187. *In vivo* Investigation of Plant-Cell Metabolism by Means of Natural-Abundance ^{13}C-NMR Spectroscopy

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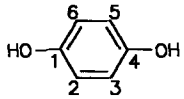
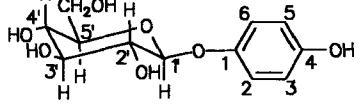
Based on the natural abundance of ^{13}C, *in vivo* ^{13}C-NMR was used for the first time to monitor the metabolism of sucrose and hydroquinone (**1**) in cell suspensions of the plant *Rauwolfia serpentina* (L.) BENTH. ex KURZ. Cells converted sucrose extracellularly into α-D- and β-D-glucose as well as into β-D-fructofuranose and β-D-fructopyranose, respectively. The sugar mixture was completely taken up by the cells after 4 days. Hydroquinone fed at that time resulted in optimum conversion into its β-D-glucoside arbutin (**2**) within 10 h. A further metabolite, the primeveroside (**3**) of hydroquinone, appeared as a trace compound after 10 h. The formation of this diglycoside can be increased by further addition of sucrose.

Introduction. – Mechanisms of metabolic sequences are usually investigated *in vivo* using precursors labeled with radioactive or stable isotopes under cell-destructive conditions. Many attempts were made in the past to apply NMR techniques to study metabolic phenomena in intact plant cells. Most of them involved ^{31}P-NMR to examine the rate of ATP synthesis, determining concentrations of phosphate metabolites or measuring changes of cytoplasmic and vacuolar pH [1–3]. *In vivo* ^{15}N-NMR spectroscopy was successfully applied to detect the flux of amino acids in the green algae *Chlorella fusca* [4], ^{14}N-NMR experiments to analyze exchange rates of NH_4^+ ions between cytoplasmic and vacuolar NH_4^+ pools [5], and ^{133}Cs as a probe to investigate subcellular compartmentation and ion uptake in maize root tissue [6].

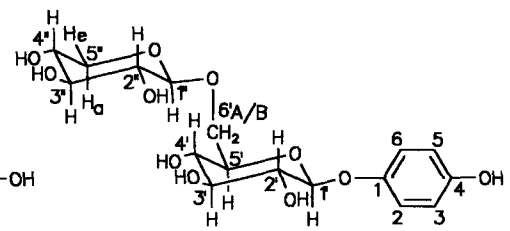
Because of the relatively low sensitivity of ^{13}C-NMR, experiments concerning plant-cell metabolism were exclusively designed for the employment of ^{13}C-enriched compounds. In fact, only a few reports have appeared in this field during recent years, *e.g.* the *in vivo* hydroxylation of [^{13}C]capsidiol by *Capsicum frutescens* followed by isolation and ^{13}C-NMR analysis of 13-hydroxy-[^{13}C]capsidiol [7], the degradation of [^{13}C]reticuline [8],



Hydroquinone (**1**)



Arbutin (**2**)



4-Hydroxyphenyl primeveroside (**3**)

the fate of [^{13}C]reticuline in the biosynthetic pathway of protoberberine alkaloids, and the transformation of [*N*-$^{13}CH_3$]reticuline into pycnarrhine [9]. A broader application of this methodology clearly depends on a high sensitivity which can be attained by using high-field NMR instruments and highly enriched ^{13}C-precursors and/or plant cells with extraordinary metabolic activity. Our recent work in the field of biotransformations has demonstrated that cell suspensions of the Indian medicinal plant *Rauwolfia serpentina* (L.) BENTH. ex KURZ are an excellent system for the *in vitro* and *in vivo* glycosylation of phenolic compounds [10–12].

In this paper, we report on the metabolism of sucrose and hydroquinone using *in vivo* ^{13}C-NMR under conditions of natural ^{13}C-abundance in intact plant cells.

Results and Discussion. – We expected that *Rauwolfia* cell suspensions exhibiting a high biotransformation of hydroquinone (**1**) would be an efficient system for the study of biosynthetic processes under *in vivo* conditions by NMR. First experiments were designed using high sucrose concentrations (10 g/100 ml) and 7 days old cells in LS medium [13] to produce good signal/noise ratios, and to determine the background spectrum of

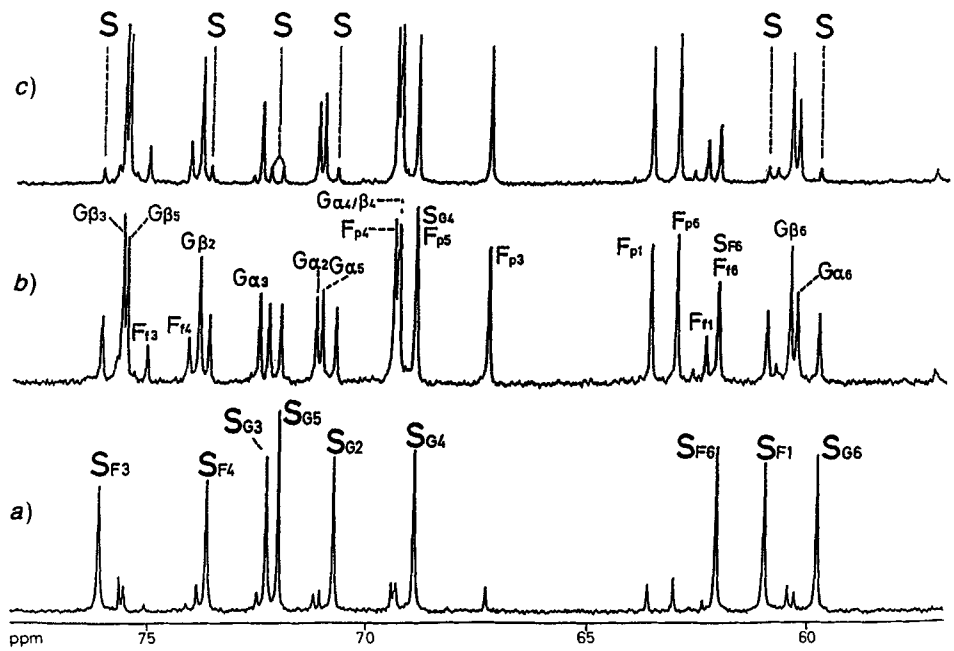


Fig. 1. *100.6-MHz ^{13}C-NMR Spectra from 7-days-old cells in fresh LS medium with high sucrose concentration* (10 g/100 ml): a) *Spectrum measured directly after cells were transferred to fresh nutrition medium* (S_{F1}–S_{F6}: C(1) to C(6) of the fructose unit of sucrose; S_{G1}–S_{G6}: C(1) to C(6) of the glucose unit of sucrose; not shown: S_{F2} (103.3 ppm), S_{G1} (91.8 ppm), and S_{F5} (81.0 ppm)). b) *Spectrum obtained after 24 h of incubation* (F_{p1}–F_{p6}: C(1) to C(6) of β-D-fructopyranose (F_{p2} at 97.8 ppm not shown); F_{f1}–F_{f6}: C(1) to C(6) of β-D-fructofuranose (F_{f2} (101.2 ppm) and F_{f5} (80.3 ppm) not shown); $G_{\alpha1}$–$G_{\alpha6}$: C(1) to C(6) of α-D-glucose ($G_{\alpha1}$ at 91.8 ppm not shown); $G_{\beta1}$–$G_{\beta6}$: C(1) to C(6) of β-D-glucose ($G_{\beta1}$ at 95.6 ppm not shown)). c) *Spectrum measured after 48 h of incubation, indicating a 90% conversion of sucrose* (S) *to its monosaccharides*. All spectra were taken with 20% D_2O and represent 9800 accumulations with a digital resolution of 0.76 Hz/data point, pulse repetition time was 1.3 s, resulting measurement time 3.5 h.

the cells, the line broadening by components of the nutrition medium, and to investigate the stability of sucrose under these *in vivo* conditions. After 3.5 h acquisition time, the ^{13}C-NMR spectrum of sucrose was obtained with only small signals (< 10%) belonging to α-D- and β-D-glucose, β-D-fructofuranose, and β-D-fructopyranose (*Fig. 1a*). After 24 h, however, the sucrose was already converted by the cells to yield more than 50% of these monosaccharides (*Fig. 1b*). This conversion was *ca.* 90% after 48 h as, illustrated by the spectrum shown in *Fig. 1c*. These experiments clearly demonstrated that the quality of ^{13}C-NMR spectra is sufficient to investigate the metabolism using unlabeled sucrose. They also indicated that there is no background spectrum of the cells which would interfere with the interpretation of the data and that the *Rauwolfia* cells transform nearly all of the disaccharide into the previously mentioned four monosaccharides within two days. It was, however, impossible to decide by ^{13}C-NMR whether the saccharides were measured within the cells and/or as components of the nutrition medium. A series of further experiments was conducted to get more detailed information on this point. Cells were grown under standard conditions in LS medium with the usual sucrose concentration (3 g/100 ml). The metabolism of sucrose was followed by measuring the monosaccharide content of the nutrition solution by ^{13}C-NMR after centrifugation of the cell suspension (see *Exper. Part*). The signal intensities of the C(1) of sucrose, β-D-glucose, β-D-fructofuranose, and β-D-fructopyranose were used to obtain semi-quantitative data (±10%). As illustrated in *Fig. 2*, sucrose in the nutrition medium was hydrolyzed within *ca.* 35 h of growth. During this time the monosaccharides accumulated at the expense of the disaccharide. After *ca.* 4 days, nearly all monosaccharides disappeared from the nutrition medium.

These results are interesting from several points of view. They indicate that the cells convert sucrose in large quantities due to the enzyme invertase. This enzyme could be

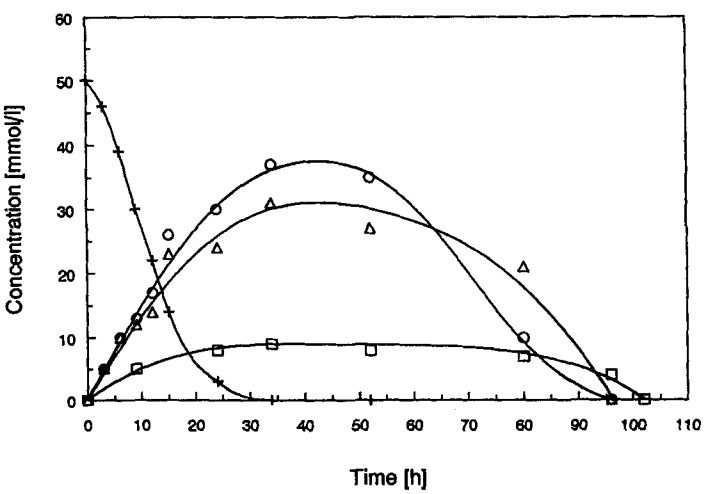


Fig. 2. *Time course of the sucrose uptake and metabolism by* Rauwolfia *cell suspensions.* The remaining concentrations of different saccharides in the nutrition medium are shown, determined on the basis of peak intensities in ^{13}C-NMR spectroscopy. Sucrose, + ; β-D-fructofuranose, □ ; β-D-fructopyranose, Δ ; β-D-glucose, ○ ; not illustrated is the concentration of α-D-glucose, which is in the anomeric equilibrium of 1:2 with β-D-glucose during the whole experiment.

located inside the cells as a soluble enzyme or at the cell wall as a bound protein. Both forms of enzyme are well known to occur in plant cells [14] [15]. When we incubated a cell wall preparation from the *Rauwolfia* cells with sucrose, we observed, again by ^{13}C-NMR, a sucrose/glucose ratio in the supernatant of *ca.* 2:1 after only 90 min of incubation. After 360 min, all the sucrose was converted to the same saccharides as discussed above, indicating that a cell-wall-bound invertase is responsible for the appearance of the monosaccharides in the surrounding nutrition medium under *in vivo* conditions. This fact, however, does not exclude the occurrence of an additional cytoplasmatic invertase. This mixture of saccharides is accumulated inside the plant cells as demonstrated by *Fig. 3a* showing a spectrum of cells grown for 5 days under standard conditions. To prove that no significant exchange of the saccharides between the *Rauwolfia* cells and the surrounding nutrition solution takes place during the NMR experiment, we analyzed the supernatant of the measured cells. In the obtained ^{13}C-NMR spectrum (*Fig. 3b*), no signals

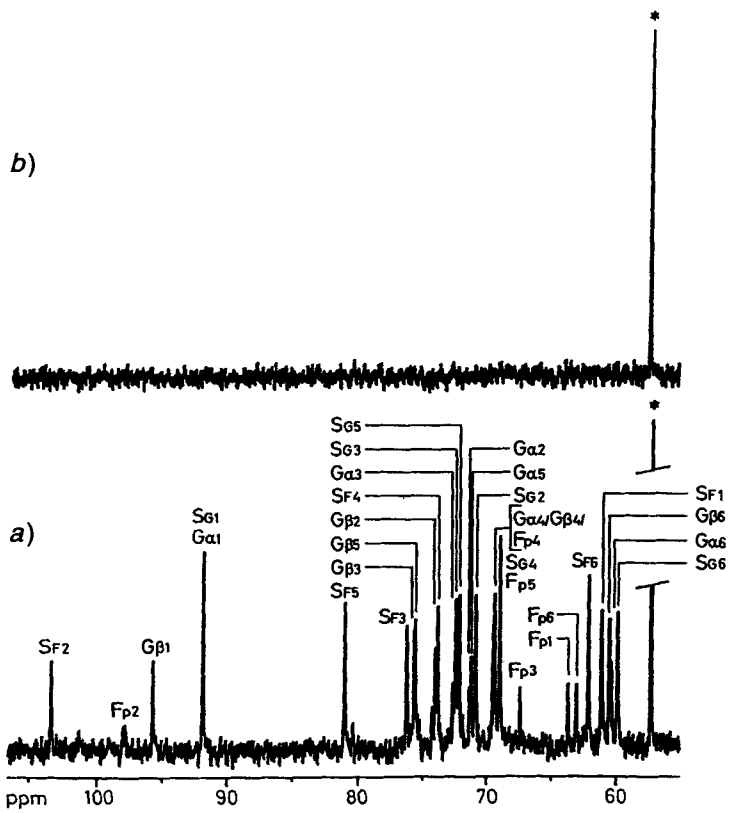


Fig. 3. *100.6-MHz ^{13}C-NMR Spectrum* a) *of densely packed cells of a suspension culture of* Rauwolfia serpentina (S_{F1}–S_{F6}: C(1) to C(6) of the fructose unit of sucrose; S_{G1}–S_{G6}: C(1) to C(6) of the glucose unit of sucrose; F_{p1}–F_{p6}: C(1) to C(6) of β-D-fructopyranose; $G_{\alpha 1}$–$G_{\alpha 6}$: C(1) to C(6) of α-D-glucose; $G_{\beta 1}$–$G_{\beta 6}$: C(1) to C(6) of β-D-glucose) *and* b) *of the supernatant taken from sample* a) *after resuspension of the cells in the NMR tube and settling of the cells.* Both spectra were taken after 8400 pulses (measuring time 3 h) with a repetition time of 1.3 s and a digital resolution of 0.76 Hz/data point. Both samples contained 15% D_2O and 0.15% EtOH as an internal standard. * = Signal of C(1) of EtOH (internal standard).

were present, indicating that cells were not damaged during the measurement and that, indeed, no significant efflux of sugars did occur under these conditions.

The above results also indicated that this is the best time to feed the cells with hydroquinone (**1**), because at this stage cells seem to be adapted to their environment and have accumulated enough sugars for further transformation. Moreover, the nutrition solution would be nearly free of components and would, therefore, not interfere with the resonances depending on the compounds of the feeding experiment.

A high-density cell culture of 250 g of fresh cells, which was grown in LS medium for 7 days, was inoculated with fresh nutrition medium (150 ml) for 4 days. Thereafter, sucrose-free medium containing 500 mg of **1** in a total volume of 50 ml was added. The biosynthesis of the glucoside of **1**, arbutin (= 4-hydroxyphenyl β-D-glucopyranoside; **2**), in this mixture could be observed directly by *in vivo* ^{13}C-NMR. For such an *in vivo* experiment, in an interval of 2 h, samples of the cells were aseptically transferred into a 10-mm NMR tube, and a ^{1}H-decoupled ^{13}C-NMR spectrum could be obtained after 120 min of measuring time. Under these circumstances, an excellent signal/noise (S/N) ratio of *ca.* 15:1 allowed us to follow very simply the dynamics of arbutin formation. The time course of the increase of the signals of the aromatic part of **2**, especially of C(3)/C(5) (at 116.0 ppm) and C(2)/C(6) (at 118.2 ppm), as well as of all resonances of the glucose moiety, was easily recognized. At time 'zero' (10 min after the addition of **1**, 2 h measuring time), the signal for **1** at 116.2 ppm was still the major peak in the whole spectrum, indicating a relative slow metabolism at the beginning of the experiment. The resonances of C(3)/C(5) and of C(2)/C(6) of **2** were, at that time, at the limit of detection (S/N *ca.* 2:1; *Fig. 4a*). The signals for the glucosidic part of **2** could not clearly be distinguished from the sucrose, fructose, and glucose ^{13}C-resonances, because the latter compounds were still present in much higher concentration than **2**.

After 2 h of incubation, all signals of arbutin (**2**) could be detected (only those of C(2)/C(6), C(3)/C(5) and C(1′) are shown in *Fig. 4b*). Because the supernatant of the cells did not contain detectable amounts of **2** under the same conditions of measurement, it is certain that the compound is accumulated intracellularly by *Rauwolfia* cells.

Two hours later, the signal for C(2)/C(3)/C(5)/C(6) of hydroquinone (**1**) and the signals for C(2)/C(6) and C(3)/C(5) of its glucoside showed nearly the same intensities (*Fig. 4c*). Most of **1** was glucosylated after 6 h which indicates a relatively rapid synthesis of **2** under these conditions (*Fig. 4d*). At this time, the signals for C(1′) (101.0 ppm), C(2′) (72.8 ppm), C(3′) (75.8 ppm), C(4′) (69.3 ppm), C(5′) (75.4 ppm), and C(6′) (60.4 ppm) of **2** reached intensities which were comparable to all other resonances and, therefore, easy to distinguish from them.

Arbutin biosynthesis was nearly complete after 8 h and fully completed in 10 h of incubation (*Fig. 4e,f*). At this stage, all major signals of the spectrum belonged to **2**. When, after each experiment, the surrounding nutrition medium was measured by NMR, the decrease of **1** could be followed indicating that, after 6 h, more than 50% and, after 10 h, more than 95% of **1** was taken up by the cells. A similar time course could be measured for the changes of endogenous sucrose concentrations (*Fig. 4a–f*, signal at 103.3 ppm). After 10 h, the sucrose was metabolized to an extent of > 70%. In addition to that, these experiments also clarified that arbutin (**2**) seems to be always completely retained by the cells. In none of the control NMR experiments was the glucoside found in the supernatant nutrition medium of the cells. More sensitive HPLC analyses of the

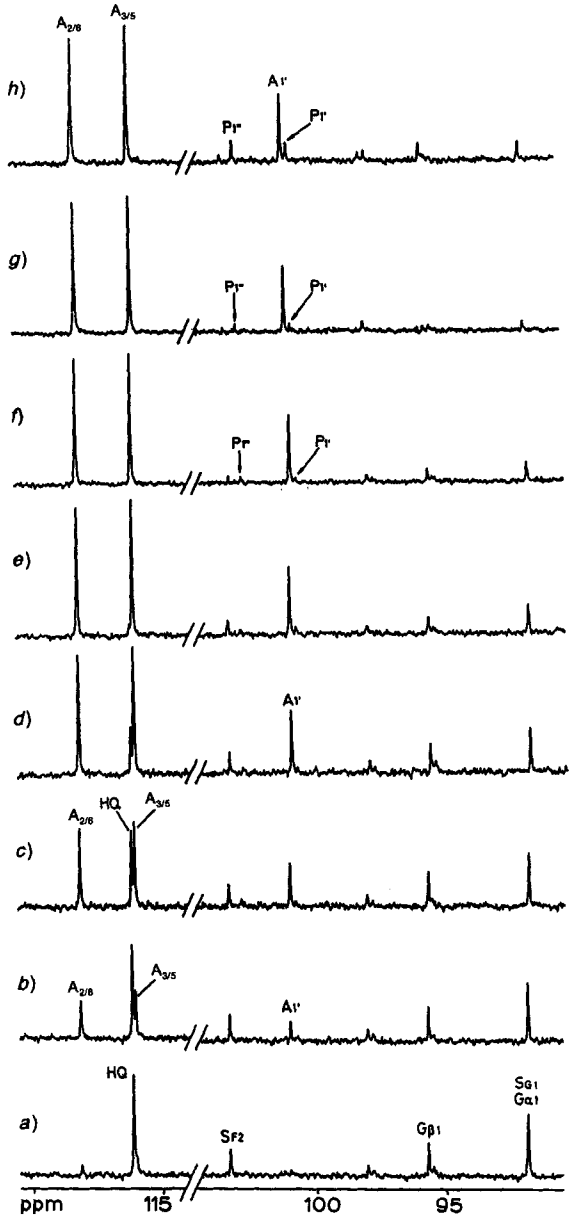


Fig. 4. *Time course of the biotransformation of hydroquinone* (**1**) *to arbutin* (**2**) *shown by 100.6-MHz* ^{13}*C-NMR spectroscopy of cells from suspension cultures of* Rauwolfia serpentina: *Spectra recorded sequentially starting* a) *10 min* (time 'zero'), b) *2 h*, c) *4 h*, d) *6 h*, e) *8 h*, f) *10 h*, g) *32 h, and* h) *196 h after addition of 500 mg of* **1** *to 250 g of wet cells cultivated for 4 days in LS medium* (500 ml total volume). After 104 h of growth time, additional 8 g of sucrose were added to the cell suspension. All measured samples contained 15% D_2O. Each spectrum represents 5600 accumulations (measuring time 2 h) with a pulse repetition time of 1.3 s and a digital resolution of 0.76 Hz/data point. Abbreviations used: HQ: hydroquinone (**1**); S_{F2}, S_{G1}: C(2) of fructose unit and C(1) of the glucose unit of sucrose, resp.; $G_{\beta 1}$: C(1) of β-D-glucose; $G_{\alpha 1}$: C(1) of α-D-glucose; $A_{3/5}$, $A_{2/6}$: aromatic C-atoms of arbutin (**2**); $A_{1'}$: C(1′) of the glucose unit of **2**; $P_{1'}$, $P_{1''}$: C(1) of the glucose unit and C(1) of the xylose unit of 4-hydroxyphenyl primeveroside (**3**).

surrounding medium demonstrated that less than 0.5% of **2** was present in the supernatant of the *Rauwolfia* cells, even after feeding times of > 3 days.

The above described time course of hydroquinone metabolism could also be seen nicely at the level of the monosaccharides (data not shown). In comparison with the data at the beginning of the experiment (*Fig. 4a*), the intensities of all C-signals of fructose and glucose decreased dramatically after 32 h. Whether the monosaccharides were metabolized or transformed into polymeric compounds could not be decided from the obtained spectra because the limit of detection was insufficient to monitor such transformations.

In our previous investigation of hydroquinone metabolism, we observed a second glycoside which was synthesized as a by-product in *Rauwolfia* cell suspensions [10]. The structure of this novel compound was the primeveroside **3** of hydroquinone (= 4-hydroxyphenyl 6-*O*-(β-D-xylopyranosyl)-β-D-glucopyranoside). Until now, the time scale of the biosynthesis of this diglycoside has not been clear. Because **3** exhibits striking ^{13}C-NMR signals at 100.8 and 102.9 ppm for C(1′) and C(1″), respectively, which do not overlap with resonances of **1**, **2**, or the above mentioned sugars, we used these signals to monitor the biosynthesis of **3** by *in vivo* ^{13}C-NMR. In fact, the formation of this diglycoside could be followed although the accumulation rates and the signal intensities were significantly lower than for arbutin (**2**). After 10 or 32 h (see *Fig. 4f,g*) primeveroside **3** appeared in the *Rauwolfia* cells, but the amounts were extremely low for detection by ^{13}C-NMR. However, a pronounced dynamic increase of this diglycoside could not be determined NMR spectroscopically within the time scale employed here. When cells were measured 3 days later, the ratio of **2** and **3** did not vary significantly (data not shown). From *Fig. 4g*, one can conclude, that nearly all of the monosaccharides disappeared after a time of 32 h of metabolism. This observation suggests that cells at that time might be deficient in monosaccharides and that no further biosynthesis of the diglycoside **3** can occur. This assumption became true when new sucrose was added to the cells 104 h after the beginning of the experiment. Indeed, after additional 4 days, an increase in the concentration of **3** could be recorded by *in vivo* ^{13}C-NMR (*Fig. 4h*), but this result also indicated that the biosynthesis of the primeveroside **3** is not very efficient. Provided that only unlabelled precursors are employed, we still believe that the biosynthesis of such primeverosides under the applied conditions might be elucidated by *in vivo* ^{13}C-NMR although the synthesis is very slow.

The results obtained in this study for the biosynthesis of arbutin **2** demonstrate for the first time that ^{13}C-NMR can be successfully applied to follow directly biosynthetic reactions in living plant cells, even if the precursors are not labelled. They also indicate that the method could be simply applied for further optimization of arbutin (**2**) production, which is interesting from the biotechnological point of view because **2** is an inhibitor for melanin biosynthesis [16]. The described experiments are promising enough to prompt us to tackle also the dynamics of diglycoside biosynthesis using the natural abundance of ^{13}C in the appropriate precursors. For such experiments, higher-field NMR instruments (500 or 600 MHz) are required and a more optimized cell system. The corresponding investigations are now in progress.

Experimental Part

Plant-Cell Culture. Cell-suspension cultures of *Rauwolfia serpentina* were cultivated in 1-l *Erlenmeyer* flasks at 25° and 100 rpm (gyratory shaker) under light (600 Lux) using LS medium [13].

Cell-Wall Preparations. Cells cultivated for 7 days in LS medium were harvested by suction filtration and frozen with liquid N_2. Of the frozen cells, 10 g were homogenized for 2 min using an 'ultra-turrax' homogenizer and 10 ml of distilled H_2O. Similar to previously described methods [17], the mixture was centrifuged (5 min, 500 *g*), and the precipitate was washed 3 times with 4 ml of distilled H_2O. The obtained precipitate was resuspended in distilled H_2O up to a volume of 10 ml. In a total volume of 1 ml, 250 µl of this suspension were incubated at 30°. A standard incubation mixture contained 5 mg of sucrose. Incubation times varied from 90 to 360 min. For spectroscopic measurements, cell-wall fragments were centrifuged off, and 0.5 ml of the supernatant were transferred to a 5-mm NMR tube and measured under standard conditions.

Quantification of Saccharides. Rauwolfia cells grown for 7 days in LS medium were transferred to a 1-l *Erlenmeyer* flask containing 250 ml of fresh LS medium. From this flask, medium samples were taken frequently over a period of 120 h. The samples were centrifuged (10000 *g*), and 4 ml of the supernatant were analyzed NMR-spectroscopically under standard conditions.

The following ^{13}C-NMR signals of the different saccharides were used for quantitative determination: sucrose, 103.3 ppm; β-D-fructofuranose, 101.2 ppm; β-D-fructopyranose, 97.8 ppm; β-D-glucose, 95.6 ppm.

Standard NMR Measurements. Standard ^{13}C-NMR spectra of aq. solns. were obtained by accumulation of 8400 FID's (measuring time 3 h) on a *Bruker-ARX-400* instrument (standard *Bruker* software) using 5-mm NMR tubes. Spectrum width was 25000.0 Hz, pulse repetition time 1.3 s (pulse angle 35°), and digital resolution 0.76 Hz/data point. D_2O was added up to 20% of total volume.

Feeding Experiments. Cells which were grown for 7 days in LS medium were transferred to 150 ml of fresh LS medium (1-l flask). After a regeneration time of 4 days, a soln. of 500 mg of hydroquinone (**1**) in 50 ml of sugar-free LS medium were applied to the cell suspension.

In vivo *NMR Measurements.* After feeding of 500 mg of **1** to cell-suspension cultures of *ca.* 250 g of wet cells (500 ml of total volume), an inoculum of *ca.* 8 ml of suspended cells was transferred aseptically to a 10-mm NMR tube that contained 1.4 ml of 1% EtOH (internal standard) in D_2O. Final D_2O concentration was 15%. Cells were allowed to settle for *ca.* 10 min before starting the measurement. After each measurement of densely packed cells, cells were resuspended in the supernatant of the NMR tube and allowed to settle again. The resulting supernatant was decanted and also analyzed by ^{13}C-NMR spectroscopy. The results obtained were verified by using more sensitive HPLC analysis.

Standard ^{1}H-decoupled 100.6 MHz ^{13}C-NMR spectra were obtained on a *Bruker-ARX-400* instrument (standard *Bruker* software) with a spectrum width of 25000.0 Hz, a pulse repetition time of 1.3 s (pulse angle 35°), and a digital resolution of 0.76 Hz/data point. For each spectrum, 5600 FID's were accumulated leading to a measurement time of 2 h.

HPLC Measurements. Hitachi-Merck system connected to a *RP-18* column (*LiChroCart®, 125-4 super-sphere 100*); flow rate of 1 ml/min, using the solvent system [18] MeOH (5% in distilled H_2O) pH 2.5 adjusted with H_3PO_4 (detection at 285 nm); t_R for arbutin (**2**) 2.98, for **1** 3.88, and for 4-hydroxyphenyl primeveroside (**3**) 6.8 min.

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REFERENCES

[1] T. W. M. Fan, A. N. Lane, R. M. Higashi, *Arch. Biochem. Biophys.* **1992,** *294,* 314.
[2] V. Wray, O. Schiel, J. Berlin, *Z. Pflanzenphysiol.* **1988,** *112,* 215.
[3] M. Katsuhara, K. Kuchitsu, K. Takeshige, M. Tazawa, *Plant Physiol.* **1989,** *90,* 1102.
[4] A. C. Kuesel, W. Kuhn, J. Sianoudis, L. H. Grimme, D. Leibfritz, A. Mayer, *Arch. Microbiol.* **1989,** *151,* 434.
[5] R. B. Lee, R. G. Ratcliffe, *Planta* **1991,** *183,* 359.
[6] P. E. Pfeffer, D. B. Rolin, D. Brauer, S. I. Tu, Th. F. Kumosinski, *Biochim. Biophys. Acta* **1990,** *1054,* 169.
[7] J. B. Stothers, A. Stoessl, E. W. B. Ward, *Z. Naturforsch., C: Biosci.* **1978,** *33,* 149.
[8] S. Jendrzejewski, *Phytochemistry* **1990,** *29,* 135.
[9] B. Schneider, M. H. Zenk, in 'Plant Tissue Culture and Gene Manipulation for Breeding and Formation of Phytochemicals', Ed. K. Oono, Sato Press, Hito, Japan, 1992, p. 323.
[10] R. Lutterbach, J. Stöckigt, *Helv. Chim. Acta* **1992,** *75,* 2009.
[11] R. Lutterbach, J. Stöckigt, H. Kolshorn, *J. Nat. Prod.* **1993,** *56,* 1421.
[12] R. Lutterbach, C. M. Ruyter, J. Stöckigt, *Can. J. Chem.* **1994,** *72,* 51.
[13] E. M. Linsmaier, F. Skoog, *Physiol. Plant.* **1965,** *18,* 100.
[14] S. Amino, M. Tazawa, *Plant Cell Physiol.* **1988,** *29,* 483.
[15] H. Masuda, T. Takahashi, S. Sugawara, *Plant Physiol.* **1988,** *86,* 312.
[16] S. Akiu, Y. Suzuki, Y. Fijinuma, T. Asahara, M. Fukuda, *Proc. Jpn. Soc. Dermatol.* **1988,** *12,* 138.
[17] M. Hallaway, P. D. Phethean, J. Taggart, *Phytochemistry* **1970,** *9,* 935.
[18] S. Inomata, M. Yokoyama, S. Seto, M. Yanagi, *Appl. Microbiol. Biotechnol.* **1991,** *36,* 315.