Restoration of Serotonin Biosynthesis in Cell Suspension Cultures of *Peganum harmala* by Selection for 4-methyltryptophan-tolerant Cell Lines

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Summary

The original capacity of *Peganum harmala* cell suspension cultures for serotonin biosynthesis which had been lost during 6 years of cultivation, was restored by selection of 4-methyltryptophan-tolerant cell lines. Four cell lines were recovered accumulating 1–2% serotonin of dry mass when maintained on the growth medium, while one tolerant line was not able to form higher levels of serotonin. In contrast to non-producing cell lines the serotonin producing cell lines expressed tryptophan decarboxylase activity, were able to use tryptophan for serotonin biosynthesis, and detoxified 4-methyltryptophan to 4-methyltryptamine. The better ability of the TDC containing cell lines for detoxification of 4-methyltryptophan seems to contribute at least partially to the overall tolerance of the selected cell lines. The original β-carboline alkaloid levels were not restored by this technique.

Key words: Peganum harmala, cell suspension culture, selection, amino acid analogue tolerance, serotonin, tryptophan decarboxylase.

Introduction

It is often observed that the initial production capacity of cell cultures may decrease or even be lost during further subcultivation. Often it takes some time before a cell culture has reached its final and rather stable state under the chosen culture conditions. While the formation of secondary products decreases, the growth rate often increases. Generally it is assumed that the cells dedifferentiate more and more with prolonged cultivation time. As formation and accumulation of many secondary metabolites indicate cell specialization (Luckner 1982), it seems logical that with progressing dedifferentiation parts of the biosynthetic potential of cell cultures are lost or not expressed. For the production of secondary metabolites by plant cell cultures rapidly growing and highly productive, or easily inducible cells, are required. However, this requirement is only met by a very few spontaneously high yielding cell culture systems (Berlin 1986). To change this situation the biochemical reasons for the switch off of secondary pathways during dedifferentiation have to be elucidated. Such knowledge may give hints of how to influence regulatory controls of secondary pathways in a desirable way.

A good model system for such studies is the biosynthesis of serotonin and β-carboline alkaloids in *Peganum harmala* cell suspension cultures. Initially our *Peganum har-*

mala cell cultures accumulated up to 1% harman alkaloids and serotonin in the growth medium (Sasse et al. 1982a), with the highly productive cell lines showing some morphological differentiation at the end of the growth cycle. Over the years the growth rate increased and the cell suspensions have become finely dispersed (Berlin and Sasse 1987). However, these better growing cell suspension cultures only produced traces of serotonin and harman alkaloids. High, low, or even non-producing cell lines of *Peganum harmala* were equally well able to transform added tryptamine to serotonin (Sasse et al. 1982b, 1987). A correlation between tryptophan decarboxylase (TDC) activity and accumulation of serotonin and β-carboline alkaloids was demonstrated (Sasse et al. 1982b). TDC activity of well growing fine suspension cultures, however, was hardly measurable and poorly inducible in production media (Berlin and Sasse 1987). Thus it was clear that the lack of *de novo* synthesis of serotonin was only caused by the lack of TDC activity. To restore the original capacity of *Peganum* cells for serotonin biosynthesis, new approaches have to be tested. At present three alternatives seem to be promising: a) introduction of expressed TDC activity by genetic transformation as outlined recently (Berlin et al. 1985); b) search for suitable elicitors of TDC which have been shown to increase serotonin levels up to 1.5% of dry mass (Berlin and Sasse 1987); and c) biochemical selection of cell types with high TDC activity (Sasse et al. 1983b). Here we report on investigations aimed at overcoming the lack of *de novo* serotonin biosynthesis by selection for 4-methyltryptophan (4-MT)-tolerant cell lines. This tryptophan analogue was chosen, as it can be detoxified by decarboxylation, and thus cells with higher TDC activity may have a better chance of survival (Sasse et al. 1983a).

Materials and Methods

Cell cultures: Maintenance and characteristics of the wild type cells have been described recently (Sasse et al. 1987). This cell line had been in suspension for 6 years when selection for 4-methyltryptophan (4-MT)-tolerance was started. Thirty flasks (70 ml MX-medium plus 0.5 mM DL-4-MT) were inoculated with 0.8 g cells (fresh mass) and screened for growth. After 2–3 months nearly 50% of the flasks showed sufficient growth for a further transfer to the selective conditions. Five of these flasks showed good growth during this second selective passage and were chosen for further experiments (PH 2, PH 5, PH 6, PH 13, PH 14). For screening of cell lines accumulating yellow fluorescent harmalol, tolerant cells selected in liquid medium were plated on MX-agar medium with 0.5–1.0 mM 4-MT. The most fluorescent areas of calli were repeatedly transferred, initially to medium with and later without 4-MT.

Analytical Methods: Freeze-dried cells were extracted with methanol. Serotonin was measured fluorimetrically after incubation with ninhydrin (Vanable 1963, Sasse et al. 1982a). Harman alkaloids were also measured fluorimetrically (Sasse et al. 1980, 1982a). Tryptophan and other amino acids were derivatized with o-phthaldialdehyde (Burbach et al. 1982) at pH 9.5 (0.4 M Na_3BO_3, mercaptoethanol) for HPLC chromatography using the following conditions: column Shandon Hypersil 5 μ, temperature 25 °C, detection by fluorescence, excitation at 330 nm, buffer A: 80% 0.05 M NaOAc pH 6.2, 19% MeOH, 1% THF; buffer B: 80% MeOH, 20% 0.05 M NaOAc pH 6.2. Gradient: %B 0–2 min 0, 2—10 min 5, 10–12 min 15, 12–16 min linear, 16–24 min 50, 24–34 min linear, 34–44 min 100, 44–49 min linear, in 4 min down to 0, equilibration 7 min. Flow rate 1 ml/min.

The metabolites of feeding experiments with various tryptophan and tryptamine derivatives were analyzed by GC/MS after trifluoroacetylation of the amino acid fraction of cell extracts as

recently described (Sasse et al. 1987). Thus only the retention indices on the methylsilicon phase DB1 and the mass spectra of TFAA-derivatives of new metabolites are given here. 5-Fluorotryptamine: Ri = 1775; ms(m/z): 370 (m^+, 18 %), 257 (100 %), 244 (83 %), 147 (35 %), 69 (16 %). 4-Methyltryptamine: Ri = 1910; ms (m/z): 366 (m^+, 23 %), 253 (49 %), 240 (100 %), 143 (23 %), 69 (37 %).

Enzyme Measurements: Tryptophan decarboxylase and anthranilate synthase activity were extracted and measured as described (Sasse et al. 1983 b).

Results

Selection of the lines

At an inoculum of 0.9 g *Peganum* cells/70 ml medium growth ceased completely at 0.2 mM 4-MT (Table 1). This growth inhibition was reversed by the addition of L-tryptophan to 50 % (Table 1). Cells were however not killed by the analogue as after a delay of 4–6 weeks slow growth was observed in many flasks. The tolerant cells were recovered from flasks which showed twice distinct growth on 0.5 mM 4-MT. The tolerance against 4-MT of cells grown in the absence of the analogue for two growth cycles is shown in Fig. 1. The recovering of cells with higher tolerance was not attempted as we found with other culture systems that secondary metabolism of such lines may be rather poor (Berlin and Sasse, unpublished). Though the tolerance trait seemed to be rather stable, the cells were routinely maintained on 0.1 mM 4-MT for security reasons. The recovered cell lines were also tolerant to 4-fluorotryptophan, another analogue which can be decarboxylated by TDC.

Growth and serotonin production of the tolerant lines

The 4-MT tolerant cell lines were characterized after they had been grown in the absence of the analogue for two growth cycles. A first comparison of the recovered lines with wild type cells showed a similar growth rate, but 4 of the 5 lines contained high levels of serotonin of 0.8–2 % of dry mass (Table 2). It should be noted that in the presence of the analogue, or when the cells have just been removed from the analogue, serotonin levels were distinctly lower due to insufficient tryptophan supply. One tolerant line had the same low level as the parent line. None of the tolerant lines produced more β-carboline alkaloids than wild type cells.

Depending upon the preculture the serotonin levels of the 4 serotonin producing lines varied by 100 % but lower levels than 0.5 % were never found. This was a factor

Table 1: Inhibition of *Peganum harmala* cell suspension cultures by 0.2 mM DL-4-methyltryptophan (4-MT) or DL-4-flourotryptophan (4-FT) and its reversion by L-tryptophan. Inoculum: 0.9 g/70 ml. Harvested after 14 d.

	Fresh mass (g/flask)		
	none	+ 4-MT	+ 4-FT
Control	10.8	1.0	0.9
+ 25 μM L-trp	10.1	2.1	2.4
+ 50 μM L-trp	10.7	4.1	4.6
+ 100 μM L-trp	9.5	4.9	4.7

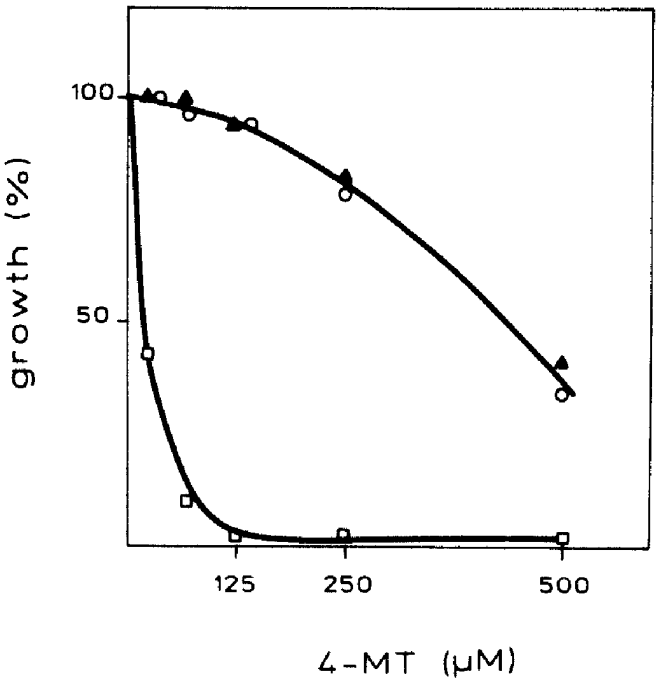

Fig. 1: Effect of 4-methyltryptophan on growth of *Peganum harmala* wild type cells (□) and on 4-MT-tolerant lines (PH 6 ○ and PH 14 ▲). Initial inoculum was 0.6 g/35 ml. Cells were harvested after 14 d. The final fresh masses of the controls (100 %) were 4.9 g for wild type cells, 4.9 g for PH 6, and 4.2 g for PH 14.

Table 2: First comparison of the 5 selected 4-methyltryptophan-tolerant *Peganum harmala* cell suspension cultures. All lines were grown for two growth cycles in the absence of the analogue before analysis. Initial inoculum was 2 g fresh mass/ 70 ml medium. Cells were harvested after 14 d.

Cell line	fresh mass g/flask	dry mass mg/flask	serotonin % dry mass	ß-carbolines % dry mass
wild type	15.2	860	0.1	0.02
PH 2	13.9	812	0.9	0.04
PH 5	14.1	750	1.3	0.03
PH 6	16.2	947	0.4	n.d.
PH 13	13.8	890	1.3	0.13
PH 14	12.5	830	2.0	0.02

of 10 greater than in wild type cells. Detailed growth and production kinetics of PH 14 cells, on the average the best producing line, and the low productive line PH 6 are given in Fig. 2. Thus, the first aim of finding some well growing and serotonin producing lines among the 4-MT tolerant cell lines was achieved.

However, our wild type cells had produced initially substantial amounts of harman alkaloids. As a positive correlation of TDC activity and harman alkaloid accumulation was indicated (Sasse et al. 1982 b), the failure of β-carboline alkaloids in the 4-MT

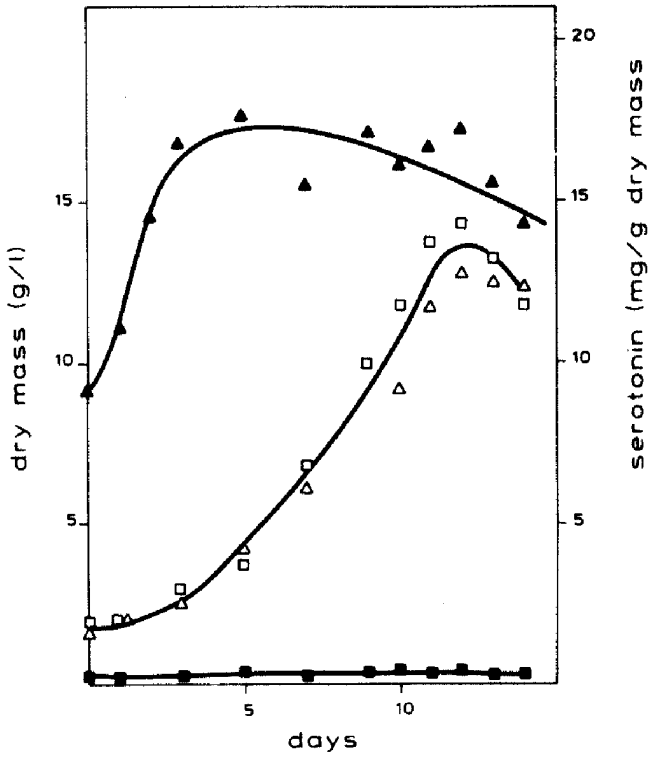

Fig. 2: Comparison of the growth (△) and serotonin accumulation (▲) of 4-MT-tolerant PH 14 cells with growth (□) and serotonin levels (■) of wild type cells.

tolerant lines was not necessarily expected. Therefore we plated the 4-MT tolerant cell lines on agar and screened individual calli for fluorescent areas (mainly for the yellow fluorescence of harmalol). Indeed yellow fluorescent calli were greatly enriched after a screening of ca. 1 year. However, growth of the lumpy callus lines in liquid medium was extremely low. Their harmalol and harmine levels were never greater than 0.1%. When growth rates increased, the yellow fluorescence decreased to the initial levels. Thus further analytical screening of the tolerant cell lines was terminated.

Tryptophan decarboxylase activity of serotonin producing and non-producing strains

According to our original assumption the tolerant lines may have in part become tolerant because of increased TDC activity and thus possess a better potential for detoxification of the analogue. Therefore the activity pattern of TDC of all lines was measured. In Fig. 3 the typical TDC activity pattern is given for the 4 serotonin producing, the non-producing line PH 6, and the wild type cells. The TDC activity of the producing cells always reached its maximum within the first 24–36 h and then declined steadily to the low level of the non-producing cells. The pattern of TDC activity of productive cells was similar to activity curves of *Peganum* cells inducible for serotonin formation when transferred to production medium (Sasse et al. 1982 b).

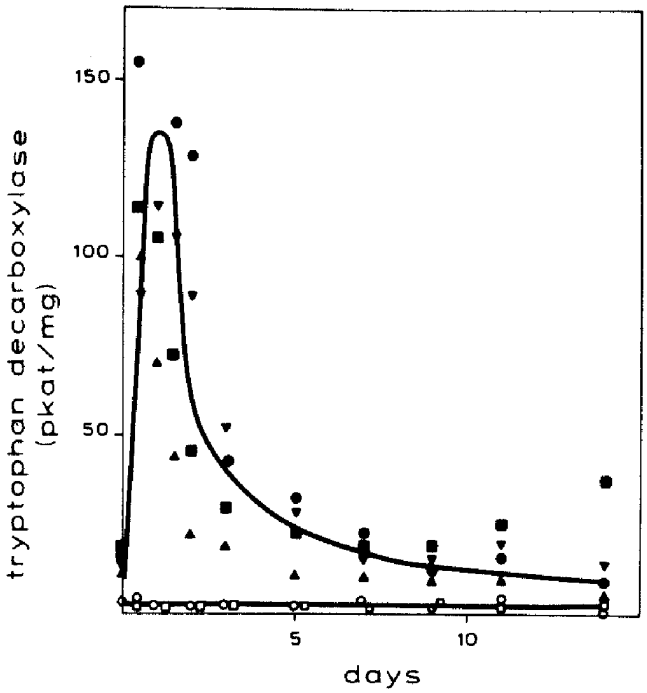

Fig. 3: Activity patterns of tryptophan decarboxylase (related to cell protein of serotonin producing cell lines (PH 2 ▲, PH 5 ▼, PH 13 ■, PH 14 ●) and non-producing cells (wild type ○, PH 6 □).

Reasons for the 4-MT tolerance of the cell lines

The main reason for tryptophan analogue tolerance in several culture systems was the overproduction of tryptophan by an altered feedback control (Ranch et al. 1983, Wasaka and Widholm 1987). Consequently, anthranilate synthase activity, which is inhibited by all toxic tryptophan analogues (Sasse et al. 1983 a), and levels of free tryptophan were measured. The comparison of free tryptophan levels of 4-MT-tolerant and sensitive *Peganum* cultures gave no indication for an overproduction of L-tryptophan (Table 3). The activity of anthranilate synthase in crude cell extracts (concentrated by NH_4SO_4 precipitation and Sephadex-PD10 filtration) of wild type and tolerant cells was very low. When specific activities of *Catharanthus roseus* extracts were set 100%, wild type *Peganum* cells contained 8.5% and the tolerant lines between 2 and 3%. These low activities and the unchanged levels of free tryptophan suggest that a different feedback sensitivity is rather unlikely. The cells may become at least partially tolerant to a toxic analogue through better detoxification (Berlin et al. 1982 b, Watanabe et al. 1982, Sasse et al. 1983 b). The pattern of TDC activity as well as the short period of high activity, make it unlikely that better decarboxylation of 4-MT is the main reason for the increased tolerance. Nevertheless the initial high TDC activity may help to lower the toxic effect of 4-MT. An indication that this may indeed be the case can be deduced from the fact that feeding of L-tryptophan increased serotonin levels of the TDC containing cell lines, while serotonin levels of wild type cells

Table 3: Specific content of free amino acids (μmol/g dry mass) of wild type and 4-MT tolerant *Peganum harmala* cell suspension cultures grown for 7 d on MX growth medium.

amino acid	wild type	PH 2	PH 5	PH 6	PH 14
Asp	6.2	7.7	4.8	4.1	6.1
Glu	14.5	15.1	16.1	9.3	14.1
Asn	13.2	10.5	4.5	7.8	1.9
Ser	10.1	20.8	14.7	9.6	13.8
Gln	13.9	26.6	16.4	12.4	18.4
Gly	2.9	2.9	2.4	2.0	1.2
Thr	5.2	9.5	6.7	5.7	7.4
Arg	1.1	2.0	1.8	2.1	2.2
Ala	8.7	13.4	8.4	6.1	6.8
Tyr	6.3	9.0	5.5	4.8	6.1
Trp	2.2	2.2	1.5	2.6	1.8
Met	3.6	0.9	0.4	0.6	0.6
Val	7.1	9.2	6.5	4.3	5.4
Phe	2.6	4.1	2.2	5.1	3.0
Ile	3.3	3.3	3.5	3.0	3.6
Leu	4.4	3.1	2.9	2.7	2.9
Lys	3.1	5.2	5.6	5.0	5.2
total	108.4	146.3	103.9	87.2	100.5

Table 4: Effect of precursor feeding on serotonin levels. The indicated amounts of tryptophan or tryptamine were added twice at day 1 and 6. Cells were harvested after 10 days. Inoculum was 1 g fresh mass/35 ml medium. Final fresh massses were between 5.2 and 6.2 g. Serotonin yields are given as g/g dry mass (A) and mg/flask (B).

	serotonin levels					
	wild type		PH 2		PH 6	
	A	B	A	B	A	B
Control	0.3	0.15	11.3	4.7	0.6	0.25
Tryptophan feeding						
+ 2 x 2 mg	0.3		13.7	5.3	1.3	0.8
+ 2 x 5 mg	0.2		16.3	6.4	0.8	0.4
+ 2 x 10 mg	0.3		21.5	8.9	0.7	0.3
Tryptamine feeding						
+ 2 x 2 mg	7.1	3.9	18.5	6.5	10.8	3.8
+ 2 x 5 mg	13.3	7.1	26.1	9.1	24.2	10.2
+ 2 x 10 mg	22.9	12.4	31.6	13.6	30.8	13.1

and line PH 6 were only increased by feeding of tryptamine (Table 4). To obtain more insight into the metabolism of tryptophan and related compounds detailed feeding experiments were made with PH 5 cells. When 6 mg tryptophan with 1 μCi ^{14}C-label was fed to 1.2 g cells/10 ml 80 % of the radioactivity was taken up after 4 days of which 60 % remained soluble. Most of the tryptophan fed was converted to serotonin (Fig. 4). Tryptamine, the intermediate of serotonin biosynthesis, also accumulated in PH 5 cells. This was the first time that we detected tryptamine in *Peganum*

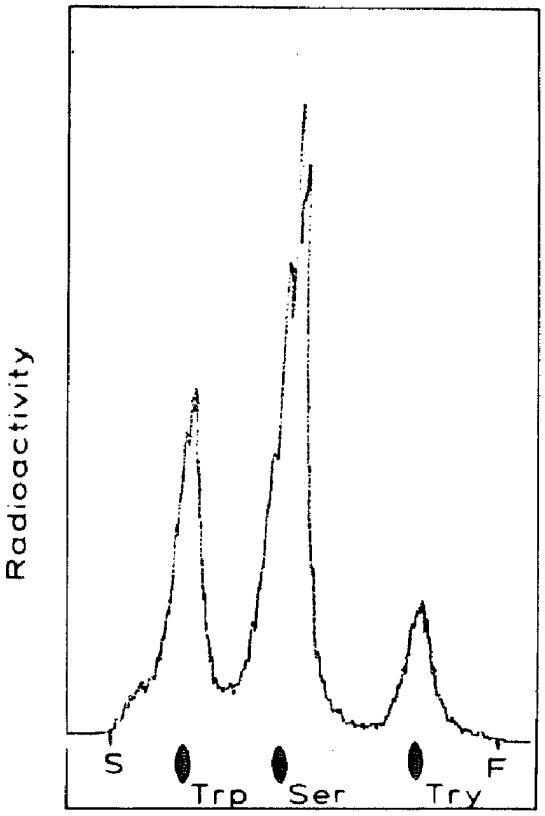

Fig. 4: Distribution of radioactivity in the amino acid fraction of cell extracts of cell line PH 5 after feeding labelled tryptophan (6 mg) for 4 days starting with inoculation. Initial biomass 1.2 g, final biomass 2.2 g/10 ml medium. Chromatographic conditions: Silica gel, solvent CH_2Cl_2 : MeOH : 25 % NH_3. S = start; F = front; Trp = tryptophan; Ser = serotonin; Try = tryptamine.

cell cultures. The *in vivo* activity of tryptophan decarboxylase activity was evidently so high that not all the tryptamine could be used directly for serotonin biosynthesis. Without feeding, or when low levels were fed, tryptamine did not accumulate, as it was immediately hydroxylated to serotonin.

The proof that added tryptophan was decarboxylated by the serotonin producing lines was only an indirect proof for the *in vivo* detoxification of 4-methyltryptophan by decarboxylation. Therefore we investigated also whether 4-methyltryptophan was metabolized via decarboxylation. Indeed the GC/MS analysis of the amino acid fraction of cell extracts showed that at least a part of the 4-methyltryptophan added to the culture medium was converted to 4-methyltryptamine. We did not detect 4-methylserotonin which may be an indication that 4-methyltryptamine cannot be hydroxylated at the 5-position. Beside 4-methyl- and 4-fluorotryptophan 5-fluorotryptophan is also substrate of the TDC. Though 5-fluorotryptophan has a higher K_m for the decarboxylation (Sasse et al. 1983 a) we used this compound because of the availability of the corresponding amine. Even this poorer substrate was decarboxylated to 5-fluorotryptamine (Table 5). Thus, not only *in vitro* but also under *in vivo* conditions the tryptophan analogues were metabolized to the less toxic tryptamine analogues. While addition of 4 mg 5-fluorotryptamine did not effect growth of PH 5 cells, the same concentrations of the DL-tryptophan analogues caused severe growth inhibition of the tolerant lines.

Table 5: Feeding of tryptophan, tryptamine and various tryptophan and tryptamine analogues and derivatives to line PH 5. The incubation time was 4 days beginning with the inoculation. The preculture was maintained on 4-methyltryptophan and had a decreased serotonin level as compared to cultures grown in the absence of the analogue.

Added Compound	Metabolite	Dry Mass mg/10 ml	Serotonin mg/g
Control day 0		86	5.0
Control day 4		131	6.3
L-Tryptophan 6 mg		166	13.4
Tryptamine 6 mg		162	21.8
DL-5-Fluorotryptophan 4 mg	5-Fluorotryptamine	121	2.3
5-Fluorotryptamine 4 mg	5-Fluorotryptamine	160	5.5
DL-4-Methyltryptophan 4 mg	4-Methyltryptamine	112	4.0

A third reason for acquired tolerance could be a decreased uptake of the toxic analogue (Berlin and Widholm 1978, Furner and Sung 1983). Indeed uptake rates for tryptophan were in most experiments distinctly lower by 30–50%. Whether the lowered uptake contributes to the tolerance, remains to be clarified. Additionally the uptake rates of the 4-MT tolerant lines, but otherwise rather sensitive cultures, varied greatly. After transfer of the cells sometimes sudden browning and delay or cessation of growth of the serotonin producing cells was noted even in the absence of analogoues. This was also seen with agar cultures. The metabolic reasons for this behaviour of the serotonin producing lines are yet unknown, it was however never found with wild type or PH6 cells.

Discussion

The characterization of the non or low serotonin producing cell lines of *Peganum harmala* indicated that the lack of tryptophan decarboxylase activity was the reason for the lack of *de novo* synthesis of serotonin. This enzyme was expressed by the initially differentiated cell cultures during the normal growth cycle or was easy to induce by transfer of the cells to a production medium (Sasse et al. 1982b). With the process of dedifferentiation TDC activity decreased more and more, and the cells no longer responded to the conditions earlier developed for TDC induction. Indeed, the *Peganum* cells developed into a very sensitive line which, for example, did not even tolerate a single transfer to a 2,4-D free culture medium. The tolerant lines also respond with complete and immediate growth inhibition to the absence of 2,4-D in the culture medium. This change of culture characteristics is perhaps one reason why we were not able to induce this enzyme under culture conditions usually beneficial for such inductions.

When wild type cells were treated with various biotic and abiotic elicitors serotonin levels of up to 1.5% were found (Berlin and Sasse 1987). Thus the potential for induction of TDC was still present, but other effectors than a transfer to a growth limiting production medium were evidently required for this 6-year-old line. Instead of inducing serotonin formation by adding a suitable inductor, we felt it might be

worthwhile to select for lines where serotonin formation occurs spontaneously without changing culture conditions. Such cultures may represent an even better basis for further improvements. The selection of 4 serotonin producing cell lines out of 5 analyzed suspensions was unique confirmation of this approach. Nevertheless the general applicability of this approach has to be discussed. Was the chosen selective agent really the reason or the requirement for recovering this cell type with this pattern of TDC activity? This question cannot yet be answered, as valid control experiments would require the analysis of 5-methyltryptophan tolerant cell lines. 5-Methyltryptophan is not decarboxylated by TDC (Sasse et al. 1983 a) and should not favour selection of TDC enriched cell types. Such lines were not selected from *Peganum* cultures. However, when we compare tryptamine levels of 5-methyltryptophan and 4-methyltryptophan tolerant *Catharanthus* lines (Schallenberg and Berlin 1979, Sasse et al. 1983 b) it is evident that only selections with 4-methyltryptophan yielded cell lines with increased TDC activity and enhanced tryptamine pools. Scott et al. (1979) measured 30–40 times higher tryptophan levels in 5-methyltryptophan resistant *C. roseus*, however, tryptamine or alkaloid levels were not increased. The finding of so many serotonin producing *Peganum* cell lines by selection for 4-methyltryptophan tolerance supports the hypothesis that selection for better detoxification is a way of finding lines where the detoxifying enzyme activity is increased. The detailed characterization of the p-fluorophenylalanine resistant tobaco cell line TX4 showed clearly that the selective agent was detoxified by phenylalanine ammonia lyase and that this contributed to the overall resistance (Berlin et al. 1982 b). On the other hand it is evident that the better detoxification of the amino acid analogues by the lines described above is not the only reason for the increased tolerance. Nevertheless the finding of a toxic agent which may be detoxified by an enzyme reaction may generally be helpful in establishing cell lines where this enzyme activity is greatly increased.

An important question is whether the proposed biochemical selection is helpful to increase desired product levels of a cell culture system. We have shown that cinnamoyl putrescine levels of the p-fluorophenylalanine resistant tobacco cell line TX4 were increased up to 10-fold compared to wild type cells (Berlin et al. 1982 a). According to our assumption we selected for cell lines with increased phenylalanine ammonia lyase activity (Berlin et al. 1981). Increased levels of cinnamoyl putrescines, the main phenolic compounds of the wild type cells, were only found because the enzyme activities of related biosynthetic enzymes were also distinctly increased in resistant lines (Berlin et al. 1982 a). The indole alkaloid levels of *Catharanthus* cells, however, were not increased by selecting for cell types with increased TDC activity. As a consequence only tryptamine levels were increased (Sasse et al. 1983 b). With 4-MT tolerant *Peganum* cells we found both. We selected for cell types with increased TDC and found serotonin because the enzyme hydroxylating tryptamine at the 5-position was always present in the cells. Though increased TDC activity also seems to be required for high β-carboline alkaloid production, β-carboline alkaloid levels were not increased in the TDC enriched lines as one or all other enzymes of this particular biosynthetic pathway remained repressed. Therefore the use of the proposed selection method may be restricted to increasing a particular enzyme activity or for

increasing product levels of pathways which are co-induced with desired enzyme activity or where the other biosynthetic enzymes are already present.

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