SOME EFFECTS OF 2,4-DINITROPHENOL ON THE METABOLISM OF TOBACCO LEAF DISKS

by

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From a study of the metabolism of radioactive sugars by tobacco leaf disks PORTER AND MAY[1] concluded that when starch formation is induced by introducing hexoses or sucrose into the cell in sufficient quantity the starch and the carbon dioxide (CO_2) simultaneously formed in the dark derive from a common pool formed from the sugar. Formation of this pool requires equilibration of the fructose and glucose structures, since these contribute in equal measure to both starch and CO_2 when sucrose or invert sugar are substrates. Coupled with the well known fact that synthesis of sucrose from hexose or of starch from sugar in the dark, by leaf tissue depleted of carbohydrate, requires the presence of oxygen, these observations might be explained by assuming that an equilibrium mixture of the phosphorylated hexoses provided the common pool of material. Oxygen might then be required for the coupled reactions leading to phosphorylation of free sugar. If this were so then starch formation and presumably glycolysis should proceed in the absence of oxygen if sugar phosphates were provided as substrates. Such a hypothesis cannot be tested directly because these esters will not penetrate the cell (PORTER[2]). An alternative means would be the use of an uncoupling reagent designed to restrict phosphorylation of free sugars after they had entered the tissue. The result should be first, inhibition of starch formation, followed by inhibition of CO_2 output if the further supposition of PORTER AND MAY[1] is correct that respiration requirements are preferentially met before starch synthesis occurs. It is widely stated (SLATER[3]) that 2:4 dinitrophenol (DNP) uncouples the oxidative phosphorylation accompanying the operation of the terminal oxidase system. Accordingly the effects of DNP upon respiration and utilization of carbohydrate by tobacco leaf disks have been studied, using both starch-containing disks and starch-free disks supplied with sucrose.

The results are consistent with the hypothesis that phosphorylation of free sugar is the first step in its incorporation into the cycle leading to starch synthesis, but they are by no means definitive. Moreover they show that the action of DNP on leaf disks has features differing from those reported for other tissues, and suggest that the pathway of sugar utilization in this system may be more complex than formation of a hexose phosphate pool, with subsequent synthesis of starch and oxidation of sugar by routes depending on accepted enzyme mechanisms. A brief account of these experiments has been presented to the Biochemical Society[4].

MATERIAL AND METHODS

Tobacco plants were grown under the conditions described by PORTER AND MARTIN[5]. Leaf disks, 1 cm diameter, were cut from fully expanded leaves on the plants either after a period of exposure to light, when starch content was high, or after a period of dark to deplete them of starch and sugar. Samples of 12 disks were floated on 1 or 2 ml of water or 5 % sucrose solution and oxygen uptake was determined over 24 hour periods at 25° C and in an atmosphere of oxygen, using the Warburg technique. The total carbon dioxide (CO_2) evolved by each sample was released from the carbonate in the centre wells of the Warburg flasks and its volume measured using the apparatus designed by RUNECKLES[6]. Starch and sugar contents of the disks were also estimated. The effect upon metabolism of DNP was studied by adding to the flasks appropriate amounts of DNP to produce the desired range of concentrations before introducing the leaf disks. When radioactive sucrose was used as substrate the starch formed was isolated for counts of activity and the sugars separated by paper chromatography. The aqueous extracts prepared from the ethanol extract of disks were made to 5 or 10 ml if sugars were estimated chemically without separation, and to 2 ml for separation on paper. The treatment of the leaf disks and analytical procedures were those described by PORTER AND MAY[1].

Preliminary experiments

The effects of oxygen tension and sucrose concentration on respiration and starch synthesis were determined using samples of six starch-free disks floated on 1 ml of sucrose solutions. One series of results is presented in Fig. 1. Increasing oxygen (O_2) tension (a) led to increased starch formation and CO_2 output up to 80% O_2. Sugar accumulation increased rapidly up to 40% O_2 and then slowly. The particular experimental conditions used here probably account for the fact that respiration and starch formation continued to increase until the gas environment was almost completely O_2 (*cf.* JAMES[7]). The disks are kept in contact with the sucrose solution by the shaking of the Warburg flasks and consequently access of O_2 is mainly from solution and so is readily limited by the low solubility. Increasing the concentration of sucrose supplied (b) increased starch formation and CO_2 output up to 2.5 % sucrose, but sugar accumulation continued to increase up to 10 % sucrose. In the absence of sucrose substrate the respiration quotient (R/Q) calculated from the total gas exchange in 24 hours was 0.78. The value rose with increasing sucrose concentrations and reached a maximum of 1.02 at 2.5 % sucrose. It is concluded that sucrose competed successfully with the non-carbohydrate substrate utilized in its absence, and displaced this substrate more effectively as the external concentration is increased, until at about 2.5 % sucrose, sugar becomes the sole respiratory substrate (*cf.* PORTER AND MAY[1]).

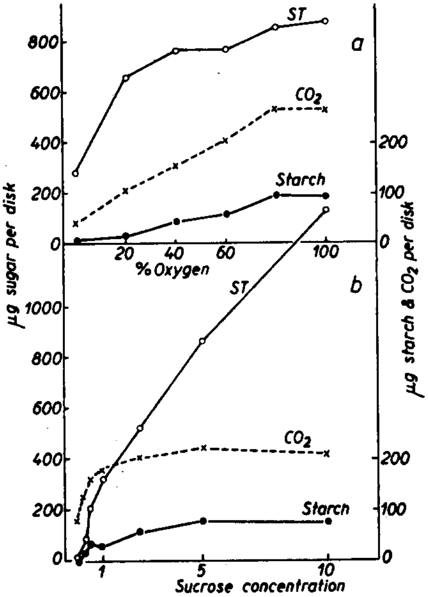

Fig. 1. Effects upon sugar accumulation, starch formation, and CO_2 output by tobacco leaf disks of (a) oxygen tension with 5 % sucrose, (b) sucrose concentration with 100 % oxygen. ST. total sugar, sucrose, fructose and glucose. Conditions as set out in text.

SIMON AND BEEVERS[8] have drawn attention to the fact that the amount of a weak acid entering a cell is dependent upon pH and that for maximum entry it is desirable to have a high proportion of undissociated molecules. The range of DNP concentrations used was from $10^{-7}M$ to $5 \cdot 10^{-4}M$ and consequently the sucrose solutions were buffered with $2 \cdot 10^{-3}M$ acetate at pH 4.6, in order to control the dissociation of DNP. Low buffer concentrations are necessary because the presence of salts in concentrations above about $10^{-2}M$ in the external solution depresses starch formation (PORTER[2]).

The standard experimental conditions were therefore 12 leaf disks, 1 cm diameter (dry weight 14 to 20 mg) in 2 ml of 5 % sucrose solution (or water) at pH 4.6 in $2 \cdot 10^{-3}M$ acetate in an atmosphere of O_2 at 25° C, for 24 hours. The conditions were modified only by addition of appropriate amounts of DNP.

RESULTS

Oxygen consumption and carbon dioxide output

The O_2 consumption of starch-free disks supplied with sucrose at four DNP concentrations is shown in Fig. 2. In the absence of DNP O_2 uptake increased for 8 to 10 hours as the sucrose penetrated across the disks, after which a steady state was reached. At $5 \cdot 10^{-5} M$ DNP an increased rate of uptake was established during the first hour, followed by a further increase presumably due to increasing sugar content up to about 8 hours, after which a steady state above that of the control samples was maintained. At $2 \cdot 10^{-4} M$ and $5 \cdot 10^{-4} M$ DNP there was also an initial increase in uptake but this was quickly followed by a fall to a new steady state or at $5 \cdot 10^{-4} M$ to nearly zero. At the higher concentrations therefore there is progressive damage to the respiratory system.

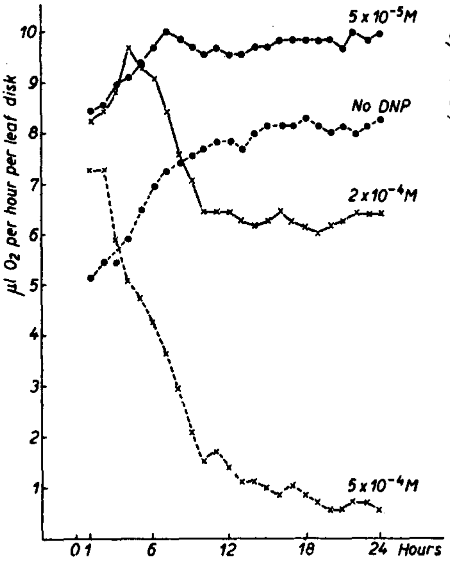

Fig. 2. Hourly uptake of oxygen by tobacco leaf disks in the presence of DNP. Conditions as set out on p. 101.

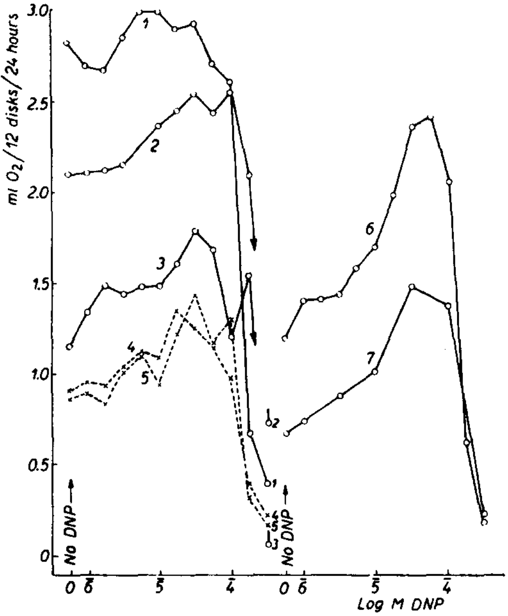

Fig. 3. Oxygen uptake in 24 hours by tobacco leaf disks in the presence of DNP. 1, 2, 3 = Starch-free disks floated on sucrose. 4, 5 = Starch-free disks floated on water. 6, 7 = Starch-containing disks floated on water. Conditions as set out on p. 101.

With starch-containing disks or starch-free disks not given a sucrose substrate, the rates of O_2 uptake in the absence of DNP were constant or fell slightly throughout the 24 hour period. The effects of DNP were similar to those illustrated in Fig. 2 but superimposed on a constant and not a rising respiration rate. It is clear therefore that low concentrations of DNP increased respiration rates, but at higher concentrations it was progressively inhibited. The mean effects over 24 hours for seven experiments are shown in Fig. 3. Curves 1, 2 and 3 are for starch-free disks supplied with sucrose, curves 6 and 7 for starch-containing disks, and curves 4 and 5 for disks without carbohydrate substrate. All three types of disks responded in a similar manner to DNP. Oxygen consumption was enhanced at low concentrations, and a maximum was reached between 10^{-5} and $10^{-4}M$. At higher concentrations it was inhibited and finally suppressed. The respiration rate in the absence of DNP for the three experiments in which disks were supplied with sucrose were very different and reflect differences between plants, leaf age and such factors not here controlled. There was also a considerable difference for the two experiments with starch-containing disks, but in the absence of carbohydrate substrate the two experiments recorded showed less variation. The increased respiration induced by DNP did not appear to be closely related to the basic rate, and was of the same magnitude for disks without carbohydrate substrate as those supplied with sucrose but for starch-containing disks the mean value was double that for the other two types. The basic rates and maximum increases due to DNP are shown in Table I.

TABLE I

MAXIMUM INCREASES OF OXYGEN UPTAKE BY TOBACCO LEAF DISKS IN PRESENCE OF 2:4 DINITROPHENOL
Maxima between 10^{-5} to $8 \cdot 10^{-5}M$ DNP (see Fig. 3). Standard conditions as set out on p. 101.

				µl oxygen/12 disks/24 hours							
Carbohydrate substrate	*None*			*Sucrose*				*Starch*			
	No DNP	*DNP*	*Increase*		*No DNP*	*DNP*	*Increase*		*No DNP*	*DNP*	*Increase*
4.* 6/ 7/54.	856	1447	591	3. 4/12/53.	1158	1789	631	6. 3/3/54.	698	1440	792
5. 31/12/54.	884	1264	380	2. 10/ 5/54.	2112	2542	430	7. 10/3/54.	1206	2409	1203
				1. 6/ 7/54.	2668	2987	319				
Mean increase			485				460				997

* Numbers are those of curves of Fig. 3.

The similarity of the response to DNP of disks without carbohydrate substrate and those supplied with sucrose together with the observation (see Fig. 2) that increased O_2 uptake was manifested during the first hour while increases due to sucrose persisted for 8 hours or more suggest that non-carbohydrate material may supply the substrate for the extra respiration in the presence of DNP. The much greater response when starch was the substrate might then be explained by supposing that such material was present in greater amounts in leaves that had not been subjected to the rather long periods of darkness necessary to remove starch before cutting the disks. Some support for this view comes from calculations of *R/Q* from the total gas exchange in 24 hours. In the absence of carbohydrate the *R/Q* is about 0.75 and the value remained unchanged at all DNP concentrations. When either sucrose or starch were substrates all values were a little greater than 1.0 with a mean of about 1.03. As the maximum CO_2 output was reached the *R/Q* began to fall and over the inhibitory phase fell to 0.85. There is therefore evidence of a change in the substrate for oxidation consistent with the increasing use of non-carbohydrate substrate, at a lower level of oxidation.

PORTER AND MAY[1] have shown in similar experiments that when radioactive sugars are supplied the relative specific activity of the evolved CO_2 is equal or nearly so to that of the sugar substrate and concluded therefrom that in the presence of sugar, oxidation of non-carbohydrate material was suppressed. If therefore increasing concentration of DNP progressively inhibits the use of sugar, the alternative substrate might compete successfully for the respiratory centres and specific activity of CO_2 would fall. The total activity moreover should show if sugar consumption was restricted.

The results of an experiment in which sucrose uniformly labelled with ^{14}C was supplied are presented in Fig. 4. Over the range of DNP concentrations at which CO_2 output is rising both total activity and relative specific activity fall, and the falls are maintained over the inhibitory range; so that at $10^{-4}M$ DNP by calculation from the activity data the proportion of CO_2 derived from sugar has fallen to 54%. It is recognised that the curves of activity show rather large fluctuations, but it seems at least justified to conclude that in the presence of DNP sugar consumption is not increased, and that some CO_2 derives from an unlabelled substrate.

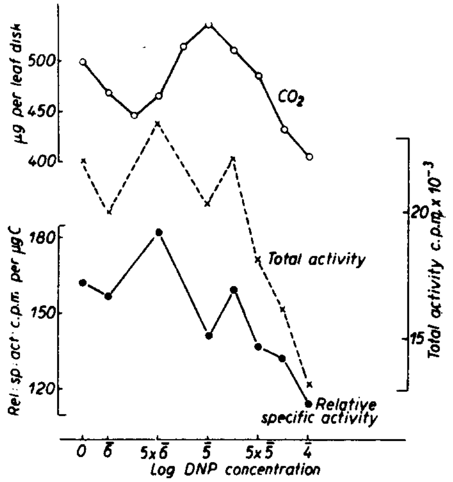

Fig. 4. Output and relative total and specific activities of CO_2 evolved by tobacco leaf disks floated on sucrose uniformly labelled with ^{14}C and in presence of DNP. Conditions as set out on p. 101.

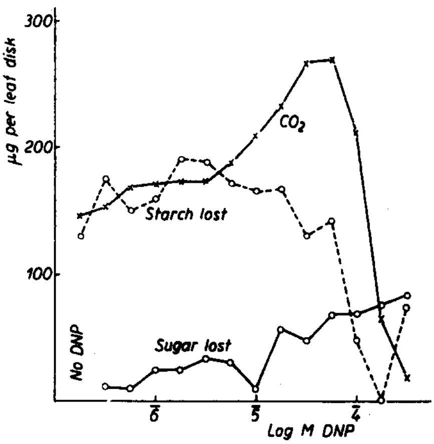

Fig. 5. Loss of starch and sugar, and CO_2 output of tobacco leaf disks floated on water for 24 hours, in presence of DNP. Initial starch content 284 µg/disk, sugar content 86 µg/disk. Conditions as set out on p. 101.

Chemical analyses

The losses of starch and sugar from leaf disks and the CO_2 output calculated as hexose on the assumption that it is derived from starch (see p. 103) are presented in Fig. 5. The data are from the experiment whose O_2 uptake is recorded in curve 4 of Fig. 3. The initial starch content was 248 µg per disk and total sugar 86 µg per disk. In the absence of DNP about half the starch was lost in 24 hours at 25° C and there was a small increase in sugar content. At low DNP concentrations the loss of starch corresponded reasonably well with the carbon dioxide output and starch was obviously the main respiratory substrate. As CO_2 output rose however the starch loss did not rise also but became increasingly inhibited between $10^{-5}M$ and $10^{-4}M$ and then rapidly prevented. At low DNP concentrations there was a small loss of sugar followed by loss of virtually all the sugar over the range at which starch loss was strongly inhibited. The increased loss of sugar was not sufficient to account for the extra CO_2, so that the chemical data here lead to the same conclusion as the data for gas exchange, namely that DNP stimulates oxidation of non-carbohydrate substrate.

The amounts of starch formed and of CO_2 evolved when sucrose was the substrate are shown in Fig. 6(b) as mean values for three experiments (*cf.* curves 1, 2 and 3. Fig. 3). There was progressive inhibition of starch formation and at concentrations of about 10^{-4} DNP it was prevented. It is clear that the reduced formation of starch was accompanied by a rising CO_2 output, but taking account of the gas exchange and radio activity data already set out it is unlikely that this inverse relation can be interpreted as a simple diversion of sucrose from synthesis to oxidation. The amounts of sugar accumulated in the disks not treated with DNP averaged 704 μg per disk and at the high concentrations of DNP 414 μg per disk. Both values are large compared with amounts of sugar normally found, usually about 100 μg per disk. Over the range of DNP concentrations used therefore there was present in the cell a relatively large amount of free sugar, so sugar supply probably does not at any time limit either starch synthesis or respiration.

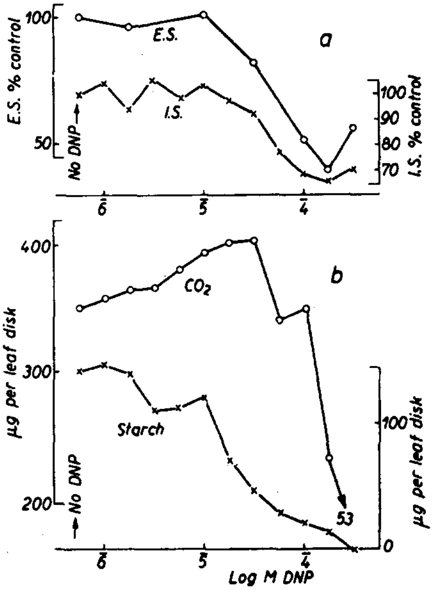

Fig. 6. (a) Loss of sugar from external solution (E.S.) and accumulation of sugar in leaf disks (I.S.). (b) CO_2 output and starch formation when starch-free tobacco leaf disks are floated on 5% sucrose for 24 hours in the presence of DNP. Mean values for three experiments. Conditions as set out on p. 101.

The effects of DNP upon sucrose uptake and sugar accumulation in leaf disks are shown in Fig. 6(a), calculated as percentages of control values. It has not been possible from such data to define precisely the fate of all the sucrose entering the disks and so determine the effect of DNP on sugar utilization directly as has been done for starch. Of the 100 mg of sucrose supplied under the standard conditions uptake in the absence of DNP for three experiments was 1392, 1078 and 1917 μg per disk. Of these rather variable amounts (see also Table I) 40 to 50% accumulated in the disks as sucrose, fructose and glucose; 25 to 30% appeared as starch and CO_2 leaving unaccounted for about 25%. The estimates of both uptake and the undetermined fraction are subject to undesirably large errors as small differences from the initial 100 mg of sucrose, and thus must be somewhat uncertain. In the earlier experiments of PORTER AND MAY[1] the recovery was somewhat better. Bearing this in mind it is tentatively concluded that both sucrose uptake and accumulation were not materially affected by concentrations of DNP up to $10^{-5} M$, but at high concentrations both processes were increasingly inhibited. The unidentified fraction also remained constant up to $10^{-5} M$, but decreased at high concentrations and may have disappeared. Sugar utilization measured as the difference between uptake and accumulation did not increase as CO_2 output was stimulated but rather tended to fall, and since at the same time possible formation of an unidentified metabolite also fell, the chemical data are at least not inconsistent with the conclusion reached from the gas exchange and activity data that the extra CO_2 did not derive from the sucrose.

At present no serious attempt has been made to resolve the question of the unidentified fraction. However it can be said that it is unlikely to be organic acid. It has been shown that small amounts of ^{14}C from the sucrose became incorporated in the organic acids of the leaf disks, and as well that the acid levels fell during the 24 hours experimental periods, but the amounts involved are too small to provide any solution to the question.

DISCUSSION

Studies of the effects of DNP in metabolism of living tissue (reviewed by SIMON[9]) show that in general synthetic processes are inhibited, while respiration rises to a maximum as DNP concentration is increased, and subsequently with still higher concentrations is inhibited and finally suppressed. The inhibition of synthesis is attributed to the uncoupling of oxidative phosphorylation which accompanies the operation of the terminal oxidase system, and provides phosphate bond energy for synthesis. The increased respiration is attributed to the maintenance of a resulting higher level of phosphate acceptor which is said to increase the glycolytic rate[10, 11] and so promote increased use of carbohydrate in respiration. The results of the present study suggest that this may not be so with leaf tissue.

Under normal conditions fully expanded leaves do not engage in active net synthesis of large molecules other than that of starch, so that when sugar is supplied to starved leaf tissue its use is largely restricted to starch and CO_2 formation. The CO_2 output under the conditions of the experiments reported here, when a large excess of carbohydrate substrate is present, is maintained at a constant level for many hours at a rate which is maximal for the tissue at the given temperature as shown by the data of Fig. 1. Using sugars labelled with ^{14}C PORTER AND MAY[1] have shown that at first CO_2 and starch formation proceed along a common pathway so that it may be postulated that these processes are limited by the amount of free sugar which can be incorporated in the common pool. The free sugar which accumulates then represents the excess entering the tissue which is not so incorporated.

In a system using exogenous free sugar as substrate, therefore, the level of phosphate donor, above that produced by glycolysis, might be expected to determine the respiration rate by controlling the amount of fructose 1:6 diphosphate available. In tobacco leaf tissue there is no doubt that starch synthesis is inhibited by DNP (see Fig. 6), but at concentrations up to about $5 \cdot 10^{-5} M$ respiration is increased, as has been reported for many other tissues. The behaviour differs from that of yeast (SIMON[10]) and of corn coleoptiles (BEEVERS[11]) in that the R/Q does not rise with increasing respiration, but remains a little above 1.0 and then falls to 0.85 with the onset of respiratory inhibition. The high R/Q's observed in yeast and corn coleoptiles was attributed to aerobic fermentation induced by increased glycolysis. In leaf disks there seems to be no evidence of such an increase in glycolysis.

In the absence of carbohydrate substrate and in an atmosphere of O_2 the endogenous respiration is about 25% of that found with sucrose, and has an R/Q 16.2 of about 0.75. As noted (p. 101) the R/Q increases when low concentrations of sucrose are supplied and at 2.5% sucrose reaches a maximum of about 1.05. These observations suggest that leaves contain appreciable amounts of non-carbohydrate material which can function as a respiratory substrate and which competes with carbohydrate for the respiratory centres. In presence of large amounts of carbohydrate oxidation of the non-carbohydrate material is virtually suppressed (PORTER AND MAY[1]). If therefore DNP, by its uncoupling action, restricts the amount of phosphate donor and reduces the use of free sugar in respiration, then the alternative substrate might again compete successfully, a situation which would account for the fall in *R/Q*, and the fall in relative specific activity of the evolved CO_2 as DNP concentration was increased. This conclusion is supported by the fact that in the absence of sucrose, respiration is stimulated to about the same degree as in its presence (Table I) without change of *R/Q*. Where carbohydrate substrate is not available, synthesis will be absent and phosphate acceptor level low. The increased oxidation of non-carbohydrate substrate might therefore be accounted for as suggested by LARDY AND WELLMAN[12] by an increase in acceptor level due to the uncoupling. If this be the case then this oxidation must involve phosphorylation.

In addition to its effects upon respiration and starch synthesis, DNP at concentrations above $10^{-5} M$ reduced the amount of sugar taken up by the leaf tissue. If the claim of ROTHSTEIN[13] in respect of yeast that phosphorylation is required to effect uptake, be also applicable to leaf cells, then the active uptake may utilize phosphate donor independently of the phosphorylation within the cell. However it seems unlikely that sugar supply is an important limiting factor in the present case, because quite large amounts of free sugar accumulate at all DNP concentrations employed.

The results of the experiments where the native starch within the tissue provided the main substrate for respiration can be interpreted in a similar manner as those where sucrose was the substrate. Here there is direct analytical evidence of the progressive inhibition of starch utilization over the phase of rising CO_2 output, accompanied by only small changes in sugar content (Fig. 5), and again there is no rise in *R/Q*.

The results may be compared with the effects of altering respiration rate by lowering the ambient temperature. In these circumstances there is an accumulation of sugar, mostly sucrose, so that starch dissolution exceeds the requirements for respiration. Restriction of respiration does not therefore necessarily prevent the passage of starch breakdown products into the common metabolic pool, where it is assumed the fructose structure arises and leads to sucrose synthesis when glycolytic rate is lowered by temperature. DNP must therefore prevent incorporation into the pool, and the conclusion must be that this process requires phosphate bond energy even when the substrate is the starch within the cell. Such a conclusion would be consistent with the long known fact that starch dissolution requires the presence of oxygen; and raises the question as to whether there is a process other than or in addition to phosphorolysis by free phosphate necessarily involved in the initiation of the breakdown of the starch grain.

Taking all the evidence together it is concluded that in tobacco leaf disks DNP does not increase glycolysis, but restricts the use of carbohydrate as a respiratory substrate. At the same time increased oxidation of non-carbohydrate material is induced perhaps by virtue of a raised phosphate acceptor level. As a result if sucrose is the substrate, first starch synthesis and, with increasing concentrations of DNP, CO_2 production from sugar are inhibited. Over the phase of rising respiration the additional CO_2 is derived from non-carbohydrate material (*cf.* SYRETT[14]). Whether or not the substances in the common pool whose formation, whether starch or sucrose is available, appears to require the phosphate bond energy is an equilibrium mixture of hexose phosphate esters, derived by direct phosphorylation, or whether more complex reactions are involved requires further investigation.

SUMMARY

1. Respiration of tobacco leaf disks in an atmosphere of oxygen at 25° C was at first stimulated and then inhibited by increasing concentrations of 2:4 dinitrophenol (DNP). Starch was at first the respiratory substrate, but at concentrations of DNP above $8 \cdot 10^{-6} M$ starch loss was strongly inhibited, although respiration was rising.

2. Leaf disks depleted of starch and supplied with sucrose as substrate showed a response to DNP qualitatively similar to that of disks from normal leaves as regards respiration, and in addition starch synthesis was inhibited. In both cases respiration quotients (R/Q) were a little over 1.0 and fell as DNP concentrations increased over $5 \cdot 10^{-5} M$. In the absence of added sucrose, R/Q was about 0.75, and respiration was again stimulated by DNP.

3. Taken together the data suggest that oxidation of starch or sucrose was gradually inhibited by DNP, and was progressively replaced by the non-carbohydrate substrate with R/Q 0.75. This conclusion is supported by the observation that when radioactive sucrose was used as substrate the relative specific activity of the CO_2 fell with increasing DNP concentration, indicating increasing use of unlabelled substrate.

4. The results are discussed in relation to the hypothesis that DNP uncouples oxidative phosphorylation.

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