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The release of ammonia from pyridine nucleotides in alkali and its importance in enzymic studies of nitrate assimilation

During an investigation of the enzymes involved in the assimilation of nitrate by higher plants[1,2] it was necessary to measure the ammonia content of enzymic reaction mixtures containing pyridine nucleotides (DPNH and DPN^+). Following the practice of earlier investigators who have reported the enzymic conversion of nitrite to ammonia with DPNH or TPNH as the electron donating cofactor[3,4] ammonia was separated from complex reaction mixtures using CONWAY'S[5] microdiffusion technique and then it was determined by the alkaline phenolate–hypochlorite method of RUSSELL[6].

Initial results with cell-free enzyme preparations from tomato seedlings[2] indicated that enzymes were present which would reduce nitrate to ammonia in the presence of DPNH and without other added cofactors. However, these results were highly variable so an investigation of the conditions contributing to this variability

TABLE I

THE EFFECT OF THREE ALKALINE REAGENTS ON THE BREAKDOWN OF PYRIDINE NUCLEOTIDES TO YIELD AMMONIA DURING 6 h MICRODIFFUSION IN CONWAY UNITS

Each CONWAY unit contained 1.0 mg of pyridine nucleotide in 1.0 ml distilled water. Microdiffusion of ammonia was brought about by 1.0 ml of alkaline reagent as shown in the table.

Pyridine nucleotide	*Alkaline reagent*	*Ammonium-nitrogen recovered (μg)*
DPN^+	0.5 N NaOH (ref. 12)	8.42
	Saturated K_2CO_3 (ref. 5)	2.00
	Saturated borate–hydroxide buffer (pH 10.1) (ref. 13)	0.63
DPNH	0.5 N NaOH (ref. 12)	1.67
	Saturated K_2CO_3 (ref. 5)	0.30
	Saturated borate–hydroxide buffer (pH 10.1) (ref. 13)	0.08

was undertaken. This investigation showed that DPN^+ was readily broken down to yield ammonia under the alkaline conditions of the CONWAY microdiffusion while DPNH was affected to a much lesser extent. The effect of three alkaline reagents frequently used in microdiffusion work on DPNH and DPN^+ is shown in Table I. The breakdown of DPN^+ increases with the length of the microdiffusion period as shown in Table II.

These results suggested that the apparant production of ammonia might actually be due to DPNH oxidase activity. That is to say, in studies of this nature the production of ammonia is normally measured as the increase in ammonia content of reaction mixtures with time as compared to control reaction mixtures having zero time conditions, *i.e.* with all pyridine nucleotides in the reduced form. However, these results show that such blank values are incorrect. The actual blank increases as the DPNH is oxidized due to the greater lability of DPN^+ in alkali as compared

TABLE II

THE BREAKDOWN OF DPN^+ TO YIELD AMMONIA DURING MICRODIFFUSION PERIODS OF VARYING LENGTHS

Each CONWAY unit contained 1.0 mg DPN^+ in 1.0 ml distilled water. The alkali used was 1.0 ml saturated K_2CO_3 (ref. 5).

Length of microdiffusion period (h)	*Ammonium-nitrogen recovered (μg)*
0	0
0.5	0.28
1.0	0.32
1.5	0.47
2.0	0.89
3.0	1.21
6.0	2.43

to DPNH so that an oxidation of DPNH could by itself appear as a production of ammonia.

Tests for the presence of DPNH oxidase activity in the preparations under investigation were made. It was found that high DPNH oxidase activity was present. These results are shown in Fig. 1. The error which these conditions can introduce into the results of investigations of enzymic ammonia production in the presence of pyridine nucleotides is an insidious one because the source of the error is itself enzymic. The apparant ammonia production, which is merely a function of DPNH oxidation itself, will vary under experimental conditions in the same way that an enzymic production of ammonia from a substrate would be expected to vary.

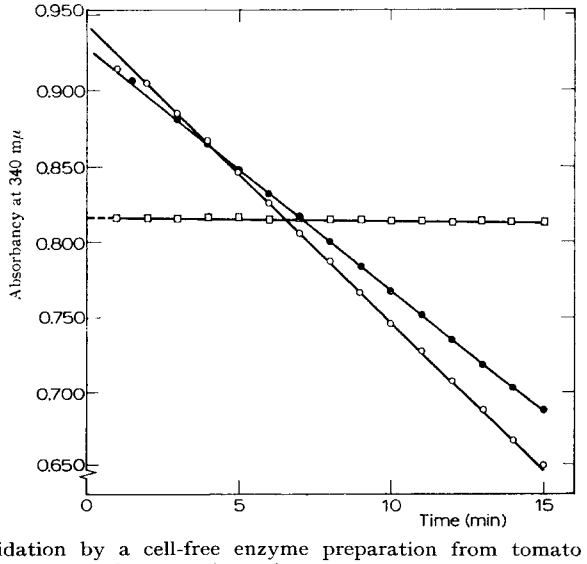

Fig. 1. DPNH oxidation by a cell-free enzyme preparation from tomato seedlings. ○—○, DPNH oxidation in a complete reaction mixture containing 90 μmoles phosphate as buffer (pH 7.5), 30 μmoles KNO_3, 0.45 μmole DPNH and 0.90 ml enzyme preparation (4.11 mg protein) in a total volume of 3.0 ml. ●—●, DPNH oxidation in the complete reaction mixture minus KNO_3. □—□, DPNH oxidation in the complete reaction mixture minus the enzyme preparation.

In order to correct for an increase in blank ammonia content of reaction mixtures with increasing levels of DPNH oxidation, the following procedure was developed. Ammonia released from control reaction mixtures in which the pyridine nucleotides were either wholly reduced or wholly oxidized was determined with every set of assays. These conditions were obtained by adding the pyridine nucleotides (the best grade obtainable from Sigma Chemical Co., St. Louis, U.S.A.) to the reaction mixtures in either the oxidized or the reduced form, at the correct level, and then determining the ammonia content immediately. These levels of ammonia were taken as the blank values for reaction mixtures with 0% and 100% DPNH oxidation respectively. These values were used to plot an ammonia blank chart with per cent DPNH oxidation as ordinate and ammonia blank as abscissa. The percent DPNH oxidation which had taken place in each reaction mixture was determined spectrophotometrically by measuring the absorbance of reaction mixtures at 340 mμ (ref. 7) at the time of their sampling for ammonia determination. With this information, the appropriate ammonia blank could be read off the ammonia blank chart. Further investigation of the cell-free preparations of tomato seedlings[1,2] showed that there was indeed no reduction of nitrate to ammonia when either DPNH or TPNH were used as electron donors. This is in agreement with the recent findings of HAGEMEN, CRESSWELL AND HEWITT[8].

The differential susceptibility of DPNH and DPN^+ to degradation in alkali has been recognized for a considerable time[9–11] but it appears that the importance of this property of the pyridine nucleotides has not been fully appreciated. Accordingly, the earlier reports of investigations purporting to show an enzymic reduction of nitrite to ammonia with reduced pyridine nucleotides as electron donors[3,4] should be interpreted with some caution.

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