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DEGRADATION OF NICOTINAMIDE ADENINE DINUCLEOTIDE IN CELL SUSPENSION CULTURES*

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Key Word Index—Leguminosae; Umbelliferae; cell suspension cultures; catabolism; NAD; trigonelline; nicotinic acid *N*-α-L-arabinoside; adenine; allantoin.

Abstract—Metabolites of [carbonyl-^{14}C]-NAD in cell suspension cultures of mung bean, soybean and garbanzo bean are trigonelline and compounds of the pyridine nucleotide cycle. Degradation of nicotinate does not occur. In parsley cell cultures nicotinate degradation and formation of nicotinic acid *N*-α-L-arabinoside were observed. These conjugates are alternative reservoir forms of nicotinic acid. The adenine moiety of NAD is degraded in cell cultures via hypoxanthine–xanthine–allantoin–allantoic acid, with accumulation of the latter two compounds.

INTRODUCTION

Plant cell suspension cultures are suitable systems for degradative studies on aromatic [1–5] and heterocyclic [6–10] compounds. In our recent investigations on the metabolism of nicotinic acid and nicotinamide in plant cell suspension cultures [7, 11–13] we have identified *N*-methylnicotinic acid (trigonelline) (**1**) and nicotinic acid *N*-α-L-arabinoside (**2**) as important reservoir forms of nicotinic acid. **1** and **2** form species-characteristic [14] metabolites in the cell cultures and their occurrence seems to be strictly alternative. The nicotinic acid moiety of **1** and **2** can be used for NAD-resynthesis [12, 13] while **2** can also be funnelled into catabolic pathways [11, 13]. Trigonelline has long been known from higher plants and seeds [15], while the arabinoside of nicotinic acid has only recently been detected in cell cultures [11, 13]. For the bioynsthesis of trigonelline and other pyridine alcaloids (i.e. nicotine and ricinine) the nicotinic acid moiety of NAD and other compounds of the pyridine nucleotide cycle can be used [16–18]. This cycle, operating in microorganisms [19], animals [20] and plants [21], assures that nicotinic acid generated by NAD degradation is refunnelled into the pyridine nucleotides. Both for plants [22] and plant cell cultures [12, 13] the metabolism of **1** and **2** seems to be linked to the pyridine nucleotide cycle. We now report on the uptake and the degradation of NAD by various plant cell suspension cultures and have studied both the pyridine and the purine moieties of NAD by using specifically labelled substrates.

RESULTS

Batch-propagated, dark-grown cell suspension cultures of garbanzo bean (*Cicer arietinum* L.), mung bean (*Phaseolus aureus* Roxb.), soybean (*Glycine max* Merr.) and parsley (*Petroselinum crispum* (Miller) A. W. Hill) were used in the late logarithmic phase. All substrates were applied in a 10^{-5} M concentration and $^{14}CO_2$ generated by the cells was collected over various periods of time. Nutrient medium and ethanol or water extracts of the cells were finally analysed for any unchanged substrate and catabolites.

Uptake of NAD by plant cell cultures

Though uptake of substrates by plant cell suspension cultures is quite often very rapid [1, 3, 7, 13] the rate of uptake of the highly charged NAD was measured. Fig. 1 demonstrates that the uptake of [carbonyl-^{14}C]-NAD by the four cell suspension cultures is comparatively very slow because the uptake of nicotinic acid and nicotinamide by the parsley cultures proceeds at least 10 times more rapidly [13]. Though the individual cell cultures showed comparable fr. wts, there are striking differences in NAD uptake. While the uptake of NAD in the parsley cultures went to completion, only 10–25% of the substrate entered the cells in the garbanzo bean and mung-bean cultures Considering the rapid penetration of

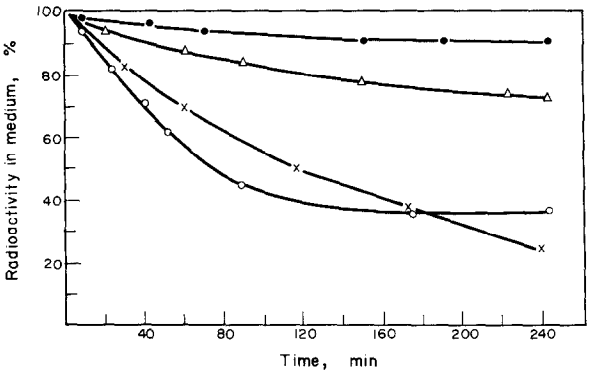

Fig. 1 Uptake of [carbonyl-^{14}C]-NAD (10^{-5} M) by cell suspension cultures of *Cicer arietinum* (●—●), *Phaseolus aureus* (△—△), *Petroselinum crispum* (×—×), and *Glycine max* (○—○) as measured by decrease of radioactivity in the medium.

*Part V in the series "Metabolism of Nicotinic Acid in Plant Cell Suspension Cultures".

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Table 1. Distribution of radioactivity (as %) in nicotinic acid and derivatives after 4 hr application of [carbonyl-^{14}C]-NAD to cell cultures

Cell culture \ Compound	Nicotinic acid arabinoside (2)	Trigonelline (1)	Nicotinic acid/ nicotinamide	NAD	Unknown compound X
Cicer arietinum	0	10	80	8	2
Phaseolus aureus	0	20	18	43	13
Glycine max	0	85	3	8	1
Petroselinum crispum	67	0	22	2	0

nicotinic acid and the even more rapid influx of nicotinamide [13], the slow uptake of NAD (Fig. 1) demonstrates that the nucleotide was obviously not hydrolysed by any exocellular enzymes. This was corroborated by incubating cell free nutrient medium of 7-day-old cultures with NAD (10^{-5} M). Electrophoretic and chromatographic analyses revealed no catabolites of NAD. Despite the low NAD uptake in mungbean and soybean cultures, the isolatable radioactivity allowed detailed analyses of cellular NAD catabolites.

Degradation of [carbonyl-^{14}C]-NAD

After application of [carbonyl-^{14}C]-NAD to the four cell suspension cultures, the formation of $^{14}CO_2$ was followed for up to 160 hr. In agreement with our previous results on nicotinic acid and nicotinamide [7, 13], only the parsley cell suspension culture produced $^{14}CO_2$ (22 %), while the three legume cultures showed negligible amounts of less than 0.1 %. During the whole experiment the $^{14}CO_2$ production in parsley was completely linear with time indicating that a main metabolite is funnelled at a constant rate in catabolic pathways.

Cell extracts of the mungbean, soybean and garbanzo bean cultures when analysed for catabolites of [carbonyl-^{14}C]-NAD by PC (solvents L_1, L_2, L_3 and L_4) and paper electrophoresis followed by scanning contained labelled NAD, nicotinic acid/nicotinamide, low amounts of an unknown compound *X* and mostly trigonelline (**1**). The nicotinic acid *N*-α-L-arabinoside (**2**) could not be found. The quantitative ratio of these metabolites after a 4 hr incubation in the three legume cell cultures is presented in Table 1. The low amounts of trigonelline in the cell cultures of *C. arietinum* and especially *Ph. aureus* (see 12] were suprising, because comparable studies with nicotinic acid in the mungbean culture had previously led to rather high values of *ca* 80 % in **1**.

Similar studies with [carbonyl-^{14}C]-NAD in parsley cell cultures resulted in the isolation of labelled nicotinic acid, nicotinamide, NAD and as predominant metabolite the arabinoside **2**; trigonelline could be excluded as catabolite in this cell culture (Table 1).

Various investigations have demonstrated that NAD is either not taken up by cells as an intact molecule [23, 24] or hydrolysed immediately during/after penetration into the cells [25]. Therefore, cell suspension cultures of *Ph. aureus* and *P. crispum* were investigated to see how rapidly and to what extent label from exogenously applied [carbonyl-^{14}C]-NAD (10^{-5} M) appeared in the cellular metabolites. These kinetic studies demonstrate that in the early phases of the experiments (after 30 min) no or extremely low amounts of NAD could be isolated while trigonelline (~25 %) or the nicotinic acid arabinoside (~65 %) were the most prominent compounds. Due to the slow penetration of label into the cells (Fig. 1) shorter incubation periods could not be chosen. The data do, however, indicate that most of the NAD is hydrolysed upon uptake and that the formation of either **1** or **2** may be linked with the uptake process. The comparably high increase in NAD radioactivity in mungbean cell cultures after 30 min seems to be mostly due to incorporation of nicotinic acid/nicotinamide as indicated by the decrease of the respective radioactivity curve. Furthermore, the data support the assumption that a rather constant ratio of nicotinic acid/nicotinamide/trigonelline or nicotinic acid arabinoside, respectively, is established in the cells [cf. 11 and 13].

Cell suspension cultures of *Cicer arietinum* L. consistently showed low levels of trigonelline together with comparably high amounts of nicotinic acid regardless of whether [carbonyl-^{14}C]-NAD (Table 1), [carbonyl-^{14}C]-nicotinic acid [13] or [carbonyl-^{14}C]-nicotinamide [7] were used as precursor. The additional application of L-methionine (10^{-4} M or 10^{-5} M) as donor for methyl groups 30 min prior to labelled nicotinic acid (or NAD) did not result in any increase in the level of **1**.

Degradation of [adenine-U-^{14}C]-NAD

Since two alternative routes exist for the metabolism and degradation of the nicotinamide moiety of NAD in the four cell suspension cultures [see 26], comparable studies with [adenine-U-^{14}C]-NAD (10^{-5} M) have shown a great similarity with respect to the adenine portion. The data in Fig. 2 demonstrate that the four cell suspension cultures all produce substantial though not quantitative amounts of $^{14}CO_2$.

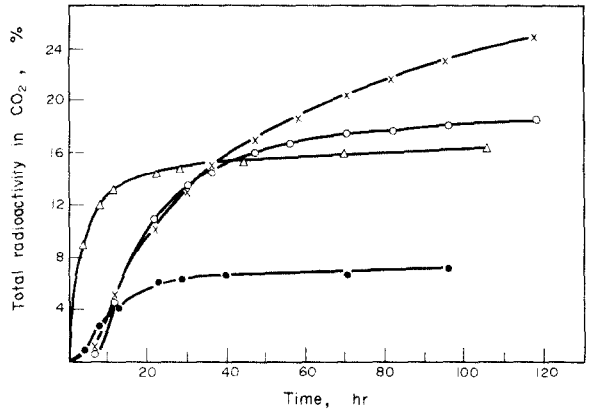

Fig. 2. Formation of $^{14}CO_2$ in cell suspension cultures of *Cicer arietinum* (●—●), *Phaseolus aureus* (△—△), *Petroselinum crispum* (×—×) and *Glycine max* (○—○) after application of [adenine-U-^{14}C]-NAD (10^{-5} M).

When cell extracts of the four cell cultures were analysed by PC (solvent systems L_4, L_5, L_6, L_7 and L_8) and paper electrophoresis, several radioactive spots could be detected. The two most important metabolites in all four cell cultures were finally identified as allantoin and allantoic acid by cochromatography, spraying with *p*-dimethylaminobenzaldehyde [27] and derivatization. Thus, allantoin was converted to 5-(ω-xanthyl-ureido)-hydantoin [28] and allantoic acid to bis-(ω-xanthyl-ureido) hydantoin [29] followed by cochromatography of these derivatives in solvent system 9 and by dilution analysis.

Due to the limited uptake of NAD by the cells, some of the other labelled catabolites of [adenine-^{14}C]-NAD were too low in radioactivity to allow further identification.

Degradation of [8-^{14}C]-*adenine*

Most of the catabolites generated from [adenine-U-^{14}C]-NAD could be obtained more readily by feeding to the cells [8-^{14}C]-adenine. This was indicated by a PC comparison (solvent system L_8) with scanning of cell extracts after application of the two labelled substrates. The much higher yield of catabolites obtained with [8-^{14}C]-adenine in comparison to NAD seems mainly to be due to the much better and more rapid uptake of adenine (10^{-5} M) by the four cell suspension cultures. In experiments similar to those described in Fig. 1, adenine was taken up to more than 90% within 60–100 min. Chromatographic (solvent systems L_4, L_5, L_6) and electrophoretic analyses of cell extracts of the four cell suspension cultures within the first two hr after adenine application showed that hypoxanthine and xanthine were intermediates in adenine catabolism. Uric acid and urea could not be determined to occur in these extracts.

Allantoin and allantoic acid were again found as the main catabolites though the latter was present in a smaller proportion than allantoin. Five hours after [8-^{14}C]-adenine uptake the greatest part of the total soluble radioactivity was accumulated in these two products. Even within 230 hr no significant further degradation of the two ureids could be found in soybean and parsley cultures. The obvious cessation of purine base degradation at the stage of allantoin and allantoic acid explains the very limited $^{14}CO_2$-production observed with [8-^{14}C] adenine. Thus, in several experiments where adenine was rapidly and quantitatively absorbed the average rate of $^{14}CO_2$ formation amounted to only 0.25% with *Cicer arietinum*, 1% with *Petroselinum crispum* and 0.6% with *Glycine max*. These values were reached within the first 12–20 hr with no further increase. The *Phaseolus aureus* cell culture, however, behaved somewhat differently because it showed a linear rate of $^{14}CO_2$ formation from [8-^{14}C]-adenine (continuously for at least 200 hr with 2% of total radioactivity in CO_2/24 hr) indicating slow but permanent degradation of allantoin and allantoic acid.

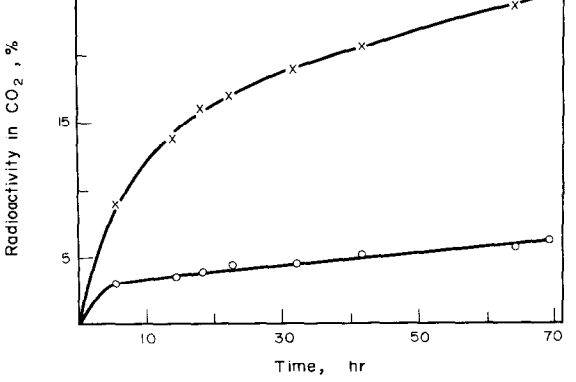

Fig. 3. Formation of $^{14}CO_2$ in cell suspension cultures of *Petroselinum crispum* (×—×) and *Glycine max* (○—○) after application of [U-^{14}C]-allantoin (10^{-5} M).

The very limited degradation of allantoin in the cell cultures was intended to be further demonstrated by feeding experiments with [U-^{14}C]-allantoin (10^{-5} M) and collection of $^{14}CO_2$. While the substrate was well taken up by the cells, Fig. 3 shows that now the soybean and the parsley cell cultures produce 5–20 times higher $^{14}CO_2$-values than expected from the above mentioned data obtained with [8-^{14}C]-adenine. The rest of the radioactivity was reisolated as unchanged substrate. This difference was repeatedly observed and shows that endogenously formed and exogenously applied allantoin are degraded to a very different extent.

DISCUSSION

Nicotinamide adenine dinucleotide when fed to plant cell suspension cultures will as in most other systems [23, 7, 20] be taken up very slowly by the cells. While our data clearly show that NAD is not cleaved by exocellular enzymes it cannot be assumed that all NAD is cleaved during uptake. The results obtained with mung bean cells indicate that either some NAD is penetrating the cells without hydrolysis or that rapid resynthesis of NAD occurs in the cells. In general, however, NAD is cleaved with the catabolites being either used for anabolic or catabolic pathways. The nicotinic acid moiety of NAD is predominantly converted to trigonelline (**1**) or nicotinic acid *N*-α-L-*arabinoside* (**2**). **1** is being formed in the 3 legume cultures while **2** only occurred in the parsley (Umbelliferae), so that there is a chemosystematic element in nicotinic acid metabolism [12, 13, 26]. Degradation of nicotinic acid seems exclusively to be associated with those cell cultures which produce **2** such as parsley. The reservoir form of **1** and **2** is indicated by the observation that constant ratios of **1** or **2**, respectively, and free nicotinic acid, nicotinamide and NAD are established in the cells [11, 13] and that **1** or **2** are constantly being converted into compounds of the pyridine nucleotide cycle [12, 13]. Application of [carbonyl-^{14}C]-NAD leads to essentially the same distribution of radioactivity as application of ^{14}C-nicotinic acid or ^{14}C-nicotinamide (Table 1) [see 11–13]. A remarkable exception is the very low production from NAD of **1** in mungbean cultures (Table 1) because nicotinic acid feeding leads to *ca* 80% trigonelline; this suggests that a good portion of NAD is penetrating into mung bean cells without hydrolysis to free nicotinic acid or nicotinamide.

In the four cell cultures investigated the adenine moiety of NAD is degraded via the sequence adenine–hypoxanthine–xanthin–allantoin–allantoic acid. This pathway is in agreement with other studies in plant systems [9, 27, 30–32] where uric acid has also not been detected as an intermediate [31, 33, 34]. Accumulation of allantoin and allantoic acid as purine catabolites and reservoir forms of nitrogen in plant cells is well known

[35, 36] and especially documented for leguminous species [37, 38]. The $^{14}CO_2$ data (Fig. 2) suggest that adenine either formed from NAD or exogenously applied is predominantly catabolized in the cell cultures. Assuming primarily cessation of purine catabolism at the stage of ureides, which is indicated by the high yields of ureides with practically no $^{14}CO_2$ formation from [8-^{14}C]-adenine, then only some 20% of the radioactivity of [U-^{14}C]-adenine should appear in CO_2. Fig. 2 shows that only the parsley cell culture produces somewhat more $^{14}CO_2$ indicating slow but steady allantoic acid degradation. It is not surprising, however, that at such a low rate of ureide degradation urea could not be found in the cell extracts. While all data point to a very limited allantoin/allantoic acid degradation in the cell cultures, the unexpectedly high $^{14}CO_2$-formation obtained with [U-^{14}C]-allantoin (Fig. 3) should best be taken as an example of cellular compartmentation. It seems to be a further case where there is a pronounced quantitative difference in the metabolism of endogenously formed and exogenously applied substrates [39, 40]. In general, our studies with NAD document that plant cell suspension cultures show a great similarity in adenine metabolism but that two groups of cultures can be differentiated with respect to nicotinic acid metabolism.

EXPERIMENTAL

Reagents. [8-^{14}C] Adenine (spec. act. 54.2 mCi/mmol), [adenine-U-^{14}C]-NAD (spec. act. 260 mCi/mmol), [carbonyl-^{14}C]-NAD (spec. act. 62 mCi/mmol) and [carboxyl-^{14}C]-nicotinic acid (spec. act. 59 mCi/mmol) were obtained from the Radiochemical Center, Amersham. [U-^{14}C]-allantoin was isolated from mung bean cell suspension cultures after application of [adenine-U-^{14}C]-NAD and purified by PC in solvents L_6, L_7 and L_8. All other chemicals were from commercial sources.

Syntheses. Allantoic acid was prepared from allantoin according to [41] and crystallized from MeOH, mp 168–173°. 5-(ω-xanthyl-ureido)-hydantoin was prepared by reaction of allantoin with xanthydrole in warm HOAc according to [28], mp 215–221°.

Cell cultures. Growth and cultivation of the cell suspension cultures have previously been described [8, 42, 43]. They were used in the late logarithmic growth phase and prior to and after the experiments, absence of microorganisms was carefully determined [43]. The application of filter sterilized substrates (10^{-5} M) to cell cultures under aseptic conditions, collection of $^{14}CO_2$ and fractionation procedures of cell extracts followed previous publications [8, 12, 13, 42].

Chromatographic solvents. PC and TLC were carried out with the solvents L_1: 60% *n*-PrOH; L_2: 85% *i*-PrOH; L_3: ammonium acetate (1 M)–EtOH, 3:7; L_4: EtOH–MeOH–H_2O, 3:1:1; L_5: *n*-BuOH–HOAc–H_2O, 4:1:1; L_6: *n*-BuOH sat. 25% NH_3; L_7: *n*-PrOH–25% NH_3–H_2O, 1:6:4; L_8: *i*-PrOH–sat. $(NH_4)_2SO_4$–H_2O, 4:160:80; L_9: MeOH. Py derivatives were detected on chromatograms under UV light (254 nm) by dark absorption and radioactive compounds according to [11].

Electrophoresis. Paper electrophoresis was carried out with potassium phosphate buffer (pH 7.5, 0.05 M) [11].

Identification of metabolites. Metabolites were identified by cochromatography in the solvents indicated, by electrophoresis and dilution analysis. Whenever necessary, suitable derivatives were prepared as mentioned in the text.

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