Phytochemistry, 1966, Vol. 5, pp. 635 to 642. Pergamon Press Ltd. Printed in England

THE EFFECTS OF GROWTH RETARDANTS ON THE RESPIRATION AND COUPLED PHOSPHORYLATION OF PREPARATIONS FROM ETIOLATED PEA SEEDLINGS

D. A. HEATHERBELL, B. H. HOWARD and A. J. WICKEN

Department of Biochemistry, Lincoln College, University of Canterbury, Canterbury, New Zealand

(*Received* 29 *September* 1965)

Abstract—The respiration of sections of roots of etiolated pea seedlings was enhanced in the presence of the growth retardant 2-chloroethyltrimethylammonium chloride (chlorocholine chloride) in concentrations between 10^{-2} M and 5×10^{-4} M. *N*-Dimethylaminosuccinamic acid, when tested over the same range, was stimulatory in the more dilute solutions but inhibitory at the highest concentration. Mitochondria were prepared from the stem and root of the etiolated pea seedlings. The highest P:O ratios (1·0–1·3) were obtained from stem mitochondria, when the mitochondria were extracted in the presence of polyvinylpyrrolidone, and when the incubation mixture contained hexokinase, glucose, cytochrome c, NAD and succinate as substrate. Fluoride inhibited phosphorylation slightly. When the mitochondria were incubated in the presence of chlorocholine chloride, the esterification of phosphorus and the P:O ratio were progressively depressed as the concentration of growth retardant increased from 10^{-8} M. Phosphorylation was abolished at concentrations above 10^{-2} M. Oxygen consumption was not affected by concentrations of chlorocholine chloride below 10^{-3} M, and was not abolished at the highest concentration tested, $6{\cdot}6 \times 10^{-2}$ M. Under the same conditions, *N*-dimethylaminosuccinamic acid depressed both the esterification of phosphorus by the mitochondria and also the P:O ratio at concentrations from 10^{-6} M to $3{\cdot}3 \times 10^{-3}$ M, at which latter concentration esterification was abolished. Oxygen consumption was unaffected at a concentration of 10^{-4} M or below, and was depressed, though not abolished, at concentrations between 10^{-2} M and $3{\cdot}3 \times 10^{-3}$ M. In the range $8{\cdot}3 \times 10^{-4}$ M to $1{\cdot}6 \times 10^{-3}$ M the consumption of oxygen by the mitochondria was stimulated. It is suggested that the uncoupling of oxidative phosphorylation may explain the effects these substances have on whole plants.

INTRODUCTION

RECENT studies in the field of plant growth regulation have revealed the existence of a group of compounds with distinct biological properties. These compounds, known as growth retardants, have been defined[1] as "chemicals that slow cell division and cell elongation in shoot tissues and regulate plant height physiologically without formative effects". In their action on the plant they therefore differ from auxins, herbicides of the growth regulator type, and germination inhibitors. Despite detailed physiological observations the biochemical effects of these plant growth retardants remain unknown,[2] though it has been suggested that they may function as uncouplers of oxidative phosphorylation.[3] The work described below was undertaken to explore this possibility, using initially sections of etiolated pea stem and root, and later mitochondrial suspensions. Two growth retardants belonging to different chemical groups were chosen for study; these were 2-chloroethyltrimethylammonium

[1] H. M. CATHEY, *Ann. Rev. Plant Physiol.* **15**, 271 (1964).
[2] K. V. THIMANN, *Ann. Rev. Plant Physiol.* **14**, 1 (1963).
[3] G. STENLID and K. SADDIK, *Physiol. Plantarum* **15**, 369 (1962).

chloride (chlorocholine chloride or "CCC"), whose action on plants was first described by Tolbert,[4] and *N*-dimethylaminosuccinamic acid ("B995").[5]

RESULTS

In manometric experiments the consumption of oxygen by excised tissue sections was almost linear with respect to time during incubation for 4 hr at 25°. Endogenous consumption by root and stem was respectively 0·5 and 0·24 μl/mg fresh wt./hr, and by root in the presence of sodium succinate 0·66 μl/mg fresh wt./hr. Because the respiration of root tissue was the greater, and because it was not greatly increased by addition of succinate, the effect of growth retardants on tissue respiration was studied using root tissue without added substrate. The results of these experiments are shown in Fig. 1.

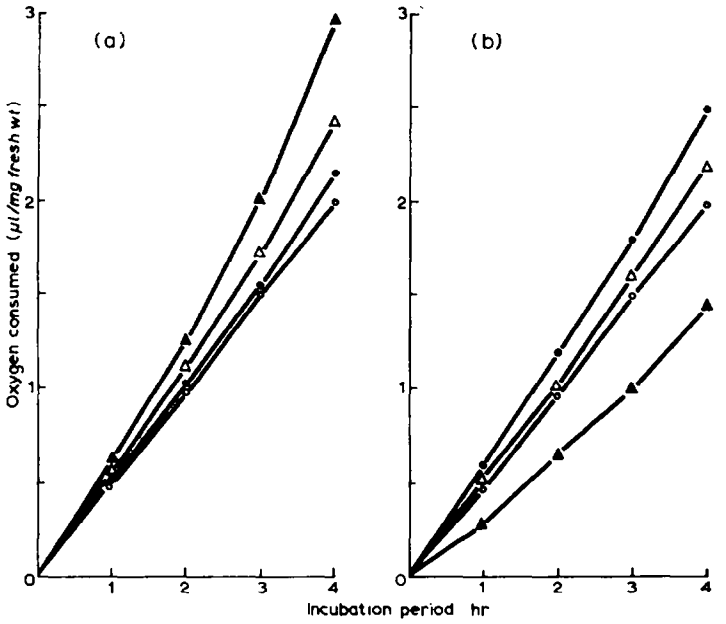

FIG. 1. THE EFFECT OF GROWTH RETARDANTS ON THE RESPIRATION OF ROOT TIPS OF ETIOLATED PEA SEEDLINGS, UNDER THE CONDITIONS DESCRIBED IN THE TEXT.

a—Chlorocholine chloride; *b*—*N*-Dimethylaminosuccinamic acid. Concentrations of growth retardants: ○, nil; ●, 5×10^{-4} M; △, 5×10^{-3} M; ▲, 10^{-2} M.

The respiration of root tissue was affected immediately upon addition of the growth retardants. There thus appears to be no barrier to the entry of the substances into the cells. All concentrations of chlorocholine chloride which were tested stimulated respiration (Fig. 1*a*). In the range studied, 0–10^{-2} M, the stimulation increased with increasing concentration, and at 10^{-2} M the respiration was approximately 150 per cent of that of the control. *N*-Dimethylaminosuccinamic acid was less strongly stimulatory, and was slightly inhibitory at the highest concentration (Fig. 1*b*).

The increase in respiration of the plant tissues caused by the growth retardants is the effect that would be expected if the substances were uncoupling oxidation from phosphoryla-

[4] N. E. TOLBERT, *J. Biol. Chem.* **235**, 475 (1960).
[5] J. A. RIDDELL, H. A. HAGEMAN, C. M. J'ANTHONY and W. L. HUBBARD, *Science* **136**, 391 (1962).

tion, and thus removing the controlling effect of the concentration of phosphate acceptor on the rate of the oxidative reaction.[6] To establish whether the increased respiration was accompanied by uncoupling it was necessary to use a mitochondrial system, because the intact tissue liberates inorganic phosphate from ATP at an appreciable rate. When mitochondria were prepared by the usual methods, those from the stem were more active in phosphorylation, and gave a higher P:O ratio, than those from the root (Table 1). Stem

TABLE 1. RESPIRATION AND PHOSPHORYLATION BY MITOCHONDRIA FROM ETIOLATED PEA SEEDLINGS

Source of mitochondria*	Incubation period (min)	(μg atoms/mg protein) P esterified	(μg atoms/mg protein) O_2 consumed	P:O ratio
Root	30	4·36	11·10	0·39
Stem	20	4·05	4·32	0·93
Stem	30	6·25	6·60	0·95
Stem	60	10·35	12·80	0·81

* The mitochondria were prepared without the addition of polyvinylpyrrolidone. The Warburg flasks contained succinate, cytochrome *c*, NAD, ATP, $MgCl_2$, KH_2PO_4, glucose, hexokinase and the mitochondrial suspension as described in the text.

mitochondria were therefore used in experiments with the growth retardants. Similar differences in mitochondria have been found by other workers for pea,[7] for mung bean,[8] and for cucumber.[3] It is clear from Table 1 that the lower P:O ratios found for root mitochondria are partly the result of the higher respiration rate of these preparations. In this respect the behaviour of the mitochondria from the two tissues corresponds with that of the tissue sections. When the incubation period was varied, 30 min gave the best results (Table 1). Although respiration was steady for an hour or more, the rate of esterification of phosphorus declined in the later stages, possibly because of the increasing rate at which phosphate was liberated from ATP as the preparation aged (Table 2).

TABLE 2. PRODUCTION OF INORGANIC PHOSPHATE FROM ADDED ATP BY MITOCHONDRIA FROM STEMS OF ETIOLATED PEA SEEDLINGS

Growth Retardant	Incubation period (min)* P_i (μmoles/flask) 0	15	30
None	0·32	0·80	1·65
Chlorocholine chloride	0·40	0·80	1·60
N-Dimethylaminosuccinamic acid	0·35	0·65	1·54

* Each flask contained cytochrome *c* (0·1 μmole), NAD (1 μmole), ATP 12 μmoles), $MgCl_2$ (5 μmoles), and mitochondrial suspension (0·4 ml) in 0·25 M sucrose, 0·1 M tris–HCl buffer, pH 7·5 (3 ml), containing growth retardant (10^{-3} M) where appropriate.

[6] H. BEEVERS, *Respiratory Metabolism in Plants*. Row, Peterson, Evanston, Ill. (1961).
[7] R. M. SMILLIE, *Australian J. Biol. Sci.* **8**, 186 (1955).
[8] J. BONNER and A. MILLERD, *Arch. Biochem. Biophys.* **42**, 135 (1953).

During experiments intended to discover the conditions in which the mitochondrial system would be most active, we made a number of observations which show the effects on oxidative phosphorylation both of the use of polyvinylpyrrolidone in preparing the mitochondria and of the presence or absence of co-factors in the final solution. These observations are summarized in Tables 1 and 3. The P:O ratio found for the mitochondria in the absence of

TABLE 3. THE EFFECT OF VARIOUS SUBSTANCES ON RESPIRATION AND PHOSPHORYLATION BY MITOCHONDRIA FROM STEMS OF ETIOLATED PEA SEEDLINGS*

Hexokinase (KM units)	NaF (μmoles)	Cytochrome *c* (μmoles)	NAD (μmoles)	(μg atoms/mg protein) P esterified	(μg atoms/mg protein) O_2 consumed	P:O ratio
80	0	0·1	1	6·81	6·01	1·13
80	3	0·1	1	4·52	7·24	0·63
80	30	0·1	1	3·81	4·46	0·86
0	30	0·1	1	3·80	5·70	0·67
0	0	0·1	1	1·31	6·60	0·20
80	0	0·1	0	4·03	5·50	0·73
80	0	0	1	2·05	4·10	0·50

* Except where mentioned in the Table, conditions were those described in the text. Polyvinylpyrrolidone was included in the medium in which the stems were ground. Each result is the mean of at least two determinations.

growth retardant varied slightly between batches, so that, for comparison between experiments, it was found convenient to express each P:O ratio as a proportion of that of the control in each experiment. The consumption of oxygen, esterification of phosphorus and P:O ratio, in the presence of different concentrations of the two growth retardants, are shown in Fig. 2 as proportions of the corresponding values found for the control suspensions. In these control suspensions, where no growth retardants were used, the oxygen consumed and phosphorus esterified were 4·4–6·6 and 4·5–7·2 μatoms/mg protein respectively in the 30 min experiments, and the P:O ratio was 1·0–1·3. P:O ratios were in general not affected by the use of polyvinylpyrrolidone in the extraction of the mitochondria, as would be expected in view of the low content of phenolic substances and phenolase in the material.

The effect of the growth retardants on the liberation of inorganic phosphate from ATP by the mitochondrial suspension is shown in Table 2. It will be seen that the substances were inactive in this respect.

In preliminary experiments in which the mitochondria were placed in the side-arm of the manometer cup, and all the other reagents were placed in the main chamber, a lag period of about 15 min followed mixing, during which respiration was low. When the mitochondria were mixed with substrate in the main chamber and incubated during the equilibration period, this lag was abolished.

Eisenhardt and Rosenthal[9] reported that an initial phase of rapid phosphorylation occupying less than 3 sec occurred in their experiments with rat-liver mitochondria. This "ATP jump" did not occur in our experiments. When two zero time flasks were used, and trichloroacetic acid was added to one before tipping, and to the other several seconds after tipping, no difference in inorganic phosphate content of the two flasks was subsequently found.

[9] R. H. EISENHARDT and O. ROSENTHAL, *Science* **143**, 476 (1964).

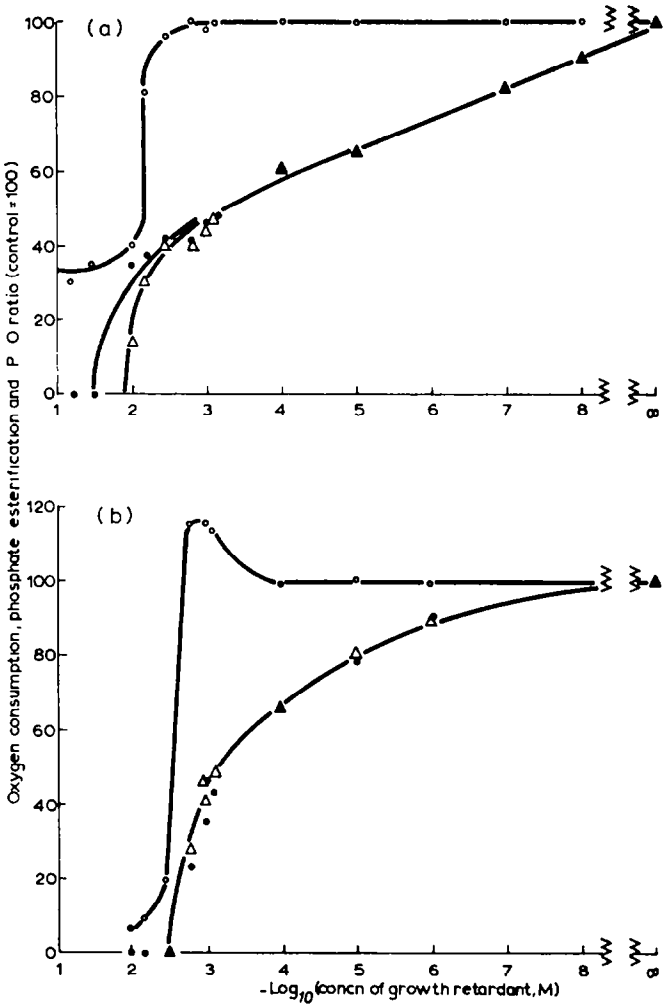

FIG. 2. THE EFFECTS OF GROWTH RETARDANTS ON THE OXIDATION OF SUCCINATE AND PHOSPHORYLATION BY MITOCHONDRIA FROM THE STEMS OF ETIOLATED PEA SEEDLINGS

Polyvinylpyrrolidone was included in the medium in which the stems were ground, and the Warburg flasks contained succinate, cytochrome *c*, NAD, ATP, $MgCl_2$, KH_2PO_4, glucose and hexokinase, as described in the text. *a*—Chlorocholine chloride; *b*—*N*-Dimethylaminosuccinamic acid. ○, Oxygen consumption; △ Phosphorus esterification; ●, P:O ratio.

In our experiments sodium fluoride, at concentrations of 10^{-2} and 10^{-3} M, slightly inhibited phosphorylation (Table 3). Switzer and Smith[10] observed the same effect on mitochondria of soybean seedlings. The liberation of inorganic phosphate from ATP by our mitochondrial preparation is considered to be of a low order, and this may account for the absence of any increase in the P:O ratio when fluoride was included in the system. The depression of phosphorylation may result from interference by the fluoride with reactions dependent on magnesium. Table 3 shows that the highest P:O ratios were obtained in these experiments when hexokinase, cytochrome *c* and NAD were all present.

[10] C. M. SWITZER and F. G. SMITH, *Can. J. Botany* **35**, 515 (1957).

The currently accepted value for the P:O ratio when succinate is oxidized to fumarate is 2·0. The highest value obtained in our experiments, 1·3, is appreciably below this, but it compares favourably with those achieved by several other workers with plant mitochondria.[3, 7, 8, 10]

The results illustrated in Fig. 2 show the similarity of the two growth retardants in their effects on the oxidative phosphorylation system of the pea stem mitochondria. Increasing concentrations up to almost 10^{-3} M have no effect on respiration, but increasingly depress phosphorus esterification. Chlorocholine chloride is the more active in this respect. Over a narrow range, $8{\cdot}3 \times 10^{-4}$ to $1{\cdot}6 \times 10^{-3}$ M, *N*-dimethylaminosuccinamic acid stimulated respiration by up to 15 per cent, but no such effect is observed in the case of chlorocholine chloride. Concentrations of the substances above 10^{-3} M inhibit respiration, and also severely depress phosphorus esterification. In neither case do the mitochondria cease to respire completely, unlike those used in experiments with 2,4-dichlorophenoxyacetic acid.[10, 11] The growth retardants resemble this substance, however, in that, at the highest concentrations used, phosphorus esterification not only ceases, but the concentration of inorganic phosphate in the medium increases during the experiment.[10, 11] An adenosinediphosphatase was found in cauliflower mitochondria[11]; such an enzyme, acting in the presence of high concentrations of growth retardant, may account for this increase in phosphate concentration. The enzyme in our preparation which releases inorganic phosphate from ATP is not stimulated by the growth retardants. "ATPase" in mitochondrial preparations appears to be affected variously by uncoupling agents; some of these compounds, for example dinitrophenol, are stimulatory,[12] and others, such as oligomycin, are inhibitory.[13]

We consider that the suppression of phosphorus esterification in our experiments, unaccompanied by any reduction of respiration, is good evidence that the effect observed is the uncoupling of oxidative phosphorylation. The increase in respiration observed in some experiments with *N*-dimethylaminosuccinamic acid lends particularly strong support to this conclusion.

The two growth retardants studied here, and especially chlorocholine chloride, are active over a very wide range of concentrations. Different effects may be exerted in different parts of this range. One effect is, of course, the suppression of phosphorus esterification without reduction in respiration, observed with low concentrations of the substances. A possibly different effect is the suppression of respiration at concentrations much higher than those which are sufficient to abolish phosphorus esterification. Some substances previously regarded as specific uncouplers of oxidative phosphorylation are now known to inhibit enzyme systems also,[14] and it is possible that the growth retardants are inhibiting respiratory enzymes in our experiments when used in the higher concentrations.

In attempting a comparison between the results of the experiments with tissue sections and with the mitochondria, it must be remembered that the former dealt mainly with root tissue and the latter mainly with stem. However, some comparisons can be made, and may be significant. In both systems *N*-dimethylaminosuccinamic acid stimulated respiration at a concentration of 10^{-3} M, and inhibited it at 10^{-2} M (Figs. 1*b* and 2*b*). This encourages us to believe that the effects observed in our *in vitro* mitochondrial system may be relevant to the activity of the growth retardants in the whole plant. Correlation between the effects of

[11] R. T. WEDDING and M. K. BLACK, *Plant Physiol.* **37**, 364 (1962).
[12] L. WADKINS and A. L. LEHNINGER, *Federation Proc.* **22**, 1092 (1963).
[13] E. RACKER and T. E. CONOVER, *Federation Proc.* **22**, 1088 (1963).
[14] D. E. GREEN, R. E. BEYER, M. HANSEN, A. L. SMITH and G. WEBSTER, *Federation Proc.* **22**, 1460 (1964).

chlorocholine chloride in the two systems is less good, in that all concentrations used on the tissue were stimulatory, but none were so for the mitochondrial suspensions, whose respiration was depressed or unaffected (Figs. 1*a* and 2*a*). It has been suggested that *in vitro* in the presence of hexokinase and glucose, the respiration rate may not always be susceptible of further increase by the action of uncoupling agents,[6] but that in the absence of these added reagents (for example, in our experiments with tissue sections), where respiration is dependent on the turnover of ATP, stimulation may be induced. This may account for the apparent discrepancy in the results of the experiments with chlorocholine chloride.

In their effects on whole plants the growth retardants antagonize the gibberellins and auxins, and so have been termed "anti-gibberellins"[15, 16] and "anti-auxins"[16, 17]. Cathey[1] has criticized these terms as assuming a biochemical role for growth retardants which has not yet been established. We suggest, in the light of our results, that these antagonistic effects may be secondary, and that the fundamental action of the substances may be the uncoupling of oxidative phosphorylation. This would be expected to reduce the amount of ATP available to the plant for cell division, cell elongation and gibberellin synthesis. The lack of toxicity of these substances may also be correlated with our findings since, despite the complete suppression of phosphorylation by the mitochondria in high concentrations of the growth retardants, the respiration was never completely abolished. This contrasts with the effects of herbicides on mitochondrial respiration, which is abolished when phosphorylation ceases.[3, 11]

EXPERIMENTAL

Pea seeds (*Pisum sativum* var. Lincoln) were soaked in running aerated tap water for 24 hr, surface sterilized by rinsing in a dilute solution of a domestic hypochlorite preparation (NaOCl approx. 0·15%), and washed with three rinses of water. The seeds were germinated in a moist, dark chamber at 24°. In all experiments 4- to 5-day-old seedlings were used. The first internodal region of the stem (hypocotyl) and the terminal portion of the root, 1·5–2 cm in each case, were taken.

The chlorocholine chloride, manufactured by Distillation Products Industries, Rochester, N.Y., U.S.A., was kindly given by Ivon Watkins, Dow Ltd., New Plymouth, New Zealand. The *N*-dimethylaminosuccinamic acid was kindly given by the manufacturer, Naugatuck Chemical Division, U.S. Rubber Co., Naugatuck, Conn., U.S.A. Stock solutions of these materials were adjusted by the addition of dil. NaOH to pH 5·3 for experiments with tissue sections or to pH 7·5 for experiments with mitochondria. Cytochrome *c* (Type II, from horse heart), hexokinase (Type III, practical grade, 40 KM units/mg) and NAD^+ (Grade III, from yeast) were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. Solutions of these were made freshly each day. Polyvinylpyrrolidone was supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks, and other chemicals used were of analytical grade.

Experiments with tissue sections. The excised segments were washed three times in 0·02 M sodium phosphate buffer, pH 5·3, blotted and weighed. The oxygen consumed by approximately 200 mg portions of tissue was measured manometrically at 25°. The main compartment of the flask contained the tissue suspended in 0·02 M sodium phosphate buffer, pH 5·3 (1·8 ml), containing sodium succinate (20 μmoles) where appropriate. The solution of growth

[15] J. A. Lockhart, *Plant Physiol.* **37**, 759 (1962).
[16] S. Kuraishi and R. M. Muir, *Plant Physiol.* **38**, 19 (1963).
[17] A. H. Halevy, *Plant Physiol.* **38**, 731 (1963).

retardant (0·2 ml) was placed in the side-arm. The centre well of the flask contained 20% KOH (0·2 ml) and the gas phase was air. A period of 10 min was allowed for equilibration before the side-arm was tipped.

Experiments with mitochondria. Suspensions of mitochondria were made by modifications of standard methods.[18, 19] The excised segments of stem or root were placed as soon as cut in a chilled solution containing 0·25 M sucrose, 0·01 M EDTA and 0·01 M tris–HCl, pH 7·5. All subsequent operations up to the placing of the Warburg flasks in the water bath were carried out at 0–4°. The plant segments were removed from the washing fluid, blotted, weighed and ground in a mortar with acid-washed sand and a fresh portion of the same solution (7 ml/g plant material). In some experiments polyvinylpyrrolidone (1% w/v) was included in this solution.[20] The suspension was filtered through a double layer of muslin and centrifuged at 1500 *g* for 10 min. The supernatant was again centrifuged, at 20,000 *g* for 20 min, and the pellet of mitochondria so obtained was suspended in 0·01 M tris–HCl buffer, pH 7·5, containing 0·25 M sucrose (0·1 ml/g original plant material). These suspensions contained between 2·3 and 3·1 mg protein/ml.

The oxidation of succinate by these suspensions was measured manometrically as soon as possible after their preparation. Except where stated otherwise, the incubation mixture contained, per flask, 60 μmoles sodium succinate, 1 μmole NAD^+, 0·1 μmole cytochrome *c*, 6 μmoles ATP, 5 μmoles $MgCl_2$, 24 μmoles KH_2PO_4, 10 μmoles glucose, 80 KM units hexokinase and 0·4 ml of the mitochondrial suspension, all in 0·25 M sucrose, 0·1 M tris-HCl, pH 7·5 (3 ml). The reaction was initiated when the glucose, hexokinase and growth retardant in the side-arm were tipped into the main compartment. The gas phase was air, and the temperature of incubation 25°. A period of 7 min was allowed for equilibration before tipping. Immediately after tipping, one of the flasks was removed and placed in ice, and the reaction arrested by the addition of 5% trichloroacetic acid. The remaining flasks were incubated with shaking for 30 min during which time the oxygen consumption was observed, and then were removed to an ice bath and 5% trichloroacetic acid was added. The contents of all the flasks were then filtered and the inorganic phosphate content of 0·02 ml portions was determined.[21]

Other methods. (1) The decomposition of ATP by a suspension of mitochondria was measured by the method of Wedding and Black.[11] (2) The protein content of the suspensions was found by adding trichloroacetic acid, and determining the protein in the precipitate[22] with bovine serum albumin as standard.

Acknowledgement—We thank Dr R. M. Allison, D.S.I.R. Crop Research Division, Lincoln, for helpful discussion and gifts of chemicals.

[18] B. AXELROD, *Methods Enzymol.* **1**, 19 (1955).
[19] P. C. JACKSON, S. B. HENDRICKS and B. M. VASTA, *Plant Physiol.* **37**, 8 (1962).
[20] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* **3**, 173 (1964).
[21] O. LINDBERG and L. ERNSTER, *Methods Biochem. Anal.* **3**, 3 (1956).
[22] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).