Radioactive Tracer Studies of the Metabolic Fates of Intracellularly Generated NADH and NADPH in Higher Plant Tissues[1, 2]

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The "division of labor" hypothesis concerning the metabolic roles of NAD and NADP was first proposed by Kaplan and coworkers (17) and it has subsequently been restated in somewhat different terms by a number of other investigators (11, 16, 20). In its present form, this hypothesis states that NAD is reduced to NADH in a large number of catabolic dehydrogenase reactions, and that this NADH is reoxidized by the mitochondrial respiratory chain to yield useful chemical energy; NADPH, on the other hand, is formed from NADP by a relatively small number of dehydrogenase reactions and is used mainly as a source of reducing power for biosynthetic reactions. Although a considerable body of indirect evidence has been marshalled in support of this theory (18, 20, 26), direct in vivo proof has been difficult to obtain since intact cells do not take up appreciable amounts of these nucleotides. An alternative approach to the problem was devised by Hoberman, who was able to generate specifically labeled NADH in vivo by supplying animal tissue slices with an appropriate deuterated substrate (15). This technique was extended by Lowenstein (19) and by Foster and Bloom (12), using tritiated substrates to study the fates of intracellularly generated $NADH^3$ and $NADPH^3$ in animal tissues. Similar techniques have been used in the present experiments in an attempt to study the metabolic roles of NADH and NADPH in intact higher plant tissues.

In experiments with animal tissues (3, 12, 19), $NADH^3$ was generated by feeding either glycerol or lactate labeled in the 2-position with tritium; $NADPH^3$ was generated by feeding glucose-1-H^3. In order to estimate the amount of tritium that enters a given cell fraction bound to the carbon (i.e., by passive, nonreductive incorporation), Foster and Bloom (12) used substrates which were labeled with both C^{14} and tritium in the same position. In the present experiments with plant tissues, ethanol-1-C^{14}, H^3 was used to generate $NADH^3$, since the ethanol dehydrogenase activity is considerably higher than the glycerophosphate and lactate dehydrogenase activities in the tissues used (24). It was assumed that the catabolism of the labeled ethanol starts with its oxidation to the level of acetate-1-C^{14} and that the 2 molecules of $NADH^3$ formed during this conversion can be reoxidized by molecular O_2 to form $H^3{}_2O$. Glucose-1-C^{14}, H^3 was used in order to generate $NADPH^3$, since any of this substrate that enters the HMP[5] pathway will give rise to $NADPH^3$ and $C^{14}O_2$. The fates of glucose-1-C^{14}, H^3 and glucose-6-C^{14}, H^3 are compared in order to estimate the amount of tritium that is incorporated from glucose broken down by glycolysis (12). The pathway of reoxidation of the $NADPH^3$ derived from glucose-1 is, of course, one of the major topics of this investigation and will be discussed at length below.

Materials and Methods

Incubation and Fractionation of Tissue. Etiolated pea seedlings (*Pisum sativum* L., var. Alaska) were grown as described previously (24) and 1-cm sections were excised from the third internode of each seedling. Three- to 5-day-old etiolated mung bean (*Phaseolus aureus* Roxb.) seedlings were obtained commercially and 1-cm sections were cut from the hypocotyl. All operations with the live tissues were carried out in a darkened room.

The tissue sections were rinsed in ice-cold distilled water and each replicate 2.0 g sample was transferred to the main compartment of a 125-ml Erlenmeyer flask with a large center well. The main compartment contained 5.0 ml of the incubation mixture, made up of 2 μc of C^{14}-labeled substrate, 10 μc of H^3-labeled substrate and enough unlabeled substrates to make a final concentration of either 1 mM or 10 mM; the center well contained 1 ml of 1 M Hyamine hydroxide (10X) in methanol. After removal of an aliquot of the incubation mixture for determination of the initial radioactivity, each flask was stoppered and incubated on a rotary shaker in the dark for 120 minutes at room temperature (24–27°). At the end of the incubation, the contents of the center well (Hyamine hydroxide containing respired $C^{14}O_2$) were transferred to a counting vial; the well was rinsed twice with 1-ml portions of a scintillation solvent

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[5] Abbreviations used: HMP, hexose monophosphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-*bis*-2(5-phenyloxazolyl) benzene; glucose-1, glucose-6, and ethanol-1, the corresponding carbon atom and the hydrogen (or tritium) attached thereto.

(see below) which were then added to the same counting vial. The incubation mixture and tissue sections were poured into a Buchner funnel and the fluid was removed rapidly by vacuum filtration. The tissue on the filter was rinsed twice with water and the filtrates combined with the incubation mixture for the determination of tritium in the water (see below). The rinsed tissue was killed with boiling 80 % ethanol. The total time elapsed from the opening of the incubation flask to the killing of the tissue was between 3 and 4 minutes.

Each tissue sample was extracted with 175 ml of boiling 80 % ethanol in a Soxhlet extractor for 18 hours. At the end of the extraction, the tissue was rinsed twice with 95 % ethanol and the combined extract and rinses evaporated to a volume of about 5 ml on a rotary evaporator at 46 to 48°. The pH of the extract was adjusted to 7.5 with N NaOH. The evaporation flask was rinsed once with 5 ml of 0.01 N NaOH, twice with 5 ml portions of H_2O and twice with 2.5 ml portions of 95 % ethanol; these solutions were combined with the evaporated extract. The combined solution was extracted with two 15-ml portions of petroleum ether (B.P. 30–60°) in a separatory funnel. The combined petroleum ether layers (the lipid fraction) were evaporated to dryness at room temperature; the residue was taken up in a scintillation solvent (see below) for counting.

The remaining aqueous layer of non-lipid material was adjusted to pH 6 with N HCl and then separated into basic, acidic and neutral fractions by ion exchange chromatography, using a slight modification of the method of Neal and Beevers (21). Each of these fractions was then evaporated to dryness on a rotary evaporator at 46 to 48°. The residues from the neutral, basic, and acidic fractions were dissolved in a few ml of 95 % ethanol, water, and N NH_4OH, respectively. Each of these fractions was evaporated to dryness as before, the residue taken up in a few ml of water, and the solution evaporated to dryness at room temperature in a vacuum desiccator over NaOH and H_2SO_4. Each of the final residues was dissolved in 1 ml of water, from which aliquots were taken for scintillation counting.

The qualitative composition of these fractions was examined by paper chromatography in standard solvent systems (27). The major identifiable components were essentially the same as those reported by Christiansen and Thimann (6–8). In the basic fraction most of the common amino acids could be detected, with glutamine, asparagine, and aspartic acid being present in the highest concentrations. Malate and citrate were the major components of the acidic fraction. The neutral fraction contained glucose, sucrose, fructose, traces of ribose and xylose, and an unidentified diphenylamine-positive compound with chromotographic properties identical with those of a noncarbohydrate compound detected recently in pea extracts (28).

The ethanol-insoluble tissue residue (the insoluble fraction) remaining after the initial ethanol extraction of the tissue was dried in air at room temperature before counting.

For the determination of the radioactivity in the water the combined incubation mixture and tissue rinses remaining after removal of the tissue were used. The solution was adjusted to pH 8 with N NaOH and made up to a known volume (usually 25.0 ml) with water. When the substrate was ethanol, 25 ml of unlabeled absolute ethanol were added to the solution and the mixture distilled in a Claisen flask until the boiling point was constant at 100° for several minutes (ca. 35 ml of distillate). A 5-ml aliquot of the remaining mixture was then used for determination of tritiated water by the double lyophilization method of Calvin et al. (5). When the substrate was glucose, the isolation of labeled H_2O by double lyophilization was carried out directly.

Counting Procedures. The Hyamine-CO_2 fraction, the lipid fraction and the insoluble fraction were all counted in a scintillation solvent containing 4 g of PPO and 0.1 g of POPOP per liter of toluene. All other fractions were counted in a solvent containing 4 g PPO and 0.1 g POPOP per liter in a 20:80 (v/v) mixture of absolute ethanol and toluene. C^{14} and tritium were counted simultaneously in a Packard Tri-Carb liquid scintillation counter by the discriminator ratio method of Okita et al. (22). For all but the insoluble fraction, counting efficiencies were determined after the addition of internal standards (toluene-C^{14} or toluene-H^3). Standards for the insoluble fraction were prepared by pipetting small amounts of either glucose-C^{14} or glucose-H^3 standards onto small disks of filter paper. The disks were allowed to dry and then laid flat on the bottom of counting vials containing the all-toluene solvent. The validity of results obtained with such standards was verified by comparison with results obtained using a gas-flow counter whose counting efficiency for ethanol-extracted dried plant tissue was known.

The fraction used to determine the radioactivity in water was consistently contaminated by small amounts of the labeled substrate, especially when the substrate was ethanol. To correct for this contamination, the fraction was counted for both C^{14} and tritium by the discriminator ratio method.

Materials. Ethanol-1-C^{14}, ethanol-1-H^3, glucose-1-C^{14}, glucose-1-H^3, glucose-6-C^{14} and standardized toluene-C^{14} and toluene-H^3 were obtained from New England Nuclear Corporation. Glucose-6-H^3 was obtained from Nuclear Chicago. Stock aqueous solutions of labeled glucose were stored in the frozen state; stock solutions of labeled ethanol were refrigerated. Hyamine hydroxide (10X, 1 M in methanol), PPO and POPOP were purchased from Packard Instruments. All other reagents were analytical grade.

The tritiated substrates were tested for nonenzymatic exchange with water in the absence of tissue under conditions identical to those used for the incubations and were found to contain the following amounts of exchangeable tritium: ethanol-1-H^3,

0.80 %; glucose-1-H^3, 0.22 %; and glucose-6-H^3, 2.89 %. All values for the tritium content of water reported in this paper have been corrected for this exchange.

Results

In the experiments reported here it is valid to compare the percentage incorporations from glucose and ethanol into the various fractions, since in all cases tested the amounts of the 2 compounds taken up by the tissue were not significantly different. The uptake in the 2-hour incubation period varied from 18 to 25 % of the labeled substrate supplied. Most of the uptake appeared to be passive, since increasing the substrate concentration from 1 mM to 10 mM resulted in a 10-fold increase in the amount of both compounds taken up.

Formation of $C^{14}O_2$ and H^3_2O. Both labeled glucose and labeled ethanol are readily converted to $C^{14}O_2$ by the pea stem sections (table I). Most, but not all, of the glucose appears to be broken down via the glycolytic pathway, since the mean C_6/C_1 ratio ($C^{14}O_2$ from glucose-6-C^{14}/$C^{14}O_2$ from glucose-1-C^{14}) was 0.79 in 7 experiments. The glucose-1 and glucose-6 metabolized by this pathway would not be expected to yield labeled CO_2 until the third turn of the citric acid cycle after the entry of the methyl-labeled acetyl CoA. In contrast, ethanol-1-C^{14} would be converted directly to acetate-1-C^{14}, which would yield $C^{14}O_2$ during the first turn of the citric acid cycle. Thus, it is not surprising that ethanol-1 is a much better precursor of $C^{14}O_2$ than either glucose-1 or glucose-6 (table I).

The metabolism of tritium-labeled glucose by pea stem sections gives rise to H^3_2O, glucose-1 being considerably more effective as a precursor than glucose-6 (table I). This suggests that the relatively small fraction of glucose-1 which is broken down via the HMP pathway is particularly effective as a tritium donor for H^3_2O formation. The amount of tritium incorporated from ethanol-1-C^{14}, H^3 into water by the pea stem sections was similar to that incorporated from glucose-1-C^{14}, H^3 (table I). Since only a small fraction of the glucose-1 that is catabolized gives rise to $NADPH^3$ (vide the C_6/C_1 ratio), while every mole of ethanol catabolized produces at least 1 mole of $NADH^3$, the similarity in H^3_2O production from the 2 substrates suggests that NADPH is oxidized more rapidly under the conditions of these experiments than is NADH. This conclusion is made even more likely if the intracellular NADPH pool is as large or larger than the NADH pool, as is the case in most higher plant tissues that have been examined (13, 29). When ethanol-1-C^{14}, H^3 and glucose-1-C^{14}, H^3 were supplied to the tissue sections at a concentration of 10 mM (rather than 1 mM), the aount of tritium incorporated into water from the former increased almost 20-fold while that from the latter increased only 5-fold (table II). This suggests that the rate-limiting factors in the oxidation of NADPH and NADH are different (see Discussion).

Table I. *Production of $C^{14}O_2$ and H^3_2O from Doubly-Labeled Ethanol and Glucose by Pea Stem Sections*

Labeled substrates were supplied as 1 mM solutions containing 2 μc of C^{14} and 10 μc of tritium. Incorporation (radiochemical yield) is expressed as percent of the supplied label incorporated per 2-g tissue (wet wt) per 2 hours. Each value is the mean of 4 or more experiments.

Substrate	Radiochemical yield (% of supplied label)		Ratio
	$C^{14}O_2$	H^3_2O	$H^3_2O/C^{14}O_2$
Ethanol-1-C^{14}, H^3	6.1	2.2	0.36
Glucose-1-C^{14}, H^3	1.2*	2.2	1.9
Glucose-6-C^{14}, H^3	0.94*	1.1	1.1

* C_6/C_1 ratio = 0.79.

Table II. *Effect of Substrate Concentration on H^3_2O Production*

Experimental details were as in table I, except that where indicated the labeled substrate was supplied at a concentration of 10 mM. The amount of H^3_2O formed is expressed as the mμmoles of the original substrate taken up that would be equivalent to the amount of tritium found in the labeled water. Each value is the mean of 2 or more experiments.

Substrate	Conc	H^3_2O Formed
Ethanol-1-C^{14}, H^3	1 mM	0.11
	10 mM	1.9
Glucose-1-C^{14}, H^3	1 mM	0.11
	10 mM	0.52

The Neutral Fraction. The incorporation of both C^{14} and tritium from glucose-1 and glucose-6 into the neutral fraction is relatively high (table III), probably due to the uptake of the intact labeled substrates into the intracellular sugar pool. The fact that the H^3/C^{14} ratios for these substrates are close to unity suggests the view that this represents a non-catabolic incorporation of intact molecules. The components in the neutral fraction were separated by paper chromatography and glucose was identified as the major labeled component; it represented over 50 % of the total C^{14} and tritium in the neutral fraction when labeled glucose was supplied. Using the data of Christiansen and Thimann (6), the intracellular sugar concentration in pea stem sections can be calculated to be around 0.1 M, assuming an average molecular weight of 180 (hexose). The present results indicate that almost half the glucose taken up is immobilized in the sugar pool. The slightly lower C^{14} incorporation from glucose-1 than from glucose-6 is probably the result of the loss of a small amount of glucose-1 carbon as CO_2 in the HMP pathway. Since this decarboxylation also results in the loss of tritium from glucose-1, with the formation of $NADPH^3$, it is interesting to note that glucose-1 is a

slightly better tritium donor for the neutral fraction than is glucose-6 (table III). This suggests that NADPH may serve as a hydrogen donor for the reductive synthesis of some component(s) in this fraction.

Ethanol-1 is also a good C^{14} and tritium precursor for the neutral fraction (table III). However, relative to the amount that is converted to CO_2, the carbon of ethanol-1 is not incorporated as efficiently into this fraction as is the carbon of glucose-1 and glucose-6. The same is true of the tritium incorporation, relative to the H^3_2O formed, although in this case the difference between ethanol and glucose is less marked. Neither ethanol itself nor any other volatile compounds would have been recovered in the fractionation scheme used in this study.

The Insoluble Fraction. The labeling patterns observed in the insoluble fraction are very similar to those seen in the neutral fraction (table III). There is considerably more incorporation of label from glucose-1 and glucose-6 than from ethanol-1. This is not surprising since some 60 % of this fraction of the pea stems is made up of polysaccharides (6), for which the sugars in the neutral fraction could serve as direct precursors. Glucose-1 is a significantly better precursor for the labeling of the insoluble fraction than is glucose-6, particularly with respect to tritium (table III). This difference may be accounted for in part by the loss of tritium and CO_2 from glucose-6 during the reactions which convert hexoses to uronic acids and pentoses. These compounds (as the nucleoside diphosphate derivatives) could then be used for the synthesis of hemicellulose and pectin (14) in the insoluble fraction. The 6-P-gluconic acid and ribulose-5-P which are formed by the dehydrogenation and decarboxylation of glucose-1 do not serve as direct polysaccharide precursors. These considerations could partly explain the higher H^3/C^{14} ratio for the incorporation from glucose-1 than from glucose-6 (table III). The fact that the H^3/C^{14} ratio for glucose-1 is significantly greater than unity suggests that some of the tritium may have entered the insoluble fraction by way of reductive synthesis using NADPH[8].

The Lipid Fraction. The metabolism of glucose and ethanol by pea stem sections would be expected to give rise to acetyl CoA and α-glycerophosphate, which could serve as precursors for lipid synthesis. The acetate (or acetyl CoA) formed from ethanol-1-C^{14}, H^3 will be $CH_3C^{14}OOH$, since the tritium is completely removed in the alcohol dehydrogenase and acetaldehyde dehydrogenase reactions. The acetate formed from glucose-1 and glucose-6, on the other hand, will be $C^{14}H^3H_2CO^{14}OH$. As expected, ethanol-1 is a much better carbon precursor for the lipid fraction than is either of the labeled glucoses (table III). On the other hand, both glucose-1 and glucose-6 are considerably more effective than ethanol as hydrogen donors. The very low H^3/C^{14} ratio for ethanol-1 incorporation into the lipid fraction (table III) shows this even more clearly. The greater effectiveness of glucose-6 than glucose-1 as a donor of both carbon and hydrogen for lipid synthesis can be explained by the fact that glucose-6 gives rise to labeled acetate (and glycerophosphate) whether it is metabolized by the glycolytic sequence or by the HMP pathway, whereas glucose-1 loses its label in the latter pathway. The fact that the absolute values for the incorporation of C^{14} and tritium from all 3 substrates into the lipid fraction are very low is probably a reflection of a low rate of lipid synthesis in this tissue.

In order to compare the effectiveness of NADH and NADPH as hydrogen donors for lipid biosyn-

Table III. *Incorporation of C^{14} and Tritium into Pea Stem Fractions*

Experimental detail as in table I. Incorporation (radiochemical yield) is expressed as percent of supplied label incorporated per 2-g tissue per 2 hours. Each value is the mean of 2 or more experiments.

Fraction	Substrate	C^{14} incorporation (%)	Tritium incorporation (%)	H^3 incorporation : C^{14} incorporation
Neutral	Ethanol-1	1.7	1.5	0.89
	Glucose-1	4.7	5.0	1.1
	Glucose-6	4.9	4.6	0.90
Insoluble	Ethanol-1	0.83	0.42	0.51
	Glucose-1	2.9	3.7	1.3
	Glucose-6	2.5	2.1	0.84
Lipid	Ethanol-1	0.099	0.0025	0.025
	Glucose-1	0.012	0.0078	0.65
	Glucose-6	0.022	0.0083	0.38
Basic	Ethanol-1	0.58	0.17	0.29
	Glucose-1	0.92	0.33	0.36
	Glucose-6	1.1	0.29	0.27
Acidic	Ethanol-1	0.67	0.26	0.39
	Glucose-1	0.67	0.17	0.25
	Glucose-6	0.80	0.55	0.61

thesis, Foster and Bloom (12) compared the tritium incorporation from glycerol-2-H^3 and from glucose-1-H^3 into lipids relative to the tritium incorporation from these substrates into water. Such a comparison is valid only if the amount of tritium that enters the lipid fraction by nonreductive incorporation is small, and the effectiveness of glucose-6-H^3 as a hydrogen donor can be used as a measure of such incorporation. However, with pea stem sections, the H^3/C^{14} ratio in the lipid fraction from glucose-6 is about 60 % of that from glucose-1 (table III), indicating that a large part of the glucose-1 hydrogen must enter the lipid fraction by passive or nonreductive incorporation. The difference in these H^3/C^{14} ratios is a measure of the amount of tritium entering lipid from glucose-1 via $NADPH^3$. This difference is some 10-fold greater than the H^3/C^{14} ratio for ethanol-1, indicating that NADPH is greatly preferred over NADH as a hydrogen donor for lipid synthesis in this tissue.

Basic and Acidic Fractions. Although the absolute incorporation into these 2 fractions varied from experiment to experiment, the relative amounts of incorporation from the 3 substrates were reproducible. The data in table III show that glucose-6 is a significantly better carbon and hydrogen donor for the acidic fraction than either glucose-1 or ethanol-1. The H^3/C^{14} ratio of 0.61 for glucose-6 indicates that 60 % of the tritium that was originally attached to the 6-position is retained in the acidic fraction. The fact that the H^3/C^{14} ratio for glucose-1 is lower (0.25) may be due to the loss of tritium during the oxidation and epimerization reactions which lead to the formation of hexonic acids. The difference is probably not due to the formation of keto acids which could readily be converted to amino acids by transamination or reductive amination, since the H^3/C^{14} ratio for glucose-6 is quite low in the basic fractions.

The basic fraction, which is composed primarily of amino acids, is not highly labeled. This fact makes it unlikely that much of the label found in the insoluble fraction (see above) is in proteins.

Mung Bean Hypocotyls. Sections of etiolated mung bean hypocotyls were supplied with C^{14}, H^3-labeled substrates by the same methods used for the pea stem segments. Table IV shows the extent of incorporation of radioactivity into the CO_2, H_2O, lipid fraction and the insoluble fraction. The results are similar in every respect to those obtained with pea stems. The C_6/C_1 ratios are almost identical. The data in the last column of table IV show that NADPH is the preferred hydrogen donor for lipid biosynthesis. Although ethanol-1 is a better hydrogen donor for water than is glucose-1, the difference between the 2 is small. The $H^3_2O/C^{14}O_2$ ratio for glucose-1 is much higher than for ethanol-1, suggesting that NADPH is oxidized by the tissue at least as readily as NADH.

Discussion

NADH and NADPH. The present results lend further support to the view that NADH and NADPH play different metabolic roles. The data presented indicate clearly that plant tissues use NADPH preferentially for the reductive synthesis of lipids, and there are suggestions that this may also be true for other cellular components. At the same time, NADPH appears to be as good a hydrogen donor for the reduction of molecular O_2 as is NADH. In this respect, the pea stem sections differ markedly from animal tissues where NADH is the preferred reductant for O_2 (3, 12).

Table IV. *Incorporation of C^{14} and Tritium into Fractions of Mung Bean Hypocotyl Sections*

Experimental details as in table I except 1-cm sections of etiolated mung bean hypocotyl were used instead of pea stem. Incorporation (radiochemical yield) is expressed as percent of supplied label incorporated per 2-g tissue per 2 hours. Each value is the mean of 2 experiments.

Fraction	Substrate	C^{14} incorporation (%)	Tritium incorporation (%)	H^3 incorporation : C^{14} incorporation
CO_2	Ethanol-1	4.2	...	...
	Glucose-1	0.54*	...	...
	Glucose-6	0.42*	...	...
H_2O	Ethanol-1	...	2.8	0.67**
	Glucose-1	...	1.8	3.3**
	Glucose-6	...	0.43	1.0**
Lipid	Ethanol-1	0.065	0.0031	0.05
	Glucose-1	0.001	0.0048	4.8
	Glucose-6	0.009	0.0036	0.40
Insoluble	Ethanol-1	0.42	0.28	0.67
	Glucose-1	0.90	1.3	1.4
	Glucose-6	0.90	0.94	1.0

* C_6/C_1 ratio = 0.78.
** The ratio given is H^3_2O; $C^{14}O_2$.

The apparent efficiency with which NADPH is oxidized by molecular O_2 in the pea stems is surprising in view of the fact that whole homogenates and mitochondria prepared from this tissue oxidize NADPH much less rapidly than NADH (23, 24). A possible explanation is that the tritium in NADPH equilibrates rapidly with NADH in the intracellular pools of the 2 nucleotides. However, such an equilibration seems unlikely in view of the fact that there is little or no NADPH-NAD transhydrogenase activity in the pea stem fractions (24). The ratios of the concentrations of NADPH to NADP and of NADH to NAD which have been reported for plant tissues (13, 29) provide little support for this alternative. Another possible explanation is that one or more of the soluble oxidase systems which are known to be present in plant extracts (4) might account for the rapid oxidation of both NADPH and NADH in vivo. Since the glucose 6-P and ethanol dehydrogenases are both soluble enzymes (24), $NADPH^3$ and $NADH^3$ generated from these substrates would be produced mainly in the cell sap. It may be that the very small amounts of $NADH^3$ and $NADPH^3$ produced in our experiments are rapidly reoxidized by a nonspecific soluble system before the $NADH^3$ can make contact with the mitochondrial respiratory chain. That this may indeed be the case is suggested by the finding that a 10-fold increase in substrate concentration results in an almost 20-fold increase in H^3_2O production from ethanol-1 and only a 5-fold increase in H^3_2O production from glucose-1 (table II). At the higher substrate concentration the soluble system(s) which oxidize $NADH^3$ and $NADPH^3$ may become rate-limiting; the excess $NADH^3$ could then be very rapidly oxidized by the mitochondrial respiratory chain, either directly (10, 23) or by means of a substrate shuttle (18).

Comparison with Other Plant Tissues. No data on tritium incorporation by other plant tissues are available for comparison with our results, but extensive studies on carbon incorporation from glucose-1, glucose-6 and ethanol-1 can be found in the literature (1, 2, 9, 25). Our glucose carbon incorporation results are in good agreement with the studies of Ap Rees and Beevers (1, 2) and Romberger and Norton (25) on carrot and potato slices, if one makes allowances for the much shorter incubation periods in our study.

On the other hand, our data on ethanol-1 carbon incorporation patterns are markedly different from those obtained by Cossins and Beevers (9) with 4-day-old pea shoots, but the experimental conditions in the 2 studies were so different that direct comparison may not be valid.

Summary

In an attempt to study the metabolic fates of NADH and NADPH in vivo in higher plants, pea stem sections were fed either ethanol-1-C^{14}, H^3 in order to generate $NADH^3$ intracellularly, or glucose-1-C^{14}, H^3 to generate $NADPH^3$. Glucose-6-C^{14}, H^3 was also fed in order to evaluate the contribution of the glycolytic pathway to total glucose breakdown. The C_6/C_1 ratio observed was 0.79, indicating a major role for the glycolytic path in glucose catabolism. Examination of the incorporation of C^{14} and H^3 into various fractions isolated from the tissue indicates that both glucose-1 and glucose-6 are good precursors for the sugar fraction and for the insoluble tissue residue, probably due to entry of intact hexose units into the intracellular sugar pool followed by incorporation of these units into polysaccharides. Ethanol-1-C^{14}, H^3 is not incorporated to any great extent into any of the tissue fractions; the label at this position is readily converted to $C^{14}O_2$ and H^3_2O, suggesting conversion of ethanol to acetate and subsequent oxidation via the citric acid cycle.

$NADPH^3$ produced from glucose-1 is a much better hydrogen donor for lipid biosynthesis than the $NADH^3$ produced from ethanol-1. In spite of the fact that mitochondria isolated from the tissue oxidize NADH very much more rapidly than NADPH, the whole-tissue experiments show no great difference between $NADH^3$ and $NADPH^3$ as hydrogen donors for cellular oxidations producing water. The $NADH^3$ and $NADPH^3$ produced in the soluble fraction may perhaps be oxidized by soluble enzyme systems.

Similar results were obtained in experiments with sections of mung bean hypocotyl.

Literature Cited

1. Ap Rees, T. and H. Beevers. 1960. Pathways of glucose dissimilation in carrot slices. Plant Physiol. 35: 830–38.
2. Ap Rees, T. and H. Beevers. 1960. Pentose phosphate pathway as a major component of induced respiration of carrot and potato slices. Plant Physiol. 35: 839–47.
3. Bloom, B. 1959. The intracellular occurrence of reduced diphosphopyridine nucleotide-coupled reactions in liver and kidney. J. Biol. Chem. 234: 2158–60.
4. Bonner, W. D. 1957. Soluble oxidases and their functions. Ann. Rev. Plant Physiol. 8: 427–52.
5. Calvin, M., C. Heidelberger, J. C. Reid, B. M. Tolbert, and P. E. Yankwich. 1949. Isotopic Carbon. Wiley, New York. p 93.
6. Christiansen, G. S. and K. V. Thimann. 1950. The metabolism of stem tissue during growth and its inhibition. I. Carbohydrates. Arch. Biochem. 26: 230–47.
7. Christiansen, G. S. and K. V. Thimann. 1950. The metabolism of stem tissue during growth and its inhibition. II. Respiration and ether-soluble material. Arch. Biochem. 26:248–59.
8. Christiansen, G. S. and K. V. Thimann. 1950. The metabolism of stem tissue during growth and its inhibition. III. Nitrogen metabolism. Arch. Biochem. 28: 117–29.
9. Cossins, E. A. and H. Beevers. 1963. Ethanol metabolism in plant tissues. Plant Physiol. 38: 375–80.

10. CUNNINGHAM, W. P. 1964. Oxidation of externally added NADH by isolated corn root mitochondria. Plant Physiol. 39: 699–703.
11. DICKENS, F., C. E. GLOCK, AND P. MCLEAN. 1959. Some problems in the choice of oxidative pathways of carbohydrate metabolism. In: Ciba Foundation Symposium on the Regulation of Cell Metabolism. G. E. W. Wolstenholme and C. M. O'Connor, eds Little, Brown, Boston. p 150–82.
12. FOSTER, D. W. AND B. BLOOM. 1961. A comparative study of reduced di- and triphosphopyridine nucleotides in the intact cell. J. Biol. Chem. 236: 2548–51.
13. FRITZ, G. J., E. R. STOUT, AND D. E. LEISTER. 1963. Estimation of nicotinamide nucleotide coenzymes in etiolated maize seedlings. Plant Physiol. 38: 642–48.
14. GINSBURG, V. 1964. Sugar nucleotides and the synthesis of carbohydrates. Advan. Enzymol. 26: 35–88.
15. HOBERMAN, H. D. 1958. Coupling of oxidation of substrates to reductive biosyntheses. I. Evidence of substrate specificity in the reductive synthesis of triose phosphate. J. Biol. Chem. 232: 9–16.
16. HORECKER, B. L. AND H. H. HIATT. 1958. Pathways of carbohydrate metabolism in normal and neoplastic cells. New Eng. J. Med. 258: 177–84 and 225–32.
17. KAPLAN, N. O., M. N. SWARTZ, M. E. FRECH, AND M. M. CIOTTI. 1956. Phosphorylative and nonphosphorylative pathways of electron transfer in rat liver mitochondria. Proc. Natl Acad. Sci. U.S. 42: 481–87.
18. KAPLAN, N. O. 1963. Multiple forms of enzymes. Bacteriol. Rev. 27: 155–69.
19. LOWENSTEIN, J. M. 1961. The pathway of hydrogen in biosynthesis. I. Experiments with glucose-1-H^3 and lactate-2-H^3. J. Biol. Chem. 236: 1213–16.
20. LOWENSTEIN, J. M. 1961. Reductions and oxidations in mammalian biosyntheses. J. Theoret. Biol. 1: 98–103.
21. NEAL, G. E. AND H. BEEVERS. 1960. Pyruvate utilization in castor bean endosperm and other tissues. Biochem. J. 74: 409–16.
22. OKITA, G. T., J. J. KABARA, F. RICHARDSON, AND G. V. LEROY. 1957. Assaying compounds containing H^3 and C^{14}. Nucleonics 15, No. 6: 111–15.
23. RAGLAND, T. E. AND D. P. HACKETT. 1961. The intracellular localization of some oxidative activities in pea stem. Biochim. Biophys. Acta 54: 577–80.
24. RAGLAND, T. E. AND D. P. HACKETT. 1964. Compartmentation of nicotinamide dinucleotide dehydrogenases and transhydrogenases in nonphotosynthetic plant tissues. Arch. Biochem. Biophys. 108: 479–89.
25. ROMBERGER, J. A. AND G. NORTON. 1961. Changing respiratory pathways in potato tuber slices. Plant Physiol. 36: 20–29.
26. SANWAL, B. D. AND M. LATA. 1962. Concurrent regulation of glutamic acid dehydrogenases of *Neurospora*. Arch. Biochem. Biophys. 97: 582–88.
27. SMITH, I., ed. 1961. Chromatographic and Electrophoretic Techniques, Vol. 1. Interscience, New York.
28. VITEK, V. 1964. Sugar-like, diphenylamine-positive metabolites in pea seedlings. Biochim. Biophys. Acta 93: 429–32.
29. YAMAMOTO, Y. 1963. Pyridine nucleotide content in the higher plant. Effect of age of tissue. Plant Physiol. 38: 45–54.