SUMMARY

Particulate fractions of wheat root protoplasm, from mitochondria to microsomes, all carry the cytochromes a_3, c, c_1, b and b_3, and probably some flavoprotein. The mitochondria fractions show more of the cytochromes a_3 and b, the microsome fraction more of the cytochromes c, c_1, and b_3, which are partly detached from the "respiratory particles". Peroxidase, DPN-linked enzymes, and more or less of the flavoprotein and cytochromes c and c_1 go into the supernatant of the fractions. The results are compared with observations on living roots.

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METABOLISM OF MALTOSE LABELLED WITH ^{14}C IN THE REDUCING GLUCOSE MOIETY, BY TOBACCO LEAF DISKS

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Metabolism of sugars labelled with ^{14}C by leaf tissues has been the subject of a number of investigations[1–7]. The results are in accordance with the view that the pathway of synthesis of sucrose and starch includes a step in which hexose units, whether supplied free or combined, become equilibrated and are only then available as substrates for respiration and further synthesis. For example when sucrose was supplied to tobacco leaf disks, there was no discrimination between the glucose and fructose moieties as substrates for starch synthesis and respiration, and sucrose was incorporated into the equilibrating system or metabolic "pool" without prior formation of free hexose[4].

A number of disaccharides with different types of linkage between the hexose units has now been tested as possible substrates for sucrose and starch formation

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in tobacco leaf tissue. Of these, namely, maltose, isomaltose, cellobiose, gentiobiose, trehalose, turanose and lactose, only maltose was effective. The utilisation of maltose has been further studied, with the object of determining whether the two glucose units, like the hexose units of sucrose, are equally available. In order to distinguish between the two glucose units, maltose has been synthesised in which only the reducing glucose moiety was uniformly labelled with ^{14}C.

Preparation of maltose labelled with ^{14}C in the reducing glucose moiety

Maltose (G $\longrightarrow$ ^{14}C/G) was prepared by the action of *B. macerans* transferase on cyclohexaamylose in the presence of uniformly labelled glucose (^{14}C/G)[8]. Cultures of *B. macerans* (National Type Culture collection 3223 and 4743) were grown on oatmeal as described by TILDEN AND HUDSON[9]. At the end of the incubation period, the cultures were centrifuged and the supernatant liquid used as the source of transferase. Enzyme activity was retained for many months if the solution was stored at 4° C. Cyclo hexaamylose was prepared by the action of the transferase on starch[10,11]. 624 mg of cyclohexaamylose and 125 mg of ^{14}C/G were dissolved in 63 ml of the enzyme solution. After 4 hours at 40° C 125 ml of ethanol were added and the mixture allowed to stand for 16 hours at 4° C. The precipitate was removed by centrifugation and the supernatant liquid evaporated to dryness over calcium chloride. The residue was taken up in water and transferred to a charcoal column. The charcoal (B.D.H. activated) was the fraction retained by a 200-mesh sieve, and was used without celite; it was washed with citrate buffer at pH 6.8 and with water before use. Glucose was eluted with water, maltose with 7.5% ethanol, and the other reaction products were recovered by elution with 50% ethanol[12]. The yield of G $\longrightarrow$ ^{14}C/G was 45 mg (activity $144 \cdot 10^3$ counts/min/mg) and the amount of ^{14}C/G recovered 44 mg (activity $327 \cdot 10^3$ counts/min/mg). After evaporation to small bulk, the solutions were stored at —20° C. Suitable amounts of inactive sugar were added when making up 5% solution for the experiments with leaf tissue.

Determination of the distribution of labelling in maltose

Maltose was reduced to maltitol using sodium borohydride[13]. Maltitol was then hydrolysed to glucose and sorbitol by heating in a sealed tube at 100° C for 2.5 hours in $N/1$ HCl. Chloride ions were removed with silver carbonate and cations by addition of a few grains of exchange resin (Zeocarb 315). The hydrolysis products were separated by paper chromatography, using as solvent methyl ethyl ketone (9 vol.), acetic acid (1 vol.), and water saturated with boric acid (1 vol.). This solvent was selected after a number of trials with solvent combinations suggested by a study of the literature[14–19]. Separation of glucose and sorbitol was effected in six hours and the R_G of sorbitol was 3.5. Their positions on the paper were defined by radioautographs or by spraying with silver nitrate[20] or sodium periodate[21]. All the radioactivity was found in the sorbitol derived from the reducing glucose moiety of maltose.

Plant material and analytical methods

Tobacco plants (White Burley) were grown as in previous experiments[22]. Leaf disks were cut from leaves just fully expanded, after plants had been kept in the dark for 40 to 60 hours to deplete them of starch. Samples of twelve disks were floated on 2 ml of 5% solutions of ^{14}C/G or G $\longrightarrow$ ^{14}C/G in stoppered weighing bottles (4 cm × 3 cm). Centre wells were fitted into the bottles and contained filter paper strips dipped in 0.2 ml 10% KOH to absorb respiratory carbon dioxide (CO_2). The volume of the bottles was large enough to maintain an adequate supply of oxygen and they were maintained at 25° C for 24 hours in dim light[23]. There were three replicates for each sugar, and the whole experiment was carried out on two occasions, making six replications in all. At the end of the 24 hours the sugar solutions contained only ^{14}C/G or G $\longrightarrow$ ^{14}C/G so that there was no hydrolysis of maltose in the external solution.

For estimation of CO_2, the contents of the centre wells, including the filter paper, were transferred to graduated tubes and made up to 3 ml. After thorough mixing 1 ml aliquots were added to 0.5 ml of $N/1$ barium chloride and the excess KOH titrated with N/HCl to the phenolphthalein end point, using an Agla micrometer syringe as a burette. Barium carbonate was then washed by centrifugation and its relative specific activity (r.s.a.) determined in the conventional manner. Allowance was made for traces of carbonate in the KOH, and for CO_2 absorbed during manipulation.

Leaf disks were removed from the sugar solutions washed free from sugar and placed in boiling ethanol. Extraction was completed and sugars separated, estimated, and their r.s.a.'s determined as already described[4], with the modifications that positions of the sugars on paper chromatograms were defined by radioautography and eluates were collected and made up to known volumes, instead of being collected directly onto planchettes. For estimation of starch, the ethanol-insoluble material was macerated with a few drops of water in a test tube fitted

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with a pestle and the starch extracted and estimated as before[4]. Portions of the extracted starch were subjected to the combined action of α and β amylases and the maltose produced isolated by paper chromatography and its r.s.a. determined. The activities of the two glucose moieties were determined as before (see p. 366).

Preliminary experiments

A series of trials in which leaf disks were floated on maltose solutions varying in concentration from 1 to 5%, in air or oxygen, and shaken in Warburg flasks or left unshaken, and in either open or closed vessels, defined conditions most favourable for starch synthesis. Starch was formed most readily with 3 to 5% maltose in air without shaking. The effect of maltose concentration, over the range 1% to 5%, on respiration and starch synthesis was small, but the amount of sugar accumulating in the leaf disks increased greatly with concentration. Shaking led in some cases to injection of the disks, and this prevented starch synthesis, but left respiration unimpaired. The behaviour of the disks also varied with the time of year at which plants were grown, spring-sown plants forming more starch than those sown in autumn. Sugar accumulated in the latter case, so that failure to form starch was not due to differences in the uptake of sugar. Chromatograms of the ethanol-soluble material in leaf disks following metabolism of radioactive maltose showed that under these experimental conditions only traces of activity were incorporated into soluble substances other than sugar.

RESULTS

Estimates of starch and the separate sugars extracted from the leaf disks after floating for 24 hours on $^{14}C/G$ or $G \longrightarrow {}^{14}C/G$, and of the CO_2 of respiration, are presented in Table I, and of the relative specific activities in Table II. With both substrates, starch and sucrose were synthesised and free fructose and glucose accumulated. The amounts of these carbohydrates and of CO_2 evolved were greater when disks were floated on glucose than when they were floated on maltose, probably because glucose enters the tissue more rapidly than maltose. With $G \longrightarrow {}^{14}C/G$ there was a little free maltose present which was found to retain the original distribution of label, *i.e.* it was unchanged $G \longrightarrow {}^{14}C/G$. The relative specific activities (r.s.a.'s) of starch and sucrose were equal to those of the two substrates, and the hexose components of sucrose had equal activities. Amounts and r.s.a.'s of the free hexoses were equal to one another and the activities were equal or nearly so to those of the substrate sugars. Maltose derived by amylolysis of the isolated starch had radioactivity equally distributed between the glucose moieties.

The r.s.a. of CO_2 was about 60% of that of the substrate sugars, as compared with values of 75% to 90% in 11 other experiments with glucose and two with

TABLE I

ESTIMATION OF SUGARS AND STARCH IN TOBACCO LEAF DISKS (1 cm DIAMETER) FLOATED FOR 24 hours ON 5% MALTOSE OR GLUCOSE SOLUTIONS AT 25° C IN AIR, AND OF THE CARBON DIOXIDE EVOLVED

Means of six replicates with standard errors.

Substrate	*Glucose ($^{14}C/G$)*		*Maltose ($G \longrightarrow {}^{14}C/G$) as hexose*	
	mg in 12 disks			
Starch	4.30	± 0.13	2.39	± 0.12
Sucrose	1.24	± 0.04	0.96	± 0.03
Fructose	0.85		0.69	
Glucose	1.18		0.54	
Maltose	—		0.30	
CO_2	3.32	± 0.08	2.45	± 0.04

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TABLE II

RELATIVE SPECIFIC ACTIVITIES OF STARCH AND SUGARS ISOLATED FROM TOBACCO LEAF DISKS (1 cm DIAMETER) FLOATED ON 5% SOLUTIONS OF MALTOSE OR GLUCOSE FOR 24 h AT 25° C IN AIR, AND OF THE CARBON DIOXIDE EVOLVED

Means of six replicates. Standard error of means ± 0.4.

Substrate	*Glucose ($^{14}C/G$)*	*Maltose ($G \rightarrow {}^{14}C/G$) (Components calculated as hexose)*
	counts/min/10^{-3} mg	
Substrate	10.3	10.9
Starch	10.6	11.3
Sucrose	9.0*	9.4*
Fructose	8.0	9.2
Glucose	7.7	10.7
Maltose	—	10.7**
CO_2	5.9	7.6
Maltose from starch	—	11.1***

* Activities of component fructose and glucose were equal.
** Activity only in reducing glucose unit.
*** Activities of two component glucose units were equal.

maltose carried out in this laboratory. Leaf material of this type depleted of carbohydrate has a CO_2 output in 24 hours at 25° C in air of about half that in the presence of sugar substrate. In the presence of $^{14}C/G$ the activity of the CO_2 increases with time, reaching a value equal to that of the $^{14}C/G$ after 8 to 15 hours (G. A. MACLACHLAN, unpublished). Sugar thus gradually replaces the non-carbohydrate inactive respiratory substrate. The low r.s.a. values for CO_2 recorded in Table II may therefore have been due to a rather slow replacement rate. In the case where $G \rightarrow {}^{14}C/G$ was the substrate, if the low value for the r.s.a. arose because the non-reducing, and here unlabelled, glucose moiety was preferentially used in respiration, there should be evidence of the retention of the labelled reducing moiety, either as sugar and starch with r.s.a.'s higher than that of $G \rightarrow {}^{14}C/G$ or in some other compounds. As noted, only traces of activity were detected on chromatograms at sites other than those occupied by the sugars, and Table II shows that none of the carbohydrates has r.s.a.'s greater than that of $G \rightarrow {}^{14}C/G$. It is therefore concluded that both glucose moieties of maltose contribute equally to respiration and that oxidation of non-carbohydrate material dilutes the ^{14}C in the CO_2.

DISCUSSION

Maltose entering tobacco leaf disks is transformed into sucrose and starch and also provides respiratory substrate. In these transformations there is no discrimination between the two glucose moieties of the maltose. Comparing the metabolism of maltose with that of glucose (Tables I and II) or of invert sugar[4], it is clear that the products are similar. Special features are that sucrose is always formed and that whatever the initial distribution of radioactivity between the hexose units of the substrate, the fructose and glucose combined in sucrose are equally labelled. Starch also derives equally from all the hexose units supplied. The results obtained with maltose are thus consistent with the hypothesis[4] that sugars are incorporated into a metabolic "pool" in which hexose units become equilibrated, or form a common intermediate, and are then available for further transformation.

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Comparing maltose with sucrose as a substrate[4], the equal utilisation of the hexose components and the accumulation of some disaccharide unchanged occurs in both cases. With sucrose, however, there appears to be no new synthesis of sucrose in the "pool", since PORTER AND MAY[4] found, using sucrose with only one moiety radioactive, that no cross-labelling appeared in the sucrose extracted from the leaf disks. The occurrence of unchanged disaccharide implies that both sucrose and maltose enter the leaf tissue intact, and that the sugar metabolised is incorporated into the "pool" without prior formation of free hexose. The mechanism of such incorporation is a matter for speculation at present, especially for maltose, which is not normally found in tobacco leaves. It may be that maltose and the hexoses are first transformed to sucrose, and that only sucrose can be used at the sites of starch formation and respiration. Such a scheme would, however, require two equilibrating "pools" for the hexose units, one at the site of sucrose synthesis and a second at the site of its further transformations, since there is no discrimination in the utilisation of the moieties of sucrose itself.

When maltose G $\longrightarrow$ ^{14}C/G is supplied, the free glucose and fructose produced have equal activities (Table II) as do the hexose components of sucrose, whereas when sucrose in which only one moiety is labelled is supplied, both sucrose and the free hexoses retain the labelling of the substrate[4]. It therefore seems likely that the free hexoses are derived exclusively by sucrose inversion, and direct hydrolysis of maltose seems excluded, since when maltose is the substrate, there is no excess of glucose over fructose. PUTMAN AND HASSID[1] and KROTKOV and his collaborators[2,3] have concluded that free hexoses were not precursors of sucrose in leaf tissue, a conclusion which is borne out by the present series of experiments.

It is generally considered that the first step in incorporation of free sugar into metabolic cycles is phosphorylation and in this connection it may be noted that a maltokinase has been reported by CRAINE AND HANSEN[24]. The randomisation of the activity of the glucose units in starch synthesised from G $\longrightarrow$ ^{14}C/G (see p. 367) makes it unlikely that synthesis from maltose proceeds by transglycosylation of the amylomaltase type, since this would involve discrimination in favour of the non-reducing moiety of the maltose. It is therefore concluded that the leaf enzyme systems convert maltose into the postulated common intermediate, and so make it available for transformation by the same routes as hexoses or sucrose. Since none of the other disaccharides tested was metabolised, it appears that there is some specificity in the system perhaps associated with the glucose unit linked through the 1 position which is common to maltose and sucrose.

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SUMMARY

Maltose (G $\longrightarrow$ ^{14}C/G) was synthesised in which the reducing glucose moiety only was uniformly labelled with ^{14}C, by the action of the transferase of *B. macerans* on cyclo-hexaamylose in the presence of uniformly labelled glucose (^{14}C/G)[8]. Tobacco leaf disks, depleted of starch, were floated on 5% solutions of ^{14}C/G or G $\longrightarrow$ ^{14}C/G for 24 hours at 25° in air. The amounts and radioactivity of the starch, sucrose, fructose and glucose and of the carbon dioxide formed were determined. The results are consistent with the hypothesis that maltose, like sucrose, enters the cell intact and is incorporated into a metabolic pool without prior formation of free hexose. The glucose components of maltose then become equilibrated and equally available for synthesis of sucrose and starch and the production of carbon dioxide. Relative specific activity of carbon dioxide varied between experiments from 60% to 80% of that of the substrate sugar. Taking all the evidence into account, it is concluded that low activity values relative to the substrate are due to oxidation of non-carbohydrate inactive material and not to discrimination between the two glucose components of the maltose.

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