.* **42**, 471 (1967).

[17] E. C. Slater, *Chem. Weekblad.* **52**, 1 (1962) quoted by Hulme and Rhodes.[18]

[18] A. C. Hulme and M. J. C. Rhodes, *Plant Cell Organelles* (edited by J. B. Pridham), p. 99, Academic Press, London (1968).

[19] T. J. Flowers and J. B. Hanson, *Plant Physiol.* **44**, 939 (1969).

With particles from tomato fruit the immediate action of TPP is to relieve a metabolic restraint so that malate is oxidizable at an increased rate.[3] The cofactor appears to be acting by the removal of an inhibitor of malate oxidation, very probably oxaloacetate. The direct addition of oxaloacetate to mitochondrial suspensions drastically reduces the rate of malate oxidation, confirming the experimental results of Drury *et al.*[20] with tomatoes and Hulme *et al.*[12] with apples. It is probable, however, that TPP is not directly involved in the decarboxylation of oxaloacetate to give pyruvate but is more likely to play a part in promoting the formation of acetyl coenzyme A, which in turn would react with a further molecule of oxaloacetate to produce citrate. Thus the indirect action of TPP on malate oxidation and the complex series of transformations on which it has an effect may explain the time lag before the cofactor exerts its full influence (see Figs. 2C and 3). The rate of penetration does not seem to be the limiting factor, for if the mitochondria are exposed to the usual concentration of TPP for 1 hr before their activity is tested in the oxygen cell they still show a rising rate of respiration.

Glutamic acid, presumably in the partially neutralized form, represents the major amino acid throughout the development of the tomato fruit,[21,22] and its concentration rises dramatically during ripening. Active glutamate-oxaloacetate transaminases are also present in mitochondria derived from tomatoes at any stage of maturation or ripening (unpublished work with R. T. Besford; see also Yu *et al.*);[23] hence this constitutes a potential system for control over the build-up of oxaloacetate as suggested for the apple by Hulme *et al.*[24] In the absence of some such mechanism, oxaloacetate accumulation in isolated mitochondria rapidly affects the activity of malate dehydrogenase by product inhibition.[25] Although it has been shown that adenosine triphosphate (ATP) accelerates the removal of oxaloacetate during the oxidation of succinate or malate by apple mitochondria (Hulme *et al.*),[12] the nucleotide is only effective against the inhibition of succinate oxidation in the case of tomato particles (Drury *et al.*).[20] This lack of effect of ATP on malate oxidation is in accord with the observation that the greater the amount of ADP given to the mitochondria the more marked is the initial inhibition in State 4 (Fig. 3A).

The work of Drury *et al.*[20] indicated that a progressive inhibition of malate oxidation by mitochondria from mature green tomatoes was prevented only by the addition of pyruvate, TPP, coenzyme A and NAD. The mitochondria used in the work presented here did not show inhibition until the green-orange stage of ripeness. In general the severity of the inhibition is related to the rate at which malate is oxidized. The response to additional TPP during malate oxidation by avocado mitochondria declined towards senescence[15] but persisted with tomato particles. Hence avocado particles, unlike those of the tomato, seem to become more self-sufficient as far as cofactor requirements are concerned towards the climacteric rise. Perhaps because avocado mitochondria from postclimacteric fruit appear to be better preserved than those from tomatoes at a similar stage, the addition of TPP and glutamate is that much more effective in avoiding inhibition in State 4.

The oxidative behaviour of particles from the green and the red parts of blotchy fruit appear to be very similar to those derived from evenly ripening fruit of the appropriate colour. It must be stressed that a number of other properties of the blotchy areas preclude them from

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[20] R. E. DRURY, J. P. MCCOLLUM and S. A. GARRISON, *Plant Physiol.* **43**, 248 (1968).
[21] M.-H. YU, L. E. OLSON and D. K. SALUNKHE, *Phytochem.* **6**, 1457 (1967).
[22] J. N. DAVIES, *J. Sci. Food Agri.* **17**, 396 (1966) and private communication.
[23] M.-H. YU, L. E. OLSON and D. K. SALUNKHE, *Phytochem.* **7**, 555 (1968).
[24] A. C. HULME, M. J. C. RHODES and L. S. C. WOOLTORTON, *Phytochem.* **6**, 1343 (1967).
[25] E. KUN, *The Enzymes* (edited by P. D. BOYER, H. LARDY and K. MYRBACK), Vol. 7, p. 149, Academic Press, New York (1963).

being considered as normal mature green fruit tissue or, indeed, as tissue from evenly ripening fruit at any stage of development.[4,26,27] Hence it is not merely that blotchy areas have been isolated from the mainstream of fruit development for one reason or the other, although the present results from mitochondrial studies might appear to support this viewpoint.

EXPERIMENTAL

Glasshouse-grown, freshly picked fruit of the "greenback-free" form of the variety "Potentate", named "Potella", were used throughout this work.

Mitochondria were prepared by the use of a previously published method[3] modified in detail and given here in summary. Outer locular wall tissue from tomatoes at selected developmental stages[28] was disintegrated in 240 ml of an isolation medium containing 0·3 M sucrose, 0·5 mM dithiothreitol, 5 mM ethylenediamine tetra-acetic acid (EDTA), pH 7·4, 10 mM potassium phosphate buffer, pH 7·4, 0·025 M TES, pH 7·6, 1 mM $MgCl_2$ and "fatty acid-poor" bovine serum albumin (Calbiochem) at the rate of 0·5 mg/ml. A stainless-steel grater with 3-mm holes was submerged in the medium, and washed tomato wall tissue grated so that the final ratio of vol. of macerate to wt. of tissue was 5:1 for small fruit, 4:1 for ripening fruit and 3:1 for ripe to over-ripe fruit. The apparent pH at 0–2° was kept close to 7·5 by the addition of 1 N KOH. The tissue suspension was gently stirred in the cold for about 45 min to help extract the particles from the cell debris, then strained through several thicknesses of a close mesh nylon cloth before centrifugation at 1000 *g* for 10 min. The supernatant liquid was poured through fine nylon net to remove floating material and centrifuged again at 12,000 *g* for 20 min. Following resuspension of the pellet in a loose-fitting Potter homogenizer, the particles were washed with 300 ml of a solution containing 0·3 M sucrose, 0·008 M TES, pH 7·6, 10 mM potassium phosphate buffer pH 7·4, 1 mM $MgCl_2$, 1 mM EDTA and albumin at the same rate as in the isolation medium, the precipitate brought down by centrifugation at 1000 *g* being rejected.

The oxidative activity of the mitochondria was measured polarographically as described by Wiskich *et al.*[14] except that albumin at the rate of 1 mg/ml was added to the medium used in the oxygen cell.

The ADP solution used as phosphate acceptor was standardized enzymatically by the coupled reaction of pyruvate kinase and lactic dehydrogenase (Boehringer Ltd.) according to the method of Bücher and Pfleiderer.[29]

Acknowledgements—The author thanks Mr. R. T. Besford for carrying out the transamination studies and ADP assays, and Mrs. J. Finney for technical assistance.

[26] G. W. Winsor, J. N. Davies and D. M. Massey, *J. Sci. Food Agri.* **13**, 145 (1962).

[27] J. N. Davies, *J. Sci. Food Agri.* **17**, 400 (1966).

[28] G. E. Hobson, *Rep. Glasshouse Crops Res. Inst.* 1958, p. 66 (1959).

[29] T. Bücher and G. Pfleiderer, *Methods in Enzymology* (edited by S. P. Colowick and N. O. Kaplan), Vol. 1, p. 435, Academic Press, New York (1955).