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TRYPTOPHAN DECARBOXYLASE, STRICTOSIDINE SYNTHASE AND ALKALOID PRODUCTION BY *CINCHONA LEDGERIANA* SUSPENSION CULTURES

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Key Word Index *Cinchona ledgeriana*; Rubiaceae; suspension culture; biosynthesis; quinoline alkaloids; tryptophan decarboxylase; strictosidine synthase.

Abstract—The activities of tryptophan decarboxylase (TDC) and strictosidine synthase (SS) have been compared with the production of quinoline alkaloids in light-grown and dark-grown suspension cultures of *Cinchona ledgeriana* transformed with *Agrobacterium tumefaciens*. Enzyme activities and alkaloid production were both substantially greater in the dark-grown cultures than in the light-grown cultures. Under the conditions of assay, SS was in all cases at least 80 times more active than TDC and did not vary very substantially over a 22-day culture period. In contrast, TDC activity in the dark-grown cells rose rapidly to a maximum after 13 days and then declined. Total alkaloid production was largely growth-related, but the cellular alkaloid content declined sharply (on account of release into the medium) within the first 4 days after subculture, prior to the rise in TDC activity. TDC activity over the 22-day period was only 2- to 3-fold greater than that required to account for alkaloid production and is therefore potentially rate-limiting under *in vivo* conditions.

INTRODUCTION

The quinoline alkaloids of *Cinchona* are known to be derived from tryptophan and the terpenoid glucoside secologanin [1]. Tryptophan decarboxylase (TDC; EC 4.2.1.27) and strictosidine synthase (SS), which condenses the decarboxylation product, tryptamine, with secologanin to form the glucoalkaloid, strictosidine, are of particular interest since they catalyse the two initial metabolic reactions committed to alkaloid synthesis and have been studied in detail in purified preparations from cell cultures of *Catharanthus roseus* [2–4]. In cultures of this species [5] and of *Peganum harmala* [6], the increased formation of indole alkaloids following transfer of the cultures to so-called 'production media' is in some cases accompanied by substantial increases in the activity of TDC, though not of SS [5], leading to the suggestion that TDC activity is an important factor regulating the biosynthesis of indole alkaloids derived from tryptophan. In cell cultures of *Cinchona pubescens* Vahl, there have been reports that exogenous tryptophan stimulates quinoline alkaloid production [7] and that this effect is also associated with a greater activity of TDC [8]. In root organ suspension cultures of *C. ledgeriana* Moens (suspensions of clusters with root-like projections), a 5-fold stimulation of quinine and quinidine production by tryptophan has recently been reported [9]: 500 mg/l. of L-tryptophan increased the levels of both alkaloids, measured at 15 days, from *ca* 100–120 μg/l. to *ca* 500–650 μg/l.

Current work in this laboratory is concerned with the physiological and biochemical limitations to quinoline alkaloid production in *Cinchona* cell cultures [10–12]. Using a suspension culture line of *C. ledgeriana* transformed with *Agrobacterium tumefaciens* (and hence capable of growth in the absence of exogenous plant growth regulators) we have demonstrated a reversible stimulation of quinoline alkaloid production by transferring the cultures to darkness [13]. Here, we examine the relationship between quinoline alkaloid production and the activities of TDC and SS over a 22-day period in dark-adapted and light-adapted cultures of this line in order to discover whether the activity of either of these enzymes could be a limiting factor in alkaloid production.

RESULTS AND DISCUSSION

The activities of both TDC and SS were substantially greater in the dark-grown cells than in the light-grown cells and in each case SS activity greatly exceeded TDC activity (Fig. 1). Whereas the activity of SS in the dark-grown cells was almost constant at about 20–30 pkat/mg of extract protein, the activity of TDC in the same cells was scarcely detectable in the inoculum, rose quite sharply to 0.34 pkat/mg of protein after 13 days and then declined to about 0.11 pkat/mg after 22 days. In the light-grown cells, SS activity varied between 2 and 5 pkat/mg of extract protein, whereas TDC was again scarcely detectable in the inoculum, rising to about 0.02 pkat/mg after 8 days and declining slightly beyond 18 days.

The total content of quinoline alkaloids (i.e. in cells and medium) was, in common with the enzyme activities, substantially greater in the dark-grown cultures than in the light-grown cultures. Expressed on a fr. wt basis, the values for total alkaloid content of 20–35 μg/g (dark-grown) and 2–5 μg/g (light-grown) did not show substantial variation over the 22-day period (Fig. 2a). The final total alkaloid contents of the culture flasks, after 22 days, were about 240 μg (dark-grown) and 24 μg (light-grown),

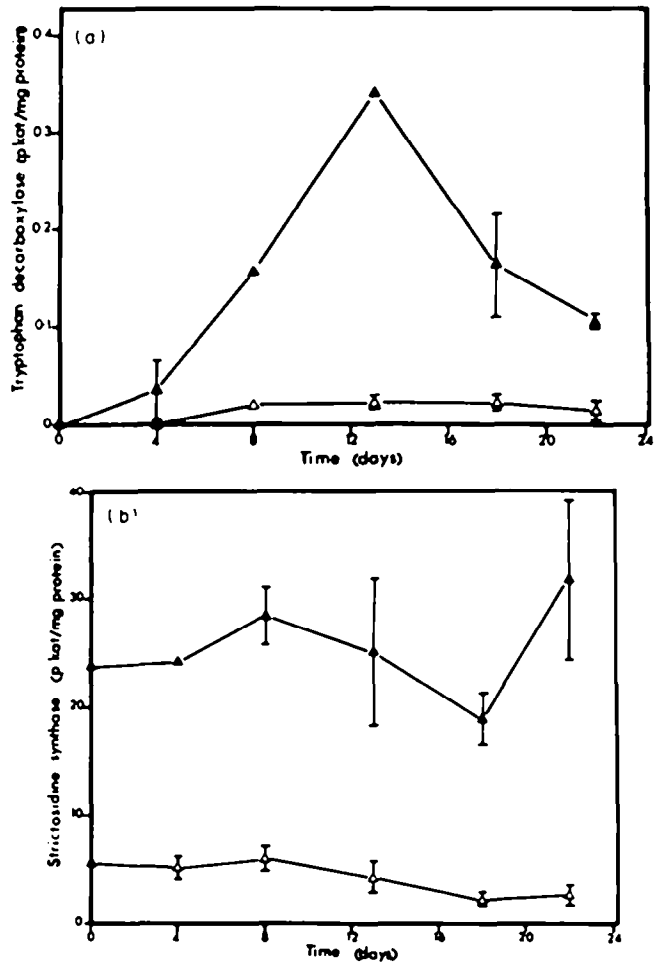

Fig. 1. Activities of (a) TDC and (b) SS in cell extracts from culture flasks grown in the light (△) and the dark (▲) over a 22-day period. Values are means ± s.d. for replicate flasks. Duplicate determinations were done on each flask.

representing an approximately 4- to 5-fold increase from the inoculum value (data not shown); since the fr. wt of the cells increased by about 3- to 4-fold over the same period, total alkaloid production was largely growth-related, as implied by the near-constant values in Fig. 2a. However, the values for the alkaloid content of the cells *alone*, expressed as μg/g fr. wt, were by no means constant over the 22-day period (Fig. 2b). The alkaloid content of the dark-grown cells decreased sharply from an inoculum value of 24 μg/g fr. wt to *ca* 8 μg/g fr. wt after 4 days and then rose approximately linearly to 22 μg/g fr. wt after 22 days; a similar, though less pronounced, pattern was observed with the light-grown cells. The initial fall in cellular alkaloid content was in each case balanced by the appearance of alkaloids in the culture medium and apparently reflected simply the release of alkaloids from the inoculum upon transfer to fresh medium (data not shown).

The contents of individual quinoline alkaloids in the dark-grown cultures (i.e. in cells plus medium), expressed as μg/g fr. wt, are given in Table 1. A qualitatively similar spectrum of alkaloids was produced by the light-grown cultures (data not shown). In the preponderance of alkaloids lacking methoxylation (cinchonine, cinchonidine and dihydrocinchonine) and the near-equivalence between 8*R*,9*S* and 8*S*,9*R* stereoisomers cinchonine and cinchonidine, the results are similar to those previously obtained with untransformed *C. ledgeriana* suspension cultures [10, 11].

From a comparison of Figs. 1a and 2a, it is possible to calculate that whilst the activity of TDC in the dark-grown cells was adequate to account for alkaloid production, it was not greatly in excess. Assuming a mean TDC activity over 22 days of *ca* 0.16 pkat/mg of protein, a mean fr. wt over the same period of *ca* 4 g and a protein content for the extracts of 1.6 mg/g fr. wt, the activity could account for the decarboxylation of *ca* 2 μmol of tryptophan, compared with a total alkaloid content after 22 days of *ca* 0.8 μmol (i.e. *ca* 250 μg, assuming an M_r of 300 for quinoline alkaloids). A similar calculation for the light-grown cultures gives a value of *ca* 0.2 μmol of tryptophan, compared with a final alkaloid content of *ca* 0.08 μmol. Therefore, whereas SS would be very unlikely to limit alkaloid formation *in vivo*, synthesis could be limited by TDC, the activity of which was at least 80 times lower (Fig. 1). This would depend upon the rates of other potentially limiting reactions in the pathway and also upon the concentration of tryptophan. In *Catharanthus*

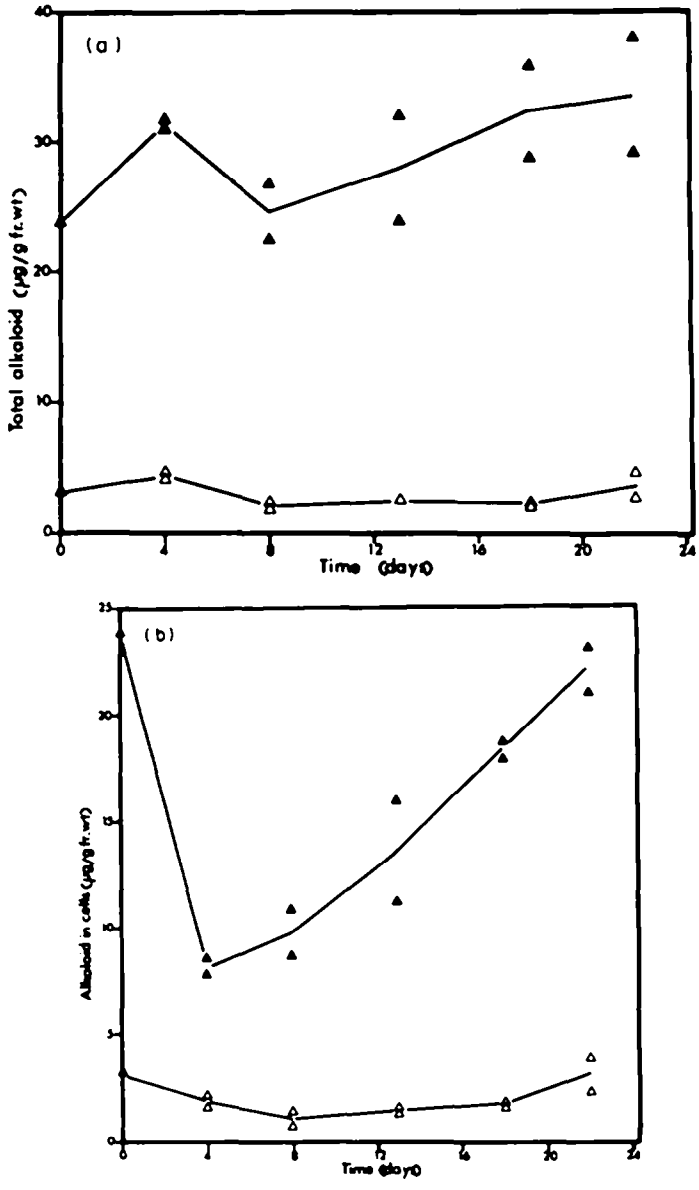

Fig. 2. Total alkaloid (a) in cells and medium and (b) in cells alone expressed per g fr. wt of cells from culture flasks grown in the light (△) and the dark (▲) over a 22-day period. Values from replicate flasks are shown. The mean total alkaloid contents of the flasks (volume 50 ml) at inoculation were 4.8 μg (light-grown) and 50 μg (dark-grown) and after 22 days were 24.4 μg (light-grown) and 241 μg (dark-grown).

roseus, the K_m value of TDC for tryptophan is *ca* 0.1 mM [2] and preliminary observations indicate a similar value for the *C. ledgeriana* enzyme (S. E. Skinner, unpublished results). This is well below the value of 0.5 mM used for assay and indicates that were the tryptophan concentration to fall below the K_m concentration, TDC would become rate-limiting.

The marked increase in TDC activity in the dark-grown cells occurred after a sharp fall in the alkaloid content of the cells (Fig. 2b), and the behaviour of the light-grown cells was similar though much less pronounced. One possible explanation is that the stimulation of TDC activity was in response to a lower intracellular alkaloid content and, conversely, that the decline in activity after 13 days was caused directly or indirectly by a rising intracellular alkaloid content; on the other hand, more transient and spectacular increases in enzyme activity following subculture to fresh medium have long been shown in other systems; for example, for the enzymes of general phenylpropanoid metabolism in parsley cell suspension cultures [14]. No effect of quinoline alkaloids on the assay of TDC activity has been observed (R. J. Robins, unpublished results); however, whether alkaloids added to *Cinchona* cell cultures can affect the extractable TDC activity has yet to be investigated.

The mechanism underlying the increased enzyme ac-

Table 1. Total contents of individual quinoline alkaloids in dark-grown cultures 0, 13 and 22 days after subculture. Values are μg of alkaloid/g fr. wt of cells and are means of duplicates (13 and 22 days) or individual values (0 days)

	Time after subculture (days)		
Alkaloid	0	13	22
Cinchonine	8.5	12.5	15.4
Cinchonidine	9.8	8.8	10.3
Dihydrocinchonine	3.6	3.3	4.0
Quinidine	0.6	1.2	1.3
Quinine	0.6	1.4	1.1
Dihydroquinidine	0.3	0.4	0.4
Dihydroquinine	0	0.4	0.5

tivities and alkaloid production of dark-grown cells in relation to light-grown cells is presently under investigation. It is possible that the levels of a number of tryptophan-related enzyme activities may be increased in dark-grown cultures. No effect of darkness on alkaloid production has been observed in cultures of the related, untransformed line (J. Payne, R. J. Robins, and M. J. C. Rhodes, unpublished results). It is not known whether the effect may be a direct or an indirect consequence of transformation by *Agrobacterium tumefaciens*.

The present observations are in some respects similar to those of Knobloch *et al.* [5], who found that TDC activity rose rapidly following the transfer of a *Catharanthus roseus* suspension culture to an alkaloid production medium whereas the SS activity remained virtually constant at a higher level. In this case, the increase in TDC activity was closely related, kinetically, to the production of alkaloids. It has recently been shown in this system that *m*RNA for TDC is detectable only in cells grown in the production medium [15]. On the other hand, the correlation between TDC activity and indole alkaloid production noted by Knobloch *et al.* [5] is by no means universal [16, 17], showing that other potentially limiting factors may be involved. One such factor is the supply of secologanin for the synthesis of strictosidine, and a stimulation of alkaloid production by the addition of secologanin to the medium has recently been observed in a culture of *C. roseus* in which no correlation between TDC activity and alkaloid production was apparent [18]. This, however, contrasts with earlier work by Zenk *et al.* [19], who failed to show any appreciable stimulation of alkaloid synthesis in *C. roseus* cultures by feeding secologanin.

In comparison with *Catharanthus*, there is less information available on the factors which limit alkaloid synthesis in *Cinchona* cultures. A stimulation of quinoline alkaloid formation by supplying exogenous tryptophan has been observed by two groups [7-9] and in one case, using *C. pubescens* cell cultures grown in air-lift fermenters, it was claimed that exogenous tryptophan caused increased TDC activity [8]. In fact, the correlation between alkaloid production and TDC activity was not consistent, since during the first 4 days of culture, the tryptophan-supplemented culture produced more alkaloid but contained less TDC activity then the unsupplemented culture.

It is clear that it is not possible to identify biochemical factors which are *universal* in limiting the production of tryptophan-derived alkaloids by individual cell cultures. This should not be surprising, bearing in mind the large number of enzyme reactions involved in the formation of strictosidine and its metabolism to indole and quinoline alkaloids and the necessity for these enzymes to be synchronously active in cultures which may show genetic and epigenetic variation.

EXPERIMENTAL

Chemicals. Secologanin was a generous gift from Dr. R. T. Brown, Department of Chemistry, University of Manchester, U.K. [*Side-chain* 2-^{14}C]tryptamine bisuccinate was obtained from New England Nuclear, Southampton, U.K.

Plant material. Dark-adapted and light-adapted cultures (line FRIN-CL2A6) of *Cinchona ledgeriana* transformed with *Agrobacterium tumefaciens* were established and maintained as described elsewhere [13]. A sterile plantlet of *C. ledgeriana* (clone QC, kindly supplied by Dr. C. S. Hunter, Bristol Polytechnic, Bristol, U.K.) was infected between nodes with *A. tumefaciens*, strain A6 (kindly supplied by Dr. J. L. Firmin, John Innes Institute, Norwich, U.K.) and the crown gall produced was aseptically excised and transferred to Gamborg B5 nutrient medium, without hormones, containing 2% (w/v) sucrose, 0.25 mg/ml vancomycin and 0.25 mg/ml carbenicillin and solidified with 1% (w/v) Difco bacto-agar. The tumour callus was maintained on this medium at 26° and subcultured fortnightly for five transfers before the antibiotics were omitted. Liquid suspension culture was established from the callus in sterile, hormone-free Gamborg B5 nutrient medium containing 2% (w/v) sucrose, and cultures were subsequently maintained in 250 ml flasks containing 50 ml of medium and an inoculum of *ca* 2 g fr. wt of cell clumps, finely chopped. Subculturing was at 28-day intervals and culture flasks were incubated at 25° with shaking. Light-grown cultures received a 24 hr cycle of 16 hr light (800 lx) and 8 hr darkness. The light-grown and dark-grown cultures were maintained (with subculturing) for at least 1 year before use.

Evidence for transformation was obtained from the synthesis of [*methionine*-^{35}S]-N^3-(1-carboxyethyl)methionine from [^{35}S]-methionine supplied to samples of callus, as described by Firmin *et al.* [20], except that analysis was performed by HPLC using a Partisil 10SAX column (Whatman Ltd., Maidstone, Kent, U.K.) eluted with a linear gradient of K-Pi, pH 5 (5–400 mM; 2 ml/min; 3%/min) and connected to a flow-through radioactivity detector. This 'pseudo-opine' methionine derivative [20] was not produced by callus of the untransformed line from which the CL2A6 line was derived.

Cell extracts were prepared from cells frozen in liquid N_2 and stored at −20°. Cells were homogenized from the frozen state in 2 vols. of chilled 50 mM HEPES–NaOH, pH 7.5, containing 5 mM dithiothreitol (extraction buffer) and 0.05 g Kollidon and 0.1 g Polyclar per g fr. wt of tissue. After passage through two layers of Miracloth, the extract was clarified by centrifugation at 0° (19000 *g*, 20 min) and 5 ml of the supernatant soln was passed through a column (250 mm × 15 mm) of Sephadex-G25 (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with the extraction buffer. The protein-containing eluate was finally concentrated *ca* 5 × in an Amicon B15 concentrator (Amicon Corporation, Lexington, MA, U.S.A.).

Enzyme assays. TDC was assayed in a vol. of 2 ml containing 50 mM HEPES–NaOH, pH 7.5, 5 mM dithiothreitol, 0.04% Na azide, 1 mM pargyline, 10 μM pyridoxal phosphate, 0.5 mM L-tryptophan and cell extract. Control mixtures lacked tryptophan or extract, or were stopped prior to incubation. Assay mixtures were incubated for 16 hr at 30° and then stopped with 100 μl 3.6 M NH_4OH. (Proportionality between tryptamine produc-

tion and both incubation period and extract concn was established.) Tryptamine was extracted with EtOAc (2 × 5 ml) and the combined EtOAc phases were evaporated under vacuum. The residue was redissolved in 0.2 ml HPLC running buffer (H_2O THF–HOAc MeCN, 455:3.6:5:45, pH 3.6) and tryptamine was determined by reverse-phase HPLC (Waters μ Bondapak C18 column, 300 mm × 3.9 mm, Waters Associates, Milford, MA, U.S.A., solvent as above, flow rate of 1.5 ml/min, detection by absorbance at 278 nm) using tryptamine as an external standard.

SS was assayed in a vol. of 0.5 ml containing 100 mM K-Pi buffer, pH 7.5, 0.1% Na azide, 25 mM δ-D-gluconolactone (freshly prepared soln), 1.25 mM secologanin; 0.25 mM [*side-chain*-2-^{14}C]tryptamine, sp. act. 4 Ci/mol; and cell extract. Control mixtures lacked secologanin or extract, or were stopped prior to incubation. Assay mixtures were incubated for 2 hr at 30° and were then stopped with 25 μl 3.6 M NH_4OH. ^{14}C-Labelled strictosidine was extracted into EtOAc (3 × 1 ml) and subsequently determined by TLC followed by liquid scintillation counting as described previously [3].

Alkaloids were extracted and determined by HPLC as described previously [10, 21].

Protein was assayed by the method of Bradford [22].

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