CHANGES IN LEVELS OF NICOTINAMIDE ADENINE NUCLEOTIDES AND KREBS CYCLE INTERMEDIATES IN MUNG BEAN LEAVES AFTER ILLUMINATION

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Summary

Changes in levels of nicotinamide adenine nucleotides were measured during a short (30 min) period of illumination following a dark period. Two phases in the time course were found. In the first phase, during the first minute of illumination, a rapid decline in oxidized nicotinamide adenine dinucleotide occurred which represented a net loss of nicotinamide adenine nucleotide. In the subsequent, second phase during illumination, a slower decline in oxidized nicotinamide adenine dinucleotide was found which was coincident with increases in nicotinamide adenine dinucleotide phosphate(s). The changes in the reduced nicotinamide adenine nucleotides were relatively small during illumination.

Changes of ^{14}C in intermediates of the Krebs cycle and related compounds, labelled with ^{14}C during a pre-incubation period in the dark in the presence of $^{14}CO_2$, were correlated with the changes in nicotinamide adenine nucleotides. Again two phases in the time course were observed. In the first minute of illumination a rapid decrease of ^{14}C in aspartate coincided with an equivalent increase in citrate. Subsequently, an apparent conversion of aspartate to malate was found.

These changes are discussed in relation to the effect of illumination on the ratio of oxidized to reduced nicotinamide adenine dinucleotides and Krebs cycle metabolism.

I. INTRODUCTION

The nicotinamide adenine nucleotides are metabolically related cofactors likely to be involved in the interactions of photosynthesis and respiration. Oh-hama and Miyachi (1960) have shown that illumination has marked effects on their levels in *Chlorella*, and very recently changes in levels of these compounds during light–dark transitions in *Beta*, *Spinacia*, and *Elodea* have been reported by Heber and Santarius (1965). Further, Graham and Walker (1962) showed that illumination of green leaves of mung bean resulted in considerable changes in the levels of intermediates of the Krebs cycle and related compounds, particularly malate and aspartate, and it was suggested that this was caused by changes in the ratio of oxidized to reduced nicotinamide adenine nucleotides. In the work now presented, reduced and oxidized NAD† and NADP† were assayed enzymatically in extracts from leaves of 7-day-old mung bean seedlings subjected to periods of darkness and illumination.

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† NAD and $NADH_2$ refer to oxidized and reduced nicotinamide adenine dinucleotide respectively; NADP and $NADPH_2$ refer to oxidized and reduced nicotinamide adenine dinucleotide phosphate respectively.

The changes in levels of the nicotinamide adenine nucleotides on illumination were correlated with changes in the levels of ^{14}C in Krebs cycle intermediates and related compounds. Thus the effects of light on nicotinamide adenine nucleotide levels are reflected in Krebs cycle metabolism.

II. Materials

(*a*) *Enzymes*

Crystalline alcohol dehydrogenase obtained from Sigma Chemical Co. was dissolved in water to give 3·56 mg/ml. Glucose-6-phosphate dehydrogenase was obtained as an ammonium sulphate suspension from Boehringer und Soehne, Mannheim, W. Germany, and 0·15 ml was diluted to 2·0 ml with 0·1M Tris.HCl buffer, pH 7·4.

(*b*) *Plant Material*

Mung bean seedlings (*Phaseolus aureus* L.) were grown from seed for 7 days on vermiculite floating on deionized water in a constant-temperature room (25±0·5°C). A 16-hr day/8-hr night regime was provided using twelve 40-W Warm White fluorescent tubes (Mazda Co., Sydney) at a distance of 50 cm.

III. Methods

(*a*) *Nicotinamide Adenine Nucleotide Measurements*

The plants were placed in a darkened room for at least 1 hr before samples were obtained by cutting off the first pair of leaves at the petiole. During sampling, illumination was provided by a green safelight with maximum emission at 520 mμ (Withrow and Price 1957). Samples comprising seven or eight leaves (total fresh weight 250±5 mg) were placed on moist filter paper with the abaxial sides uppermost. These samples were kept in the dark for 30 min and then illuminated by a 250-W reflector spotlamp 35 cm away (*c.* 30,000 lux). An intervening water-cooled heat filter, 2·5 cm in width, of 3% ferrous ammonium sulphate in 2% H_2SO_4 (Withrow and Price 1953), maintained the samples at room temperature. After the prescribed treatment the samples were rapidly transferred to liquid air and then extracted by homogenizing for 30 sec in 3 ml of ice-cold 0·1N HCl (for oxidized nicotinamide adenine nucleotides) or 0·1N NaOH (for reduced nicotinamide adenine nucleotides) contained in a Potter–Elvehjem all-glass homogenizer with a variable-speed, motor-driven plunger. The homogenates were heated in a boiling water-bath for 2·0 min, centrifuged at 30,000 *g* for 30 min at 0°C with about 5 ml of deionized water rinsings, and the pH values of the decanted supernatants adjusted by addition of 5 ml of 0·2M Tris.HCl buffer at pH 8·0 for the acid extracts and pH 7·0 for the alkaline extracts. The addition of 2 ml of 2,6-dichlorophenolindophenol at 0·35 mg/ml was sufficient to give a permanent blue colour to the supernatant which was then made up to 25 ml with ice-cold, deionized water and kept at 0°C. The pH of the final solution was 7·2–7·4 (acidic extraction) or 7·4–7·6 (alkaline extraction). The supernatants were assayed usually within 30 min of neutralization but could be stored for up to 4 hr at 0°C in the acidic or alkaline condition without significantly affecting the levels of nicotinamide adenine nucleotides.

The diluted supernatant was assayed for nicotinamide adenine nucleotides by a cyclic enzymic system, modified after that of Villee (1962) and Slater, Sawyer, and Sträuli (1964), phenazine methosulphate being used as electron carrier. The rate of decolorization of dichlorophenolindophenol, which is proportional to the concentration of nicotinamide adenine nucleotide, was measured in the interval 2–7 min after the beginning of the reaction in 1 cm light path cuvettes at 610 mμ and 25°C in a Beckman DU spectrophotometer fitted with a Gilford automatic cuvette positioner and absorbancy indicator linked to a recorder. The reaction mixtures were as follows:

(1) *For NAD and $NADH_2$ assay:* 0·1 ml of 95% (v/v) ethanol; 0·3 ml of 0·2M Tris.HCl buffer, pH 7·4; 0·2 ml of 1·2 mM 2,6-dichlorophenolindophenol; 0·5 ml of 40 mM EDTA (sodium salt neutralized to pH 7·4); and 0·1–0·5 ml of neutralized acidic leaf extract, or 0·3–1·5 ml of neutralized alkaline leaf extract; and deionized water to 2·75 ml. After standing for 8–10 min for temperature equilibration and to allow non-enzymic reactions to go to completion, 0·15 ml of 10 mM phenazine methosulphate was added followed by 0·1 ml of diluted alcohol dehydrogenase to start the reaction.

(2) *For NADP and $NADPH_2$ assay:* 0·3 ml of 10 mM D-glucose-6-phosphate; 0·3 ml of 0·2M Tris.HCl buffer, pH 7·4; 0·2 ml of 1·2 mM dichlorophenolindophenol; 0·05 ml of 20 mM EDTA (pH 7·4); 0·1–1·0 ml of neutralized acidic leaf extract, or 0·3–1·5 ml of neutralized alkaline leaf extract. The reaction mixture was made up and then treated in the same way as for the NAD assay, except that the reaction was started by the addition of 0·1 ml of diluted glucose-6-phosphate dehydrogenase.

The assay results were corrected for an "extract blank" containing the extract and all the reagents except the enzyme. "Enzyme blanks", in which all reagents and enzyme but not extract were present, did not show any significant change of extinction with time. The assay measures nicotinamide adenine nucleotides in the range 0·05–1·0 mμmole per 3 ml of reaction mixture.

In order to measure $NADH_2$, NADP, and $NADPH_2$, which were present in the extracts in exceedingly low amounts, it was usually necessary to increase the sensitivity of the assays two- or threefold by reducing the final volume of the assay to 1·5 or 1·0 ml respectively. In these assays the concentrations of all reagents were kept the same as for the 3-ml assay with the exception of the buffer, which was increased two- or threefold to maintain buffering capacity. The assays were standardized by the addition of 0·2 – 1·0 mμmole of nicotinamide adenine nucleotide to the 3-ml assay, and 0·05–0·4 mμmole to the 1-ml assay, in place of extract.

Recoveries of standard nicotinamide adenine nucleotide solutions added to the extracting medium immediately before homogenization of the tissue gave the following mean percentage recoveries and standard error of means: NAD 92·0$\pm$1·7; $NADH_2$ 95·7$\pm$1·1; NADP 94·8$\pm$6·5; $NADPH_2$ 82·5$\pm$3·1. Evidence regarding the validity of the methods used for the extraction and assay of nicotinamide adenine nucleotides will be presented in a separate paper.

(b) Radiocarbon Labelling Experiments

Six samples, each of four leaves, were together fed 120 μc of $^{14}CO_2$ obtained by injecting a solution of sodium [^{14}C]carbonate (specific activity, 22·7 mc/m-mole) into lactic acid in a central container inside a sealed, 75-ml petri dish. The initial concentration of carbon dioxide was thus about 0·20% (v/v). The leaves were kept in the dark in the presence of $^{14}CO_2$ for 2 hr. The lid was then removed from the petri dish and the leaves left in the dark for a further 15 min to remove excess $^{14}CO_2$.

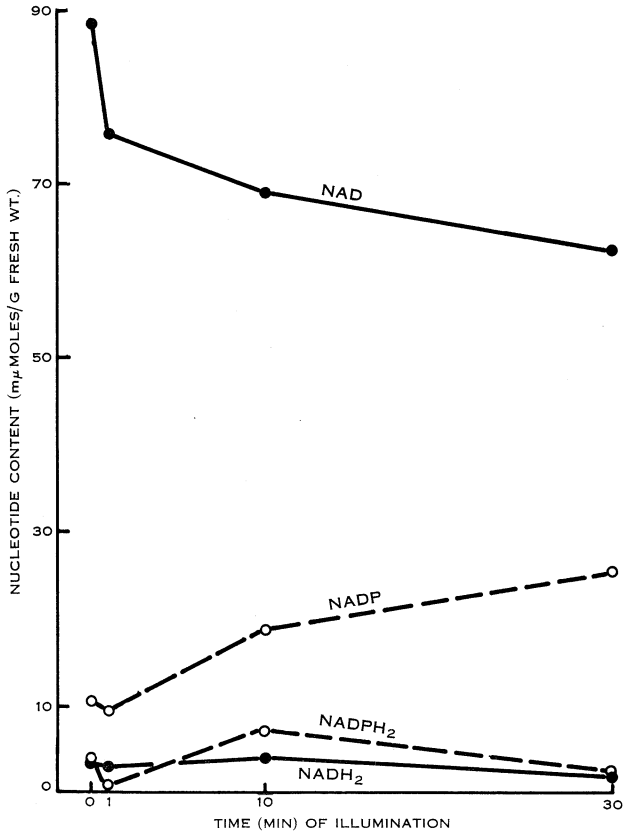

Fig. 1.—Changes in levels of oxidized and reduced nicotinamide adenine nucleotides of mung bean leaves during illumination.

They were then illuminated with the reflector spotlamp as described above. The samples were taken in the dark and after various periods of illumination. Compounds soluble in aqueous ethanol were extracted by boiling the leaves in 80% (v/v) ethanol for 3 min and then extracting them twice with 20% ethanol. Aliquots of the combined, concentrated, ethanolic extracts were chromatographed on Whatman No. 1 paper in two directions using, firstly, phenol–water and, secondly, n-butanol–acetic acid–water (Benson *et al.* 1950). Radioactive compounds, located by radioautographs, were cut out and counted in a Packard Tricarb 3000 Series liquid scintillation counter.

IV. Results

(a) *Changes in Nicotinamide Adenine Nucleotides*

Figure 1 shows typical changes in levels of nicotinamide adenine nucleotides when detached mung leaves were illuminated. Data for some other experiments are given in Table 1. Major changes were a decrease in NAD and an increase in NADP.

Table 1

LEVELS OF NICOTINAMIDE ADENINE NUCLEOTIDES IN EXCISED MUNG BEAN LEAVES DURING THE COURSE OF ILLUMINATION

Values of nicotinamide adenine nucleotides are mμmoles/g fresh weight

Nucleotide	Time of Illumination (min)	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
NAD	0 (dark)	56·4	67·7	90·0	116·0	90·1
	1	60·5	62·6	—	92·0	—
	10	59·0	60·4	68·0	—	60·8
	30	37·5	45·7	—	62·0	—
$NADH_2$	0 (dark)	4·3	5·3	—	5·9	5·6
	1	3·8	4·8	—	7·7	—
	10	5·5	3·6	—	6·9	7·7
	30	2·7	4·1	—	6·1	—
NADP	0 (dark)	29·4	—	9·7	—	—
	10	—	—	19·6	—	—
	30	42·5	—	—	—	—
$NADPH_2$	0 (dark)	10·2	—	4·0	—	—
	10	—	—	6·0	—	—
	30	16·0	—	—	—	—

The decrease in NAD usually occurred in two phases, being rapid in the first minute and subsequently slower. The initial decrease was found in only four out of six

Table 2

LEVELS OF NICOTINAMIDE ADENINE NUCLEOTIDES IN MUNG BEAN LEAVES EXCISED FROM 7-DAY-OLD SEEDLINGS AND KEPT FOR 90 MIN EITHER IN LIGHT OR DARK

Values of nicotinamide adenine nucleotides are mμmoles/g fresh weight

	NAD	$NADH_2$	NAD+ $NADH_2$	Ratio NAD/ $NADH_2$	NADP	$NADPH_2$	NADP+ $NADPH_2$	Ratio NADP/ $NADPH_2$	Total Nucleo-tides
Dark	49·0	1·4	50·4	35·0	9·7	4·1	13·8	2·4	64·2
Light	34·5	2·3	36·8	15·1	19·6	6·5	26·1	3·0	62·9

experiments reported here. (The reason for this variability is not known but may be connected with the time the leaf samples were taken during the 16-hr day/8-hr night

regime.) The initial decrease in NAD involved a net loss of nicotinamide adenine nucleotides, since no commensurate increases in $NADH_2$, NADP, or $NADPH_2$ were found. Binding of NAD in the pellet obtained on centrifuging is unlikely to account for the loss, because experiments with pepsin-treated pellets showed that insignificant amounts of NAD or NADP were liberated by such treatment.

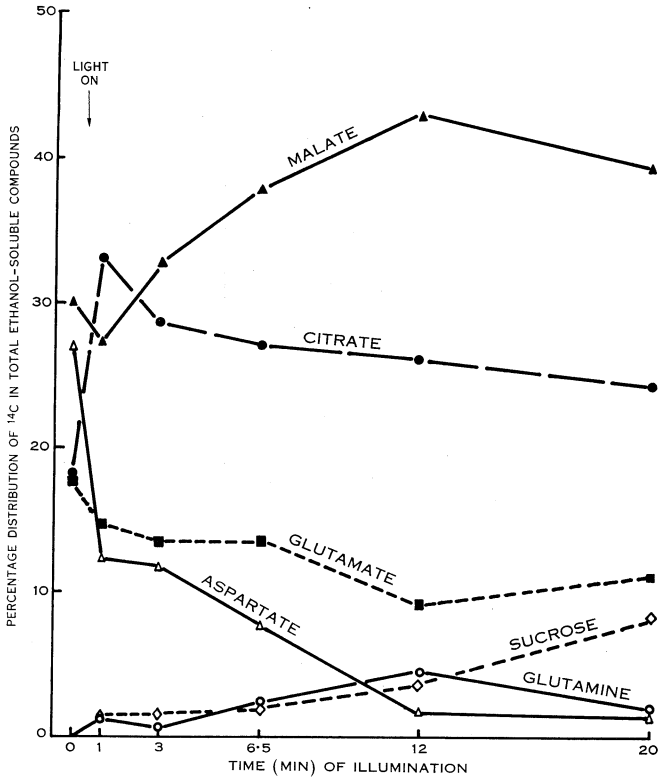

Fig. 2.—Changes in levels of ^{14}C-labelled compounds in mung bean leaves during illumination.

The slower, second phase of decrease of NAD is apparently due to a conversion to NADP, presumably as a result of light-induced NAD kinase activity (Oh-hama, Miyachi, and Tamiya 1963):

$$NAD+ATP \longrightarrow NADP+ADP.$$

The conversion of NAD to NADP is indicated more clearly in Table 2, which shows the marked changes in the levels of nicotinamide adenine nucleotides upon prolonged illumination, the leaves in this experiment being either left in darkness or illuminated for 90 min. Under either condition no change in total nicotinamide adenine nucleotide occurred, but there was apparently a stoichiometric conversion of NAD to NADP + $NADPH_2$ in the light. In addition, the ratio $NAD/NADH_2$ changed from 35·0 in the dark to 15·1 in the light, and the ratio $NADP/NADPH_2$ was slightly greater in the light than that in the dark.

(*b*) *Changes in ^{14}C-labelled Intermediates*

Figure 2 shows the relative distribution of ^{14}C in isotopically labelled ethanol-soluble compounds (as a percentage of total ethanol-soluble ^{14}C) for a typical illumination experiment. $^{14}CO_2$ was fed to samples of leaves in the dark and subsequently removed in the dark over a period of 15 min. This procedure labels mainly compounds of the Krebs cycle and closely related intermediates, e.g. aspartate and glutamate. Samples were taken during the course of 20 min illumination. The data in Figure 2 account for about 90% of the ^{14}C in ethanol-soluble compounds, and changes in other labelled compounds during illumination were slight. During the first phase seen in Figure 2, extending over the first minute of illumination, there was a very rapid decrease of ^{14}C in aspartate and a coincident accumulation of it in citrate. In the second phase, i.e. after 1 min illumination, a slow accumulation of label in malate occurred, this being approximately accounted for by a coincident decrease of label in aspartate.

V. Discussion

To account for the above results, it is suggested that the changes in levels of ^{14}C in malate, aspartate, and possibly citrate on illumination are related to the changes in the ratios of nicotinamide adenine nucleotides, particularly the ratio NAD/$NADH_2$. The latter ratio would be expected to be important in the control of the reaction catalysed by malic dehydrogenase, for which the equilibrium constant, K, given by Stern, Ochoa, and Lynen (1952) is:

$$K = \frac{[\text{oxaloacetate}][NADH_2]}{[\text{malate}][NAD]} = 2{\cdot}3 \times 10^{-5} \text{ (pH } 7{\cdot}2).$$

Hence, a decrease in NAD/$NADH_2$ on illumination would be expected to favour malate synthesis and limit the supply of oxaloacetate for the $C_4 + C_2$ condensing reaction in the Krebs cycle. Nevertheless, in the first minute of illumination such a limitation appears to be overcome, since a rapid initial depletion of [^{14}C]aspartate occurs; presumably this is through conversion of aspartate to oxaloacetate, e.g. by a transaminase reaction. Much of the ^{14}C from this source could accumulate in citrate through the change in NAD/$NADH_2$ limiting the rate of isocitric dehydrogenase (NAD-mediated) in the direction of decarboxylation. However, it is not clear why the ^{14}C from aspartate should appear in citrate rather than in malate during the first phase, since the similarity of the relevant kinetic constants (Hiatt and Evans 1960; Hiatt 1962) argues against disparate competition between malic dehydrogenase and condensing enzyme for the available oxaloacetate.

It seems unlikely that the changes in ^{14}C levels of Krebs cycle intermediates found in the first minute of illumination could be accounted for by intracellular compartmentalization of the various metabolite pools. A possible explanation is that, as the ratio of NAD/$NADH_2$ is falling during the first minute of illumination, the malic dehydrogenase reaction supplying oxaloacetate for the reaction of condensing enzyme becomes curtailed and consequently aspartate is used temporarily as an alternative source for oxaloacetate. After the first minute of illumination the ratio NAD/$NADH_2$, or perhaps more particularly the level of NAD, has become so markedly

decreased that the malic dehydrogenase step is essentially reversed and the ^{14}C-labelled pool(s) of aspartate is then converted to malate. It has been shown (Santarius and Heber 1965) that in the leaves of at least some species the level of pyruvate declines sharply during the first minute of illumination. This is consistent with the conversion of pyruvate to acetyl coenzyme A and the condensation of the latter with oxaloacetate to give citrate, and also with the rapid inhibition of glycolysis in the light (see, for example, Kandler and Haberer-Liesenkötter 1963).

The likely breakdown product of NAD, resulting in the net loss of nicotinamide adenine nucleotide during the first minute of illumination, is AMP (Kornberg and Pricer 1950). Such an accumulation of AMP and a net increase in adenine nucleotides (of about the right order of magnitude) has been found in *Coleus* leaves (Santarius and Heber 1965), in conditions similar to those of the mung bean leaves, but not in *Beta* or *Spinacia*.

It is suggested that during the second phase of ^{14}C changes the decrease in $NAD/NADH_2$ drives the malic dehydrogenase catalysed reaction in the direction of malate synthesis, and aspartate is apparently again the principal source of ^{14}C via oxaloacetate. It is emphasized that the dark fixation of $^{14}CO_2$ labels probably only one particular pool of malate and aspartate, so that it is not necessarily implied that the Krebs cycle ceases to function in the light. Alternative sources of unlabelled C_4 acids may be available for the condensation to form citrate. It is interesting therefore that Marsh, Galmiche, and Gibbs (1965) have recently shown that in *Scenedesmus*, under their conditions, the Krebs cycle functions at the same rate in the light or dark.

Our data provide support for the postulation of Graham and Walker (1962) that the reciprocal changes in the malate/aspartate ratio occurring between darkness and illumination are explainable in terms of a lower ratio of $NAD/NADH_2$ in the light than in the dark.

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