THE CLIMACTERIC RISE IN FRUIT RESPIRATION AS CONTROLLED BY PHOSPHORYLATIVE COUPLING

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Introduction

The ripening of fruit is accompanied by a group of physiological and chemical changes which are characteristic and moderately uniform as between different species of plants. Typical components of the ripening process are pectic transformations which lead to fruit softening, changes in fruit color, often with the disappearance of pigment such as chlorophyll and the appearance of new secondary pigments, changes in compounds responsible for taste or odor, transformation of reserve materials such as polysaccharides to simpler sugars, and finally, changes in the magnitude of the respiratory gas exchange. The respiration of many fruits after removal from the plant follows the general course shown for the avocado in figure 1 (**1**). Respiration drops slowly to a low point known as the preclimacteric minimum. At this point the fruit is still unripe. With the initiation of fruit ripening, rate of respiration rises from the preclimacteric minimum to the climacteric maximum. Edible maturity of the fruit coincides in many cases with the climacteric maximum in respiration, or follows this maximum by a few hours or days. Subsequent to the climacteric the respiratory rate drops steadily as the fruit passes into senescence.

We have tried to get some insight into the process of fruit ripening by study of the factors which underlie the changing rate of respiration during the climacteric cycle. The sudden rise in respiration from the preclimacteric minimum to the climacteric maximum should become understandable if one could discover the factor or factors which limit respiratory rate at the preclimacteric minimum. We have therefore studied the factors which limit respiratory rate, and in particular, the factors which might by alteration or increase bring about increases in rate of respiration of preclimacteric fruit.

Materials and methods

The study reported here has been concerned with avocado fruits of the variety Fuerte. The avocado was selected for the purpose because of the rapidity and uniformity of its climacteric cycle. These fruits remain in the preclimacteric state so long as they are attached to the tree. On removal from the tree they show a slowly decreasing rate of respiration, a decrease

which continues for approximately three days at 15° C. At this time the preclimacteric minimum is reached. Respiration then increases rapidly in rate for a further five days at which time the climacteric maximum is obtained. It is of importance for such experiments that fruits of known climacteric status be used. The exact position of each individual fruit on the climacteric curve (fig. 1) was therefore determined by following the rate of respiration of individual fruits, using a continuous flow method with the Beckman oxygen analyzer (**11**). In this way fruits within a few hours of the preclimacteric minimum or the climacteric maximum could be selected for use in initial experiments. For many experiments, however, fruits were held in the preclimacteric condition simply by placing the freshly picked fruit at 5 to 8° C and using them within a few days. Similarly, fruit in the climacteric condition were obtained by allowing ripening to take place at 25° C for five to six days.

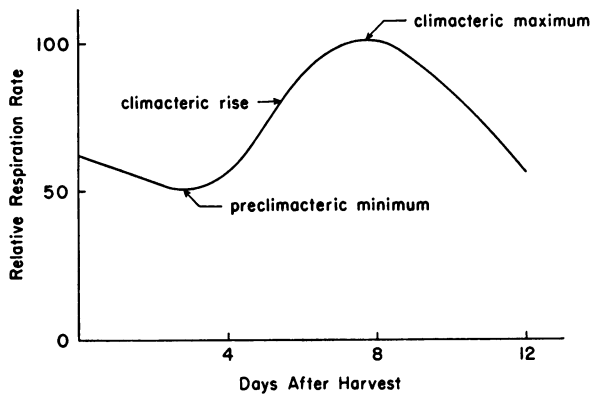

FIG. 1. Respiratory characteristics of the avocado.

Experiments with excised tissue were done with disks 1 cm. in diameter and 0.5 to 0.75 mm. thick. For this purpose a cork borer was used to remove a cylinder of fruit tissue which was then sliced with a hand microtome.

The isolation of particles capable of carrying on active respiratory metabolism was done by techniques similar to those used by LATIES (**4**) with cauliflower and by MILLERD *et al.* (**7**) with mung bean. The fruit was peeled, grated with a kitchen grater, and ground with quartz sand in 0.5 M sucrose solution. Thirty grams of tissue were ground in 50 ml. of the grinding solution. After a preliminary centrifugation at 2,000 × g for five minutes, the supernatant was centrifuged for 15 min. at 10,000 to 16,000 × g. The supernatant, including the fat accumulated at the surface, was removed by suction and the residue resuspended in 20 ml. of 0.5 M sucrose. This suspension was again centrifuged at 10,000 to 16,000 × g, the supernatant removed and the residue taken up in 3.0 ml. of 0.5 M sucrose. These operations, which are summarized in figure 2, were all carried out at 0 to 4° C. The enzymatically active particles isolated by this procedure have been

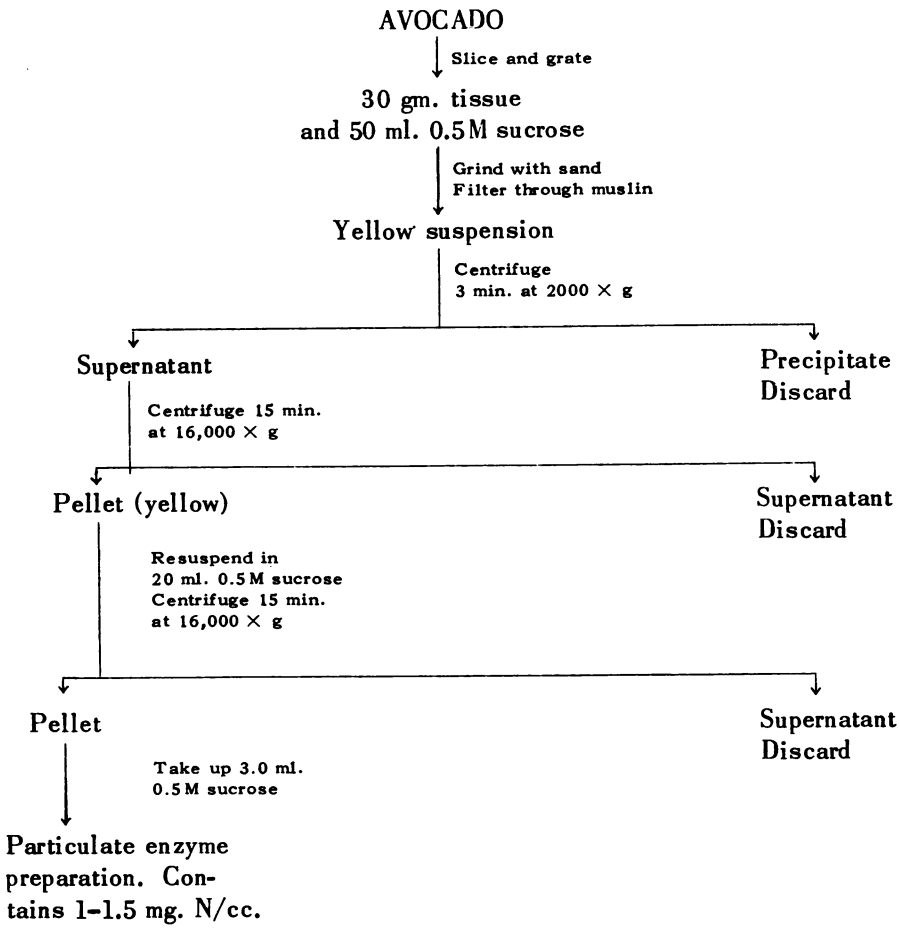

FIG. 2. Outline of procedures used in preparation of respiratory particles from avocado fruit.

shown to be similar to or identical with mitochondria (7) and will for convenience be termed mitochondria below, although no critical evidence is yet available on this point for the avocado.

Measurements of oxygen uptake of slices were measured by standard manometric procedures and at 25° C while measurements of enzymatic activity were made at 20° C.

Results

MITOCHONDRIAL OXIDATION OF PRECLIMACTERIC AND CLIMACTERIC FRUIT

The respiratory machinery of the avocado, as of other plant tissues which have been studied in detail, appears to be mediated by a particulate enzyme complex, a complex located in the mitochondria and competent to oxidize the acids of the Krebs cycle, including pyruvate. It is possible to prepare isolated mitochondria possessed of respiratory activity both from

TABLE I

OXIDATION OF ACIDS OF THE KREBS CYCLE BY CYTOPLASMIC PARTICLES OF PRECLIMACTERIC AVOCADO FRUIT. THE COMPLETE SYSTEM CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE, 0.01 M, PHOSPHATE BUFFER, PH 7.1, 0.01 M, ADENOSINE-5-PHOSPHATE (AMP) 10^{-3} M AND $MgSO_4$ 10^{-3} M.

Substrate	Concentration	O_2 consumption
		mm.³ O_2/30 min./mg. N
Citrate	2×10^{-2} M	152
α-Ketoglutarate	2×10^{-2} M	125
Succinate	2×10^{-2} M	169
l-Malate	2×10^{-2} M	47
l-Malate	5×10^{-4} M	10
Pyruvate *	2×10^{-2} M	45

* Also contains l-Malate 5×10^{-4} M.

preclimacteric and climacteric avocado fruit. The data in table I show that respiratory particles prepared from the avocado are indeed able to oxidize the Krebs cycle acids at rates which are comparable to those obtained with respiratory particles of other plants. The respiratory particles or mitochondria prepared from preclimacteric fruit, are fully as active as those prepared from climacteric fruits. The data of table II suggest, in fact, that per unit of mitochondrial nitrogen, the mitochondria of preclimacteric fruits are even more active under the conditions of the experiments of table II than are the mitochondria of climacteric fruits. There is no reason to suppose that limitation in the amount of enzyme system itself is responsible for the low respiratory rate of preclimacteric fruits.

With all of the plant mitochondria, as well as with the animal mitochondria studied to date, oxidation of substrate is linked to the uptake of

TABLE II

OXIDATIVE AND PHOSPHORYLATIVE PROPERTIES OF THE MITOCHONDRIAL SYSTEM OF AVOCADO FRUITS. THE COMPLETE SYSTEM CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE 0.01 M, PHOSPHATE BUFFER PH 7.1 0.01 M, AMP 10^{-3} M, $MgSO_4$ 10^{-3} M, NaF 0.01 M AND α-KETOGLUTARATE 0.02 M. TOTAL VOLUME 1.5 ML. ENZYME N/ML., PRECLIMACTERIC: 0.64 MG./ML., CLIMACTERIC: 0.76 MG./ML. REACTION MIXTURE. TEMPERATURE 20°C. ONE MILLILITER OF REACTION MIXTURE CONTAINS ENZYME FROM 3.3 GM. FRESH WEIGHT OF FRUIT. ALL CHEMICAL TRANSFORMATIONS ARE EXPRESSED IN TERMS OF MICROATOMS PER ML. OF REACTION MIXTURE.

Source of tissue	System	Uptake of phosphorus	Uptake of oxygen	Ratio of phosphorus to oxygen
		μ atoms/ml./30 min.		
Preclimacteric fruit	Complete	1.46	2.17	0.68
	No adenylate	0.12	0.37	0.32
Climacteric fruit	Complete	1.09	1.08	0.99
	No adenylate	0.13	0.40	0.33

inorganic phosphate and incorporation of this material into adenosine triphosphate (ATP) (**3, 6**). Rate of mitochondrial oxidation is therefore limited by availability of phosphate acceptor. The data of table II show not only that mitochondria from both preclimacteric and climacteric avocados possess the ability to carry on such coupled phosphorylation, but show also that the rate of oxidation by such mitochondria depends upon the presence of a phosphate acceptor such as adenylate. In the absence of suitable phosphate acceptor, the oxidative rate of avocado mitochondria is from one half to one fifth that found in the complete system. The experimental system for the demonstration of coupled phosphorylation with the respiratory particles of avocado (table II) is similar to that which has been used with other plant material (**3**). Inorganic phosphate is transformed in the course of coupled phosphorylation into the phosphate of ATP. This ATP is subject to degradation by adenosine triphosphatase which is present in avocado

TABLE III

EFFECT OF DNP ON THE RATE OF α-KETOGLUTARATE OXIDATION BY MITOCHONDRIA OF PRECLIMACTERIC AVOCADO. RATES ARE BASED ON THE FIRST 10 MINUTES. THE REACTION MIXTURE CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE 0.01 M, PHOSPHATE BUFFER PH 7.1 0.01 M, $MgSO_4$ 10^{-3} M, NaF 0.01 M AND α-KETOGLUTARATE 0.02 M. TOTAL VOLUME 1.5 ML. ENZYME N 1.28 MG./ML. REACTION MIXTURE. TEMPERATURE 20°C.

Adenylate concentration	DNP concentration	Oxygen uptake	Increase by DNP
		mm.³/ml./30 min.	%
None	None	38	....
	10^{-5} M	59	55
10^{-4} M	None	78	....
	10^{-5} M	92	18
10^{-3} M	None	86	....
	10^{-5} M	92	7

as in other mitochondria (**3, 7**). The action of this enzyme is however minimized by the fluoride (0.01 M NaF) present in the reaction mixture. The ATP is also subject to attack by hexokinase which is present in avocado as in other plant mitochondria (**7, 9**) and which transfers the terminal phosphate to glucose (0.01 M in the reaction mixture) to form the stable glucose-6-phosphate. Thus as oxidative phosphorylation proceeds in this system, inorganic phosphate is transformed to glucose-6-phosphate. The extent of oxidative phosphorylation is measured simply by estimation of the residual inorganic phosphate of the reaction mixture.

It is known from work of recent years that the coupling of mitochondrial oxidations to oxidative phosphorylation can be severed by certain reagents, including 2,4-dinitrophenol (DNP) (**3, 5, 10**). By decreasing or abolishing the requirement for exogenous adenylate, DNP frees the mitochondria from dependence on this material and permits rapid mitochondrial oxidation to occur even in the presence of low adenylate concentrations. Table III

shows that dinitrophenol greatly increases the rate of oxidation of avocado mitochondria in the absence of adenylate. With these mitochondria, as with those of other tissues, dinitrophenol also decreases or abolishes the oxidative incorporation of phosphate into organic form (fig. 3). The effects of DNP with mitochondria of avocado as with other mitochondria are therefore two-fold and consist both in emancipation of the respiratory oxidation from the adenylate requirement and in suppression of the normal energy transfer from respiration through ATP.

Factors limiting respiratory rate in tissue slices

Dinitrophenol is not only effective *in vitro* but also may be used *in vivo* as a reagent for assessing the extent to which respiratory rate of a tissue is limited by the phosphorylative system (**2, 8**). In tissues which are limited

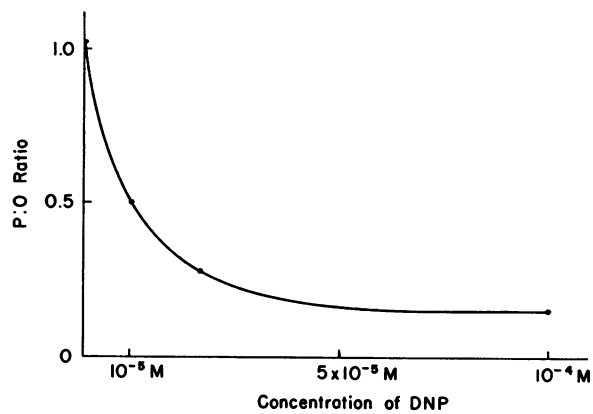

Fig. 3. Effect of dinitrophenol (DNP) on phosphorylative efficiency of α-ketoglutarate oxidation by avocado mitochondria. The complete system contains enzyme, sucrose 0.3 M, dextrose 0.01 M, phosphate buffer pH 7.1 0.01 M, AMP 10^{-3} M, $MgSO_4$ 10^{-3} M, NaF 0.01 M and α-ketoglutarate 0.02 M. Total volume 1.5 ml. Enzyme N 0.59 mg./ml. reaction mixture. Temperature, 20° C.

by the amount of adenylate, and hence, by capacity of the phosphorylative system, dinitrophenol brings about large increases in respiration. In tissues which are not limited by adenylate, but are limited by other matters, such as substrate, *etc.*, dinitrophenol is without such an effect on respiratory rate. The data of table IV show that dinitrophenol exerts a marked effect on the rate of respiration of avocado slices provided only that these slices are taken from preclimacteric fruit. With slices taken from climacteric or senescent fruit, on the contrary, dinitrophenol does not increase respiratory rate. It is noteworthy, however, that the climacteric rise in respiratory rate is not nearly as marked when measured with slices as it is with the intact fruit from which the slices are made. The slices respire more rapidly than an equal amount of tissue in the intact fruit, and the difference is more marked for the preclimacteric than for the climacteric fruit. The basis of

this behavior is still unknown. It does appear from this experiment, however, that the climacteric rise in respiration in the avocado is in some manner associated with changes in the phosphorylative coupling system. The phosphorylative coupling system appears to limit respiratory rate in tissue from the preclimacteric fruit but does not limit respiratory rate in tissues from fruit at the climacteric maximum.

Native uncoupling agents

Let us now suppose that the normal climacteric rise in respiration is indeed to be attributed to the removal of a limitation previously imposed by the capacity of the phosphorylative system. In what ways might removal of this limitation be achieved in the ripening fruit? One possibility

TABLE IV

EFFECT OF DNP ON THE RESPIRATION OF SLICES MADE FROM PRECLIMACTERIC AVOCADOS AND SLICES MADE FROM CLIMACTERIC AVOCADOS. TEMPERATURE 25°C.

Stage of fruit used as source of tissue	Number of measurements	O_2 uptake of whole fruit	O_2 uptake of slices				
			Control	DNP 10^{-5} M	Per cent. increase	DNP 10^{-4} M	Per cent. increase
		mm.³ O_2/gm./hr.	*mm.³/mg. dry wt./hr.*				
Fresh from tree	5	....	0.64	1.12	75	1.42	122
At climacteric minimum	7	25	0.68	1.11	63	1.36	100
On climacteric rise	1	72	0.73	0.90	23	0.85	16
At climacteric peak	6	93	0.60	0.59	−2	0.55	−8

is that during the ripening process, additional amounts of adenylate might appear within the fruit, or alternatively, that ripening involves the expenditure of energy and that utilization and turnover of adenylate is thus increased. A second general possibility would evidently involve the production during ripening of materials which, like DNP, would decrease the dependence of fruit respiration on the phosphorylative system. The implications of these two alternatives for our understanding of the process of fruit ripening are considerable. According to the first alternative, ripening should be regarded as a positive process involving the doing of some sort of biological work. The increased rate of respiration in ripening fruit would be a measure of the increased rate of utilization of ATP in the execution of this work. According to the second view, on the other hand, fruit ripening would consist of a generally degenerative process, and would be based on

TABLE V

EFFECT OF A SUBSTANCE OR SUBSTANCES FROM CLIMACTERIC AVOCADOS ON RATE OF MITOCHONDRIAL OXIDATION AND OXIDATIVE PHOSPHORYLATION. THE TEST SYSTEM IS MITOCHONDRIA OF MUNG BEAN. THE COMPLETE SYSTEM CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE 0.01 M, PHOSPHATE BUFFER PH 7.1 0.01 M, AMP 10^{-3} M, $MgSO_4$ 10^{-3} M, NaF 0.01 M AND α-KETOGLUTARATE 0.02 M. TOTAL VOLUME 1.5 ML. ENZYME N 0.32 MG./ML. REACTION MIXTURE. TEMPERATURE 20°C. SUPERNATANT WHERE USED WAS ADDED AT THE RATE OF 1 ML. PER 3 ML. OF TOTAL REACTION MIXTURE.

System	Added supernatant from	Uptake of phosphorus	Uptake of oxygen	Ratio of phosphorus to oxygen
		μ atoms/ml./30 min.		
Complete	None	1.16	1.23	0.94
No adenylate	None	0.11	0.35	0.31
No adenylate	Climacteric fruit	0.06	1.25	0.05
No adenylate	Preclimacteric fruit	0.04	0.30	0.13

the dissociation of respiration from phosphorylation and energy transfer. According to this view fruit ripening would result from diminution of the ATP supply to a level inadequate to maintain the metabolic level characteristic of the unripe fruit. The evidence to be presented below suggests that the latter alternative is the more plausible and that fruit ripening, in the avocado at least, may perhaps be regarded as an uncoupling process.

That substances which are capable of replacing or supplanting adenylate in increasing rate of mitochondrial oxidation are produced in avocado fruit during ripening is shown in the data of table V. These substances are found in the supernatant which remains after the removal of the mitochondria by centrifugation at 16,000 × gravity. Their effects may be demonstrated with

TABLE VI

PROPERTIES OF SUBSTANCES IN CLIMACTERIC AVOCADO SUPERNATANT ON OXIDATION AND PHOSPHORYLATION BY MUNG BEAN MITOCHONDRIA. THE COMPLETE SYSTEM CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE 0.01 M, PHOSPHATE BUFFER 0.01 M, AMP 10^{-3} M, $MgSO_4$ 10^{-3} M, NaF 0.01 M AND α-KETOGLUTARATE 0.02 M. TOTAL VOLUME 1.5 ML. ENZYME N 0.32 MG./ML. REACTION MIXTURE. TEMPERATURE 20°C.

System	Supernatant	Uptake of phosphorus	Uptake of oxygen	Ratio of phosphorus to oxygen
		μ atoms/ml./30 min.		
Complete	None	1.16	1.23	0.94
No adenylate	None	0.11	0.35	0.31
No adenylate	Climacteric fruit	0.06	1.25	0.05
No adenylate	Same heated	0.48	0.76	0.63
No adenylate *	Same dialyzed	0.22	1.34	0.16

* Data from comparable but separate experiment.

mitochondria of either preclimacteric or climacteric fruit or alternatively with unrelated mitochondria. The data in tables V and VI refer to the effects of avocado supernatants on the mitochondrial system of mung bean, a system which has been extensively studied and standardized (**3, 6, 7**). It is clear that the supernatant of the climacteric fruit causes a greatly increased oxygen uptake in the adenylate deficient mitochondrial system. The supernatant can evoke maximum oxidative rate from mung bean mitochondria even in the absence of exogenous adenylate. Phosphorylation is at the same time essentially totally suppressed. These effects are not exerted or are exerted to only a slight degree by the supernatant from preclimacteric fruits. Results entirely similar to those of table V were obtained in experiments in which supernatants of preclimacteric and climacteric avocados were supplied to avocado rather than mung bean mitochondria.

The data of table V clearly suggest that mature avocado fruits contain a principle or principles which like DNP act to decrease the degree of the dependence of respiratory oxidation on coupled phosphorylation. Further information on the nature of this principle is contained in the data of table VI. The striking effect of the supernatant of ripe avocados on the rate of mitochondrial oxidation appears to be due to at least two materials. The first is heat stable and acts qualitatively like adenylate itself. This factor alone causes a modest increase in oxygen uptake coupled with an appreciable phosphorylation. It is to noted that this factor is found in preclimacteric as well as in climacteric fruit. The second factor is heat labile and nondialyzable and may well be a protein. To this factor may be attributed the bulk of the supernatant effect. An attractive although hypothetical possibility is that the uncoupling may be owing to a soluble ATPase, since ATPase activity like the uncoupling activity develops in the avocado during the course of ripening.

Discussion

The evidence presented above suggests that respiratory rate in the unripe avocado is limited by the capacity of the phosphorylative system; by availability of acceptor for organic phosphate produced during oxidative phosphorylation. As the respiratory rate of the fruit rises from the preclimacteric minimum to the climacteric maximum, this limitation is gradually removed. A preliminary investigation of the factors involved has shown that the ripe, but not the unripe, fruit contains soluble materials which possess the ability to uncouple respiratory oxidations from their associated phosphorylative systems. These facts taken together suggest that ripening in the avocado may be in fact an uncoupling process and that the varied chemical changes which accompany ripening may result from this uncoupling. It will be evident that a critical test of this hypothesis may be based on determination of whether or not known uncoupling agents such as DNP are themselves able to induce the chemical changes characteristic of ripening.

Summary

This work concerns the climacteric rise in respiration of ripening fruit. The characteristics of the respiration of preclimacteric fruit have been contrasted with those of climacteric fruit. The respiration of the avocado fruit appears to be mediated by an organized respiratory complex. The active respiratory particles have been isolated from both preclimacteric and climacteric fruit.

The oxidation of substrate by the respiratory particles of avocado is coupled to the oxidative production of ATP. Rate of substrate oxidation by the respiratory particles is dependent upon the concentration of exogenous phosphate acceptor; adenylate. Although rate of substrate oxidation is normally limited by the capacity of the phosphate accepting system, this limitation can be removed by agents such as DNP which sever the coupling of oxidation to phosphorylation.

With the aid of the uncoupling agent, DNP, it has been shown that the rate of respiration of the preclimacteric but not of the climacteric fruit is limited by the capacity of the phosphate transfer system. The fact that ripening avocado fruits contain a substance which like DNP acts as an uncoupling agent suggests that both the climacteric rise in respiration and the chemical changes of fruit ripening may be owing to uncoupling: a decrease in synthesis or availability of ATP within the fruit.

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