174. Alkaloid Production in *Catharanthus roseus* Cell Cultures. VII.[1]) Effect of Parameter Changes and Catabolism Studies on Cell Line PRL No. 953.

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Summary

Catharanthus roseus cells suspended in production medium showed the presence of four *Aspidosperma*-type alkaloids, however, no vindoline. Cells grown in media with the pH adjusted to 7.0 produced 3.3 mg total alkaloids/g dry weight. At pH 5.0, 1.7 mg of alkaloid/g dry weight was produced. (*S*)-Adenosyl-methionine did not stimulate the production of vindoline. When added to cell suspensions for 21 days, vindoline and catharanthine were degraded to non-alkaloidal substances, not dimerized to bisindole alkaloids.

Catharanthus roseus, a source of antitumor agents including the bisindole alkaloids vinblastine (**1a**) and vincristine (**1b**), has been subjected to *in vitro* cell culture for over 30 years [3]. So far attempts to detect bisindole alkaloids in cell cultures have failed. An alternative goal would be the production of vindoline (**2a**) and catharanthine (**3**). Both are monomeric alkaloids of *C. roseus* and it is possible to synthesize known and novel bisindole alkaloids from these two compounds [2]. Catharanthine has been isolated from cell line PRL No. 200 and fully characterized [9]. So far, the only claims of vindoline production from cultured cells have been made on the basis of either Rf and color on TLC. plates [5] or incorporation of radio-labelled precursor [6] and in yields *ca.* 500 times lower than obtained from *C. roseus* plants.

Cell line PRL No. 953 has been analyzed earlier [10]. It did not show catharanthine and vindoline accumulation. Out of 13 alkaloids identified, 7 belonged to *Aspidosperma*-type alkaloids of which vindoline is a member. This result led to several experiments designed to stimulate vindoline synthesis. The approach taken was to change the acidity of the culture medium from pH 5.5 to pH 7.0 and

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add (*S*)-adenosyl-methionine to the medium in order to provide for indole *N*-methylation in vindoline synthesis. It has been shown elsewhere [11] that the alkaloid yield is affected by the initial pH of the medium and earlier experiments with cell-free systems revealed that the presence of (*S*)-adenosyl-methionine is of great importance in the biosynthesis of vindoline [12]. Failure to detect vindoline and catharanthine accumulation in cell line PRL No. 953 required several exploratory feeding experiments even though results might not fully reflect intracellular metabolism.

The *Catharanthus roseus* (L.) G. DON. cells employed were subcultures of a line coded PRL No. 953. This line originated from anther wall callus initiated in August 1978. Serially cultured callus No. 953 was suspended in liquid 1-B5C medium [13] and transferred to fresh medium at intervals of 4 days over a period of 2 months prior to use. The suspensions were kept on gyratory shakers at 27° C in continuous light (10 $W.m^{-2}$). Aggregates in the suspension consisted of *ca.* 30 cells.

For experiments on the effect of pH and of (*S*)-adenosyl-methionine, 50 ml of cell suspension, *i.e.* 8–10 g of cells, were added to 250 ml of production medium [4] in 1-l-*Erlenmeyer* flasks. The pH of the media was adjusted prior to autoclaving and maintained constant during the culture period by adding, if necessary, 0.2N KOH. Samples were withdrawn at various intervals and analyzed for mitotic acitivity.

Vindoline and catharanthine were obtained from *Eli Lilly & Co*, Indianapolis, Indiana 46206, U.S.A. Both compounds were sterilized by filtration. They were added to 300-ml-cell suspensions at the time when the mitotic activity of the cells had ceased. [Ar-^{3}H]-vindoline and [Ar-^{3}H]-catharanthine were likewise administered. These compounds had been prepared by a tritium exchange method [19–21].

Upon terminating the experiments the cells were freeze-dried, weighed, extracted and analyzed by TLC., and HPLC. methods as described earlier [7] [8]. Cells exposed to tritiated vindoline and catharanthine were processed as described below.

Subcultures of *C. roseus* cell line PRL No. 953 were grown for 4 weeks in production medium adjusted to pH 5.5 or pH 7.0. Upon examining TLC. and HPLC. data the cells were found to have accumulated ajmalicine (**4**), strictosidine (**5**), yohimbine (**10**), isositsirikine (**11**), lochnericine (**9**), hörhammericine (**7**), hörhammerinine (**8**) and vindolinine (**6**). Vindoline and catharanthine were not detected in this material.

When production medium adjusted to pH 5.5 or pH 7.0 and supplemented with various amounts of (*S*)-adenosyl-methionine was inoculated with cells and incubated for a period of 4 weeks, the levels of alkaloids indicated in *Table 1* were

Table 1. *Effect of pH and (S)-adenosyl-methionine on the accumulation of alkaloids in periwinkle cells* (Control experiments repeated 3 times, extreme data in brackets)

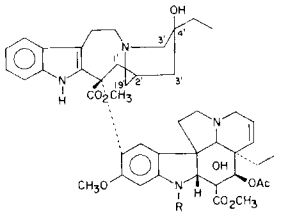
		Amount (mg) of (*S*)-adenosyl-methionine	Wt. of freeze-dried cells (gm)	Wt. of crude alkaloid fraction (mg)	% yield of alkaloid from cells	Specific yield mg alkaloid/ gm dried cells
pH 5.5	1	0	7.68	13.28	0.17 (0.09–0.20)	1.73
	2	10	7.45	10.0	0.13	1.34
	3	20	8.95	10.0	0.11	1.12
	4	30	6.47	9.0	0.14	1.39
	5	40	7.02	12.0	0.30	2.99
pH 7.0	1	0	7.39	24.25	0.32 (0.24–0.38)	3.28
	2	10	7.81	31.0	0.39	3.97
	3	20	8.35	31.0	0.37	3.71
	4	30	10.75	30.0	0.28	2.79
	5	40	9.01	33.7	0.37	3.74

Table 2. *Recovery of added vindoline and catharanthine after incubation with cell suspensions over various periods of time*

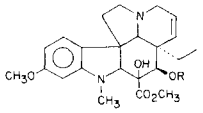
Incubation Time	Precursor	% Recovery
24 h	catharanthine (25 mg) and vindoline (25 mg)	100
48 h	catharanthine (25 mg) and vindoline (25 mg)	100
72 h	catharanthine (25 mg) and vindoline (25 mg)	100
21 days	catharanthine (200 mg)	12
21 days	vindoline (200 mg)	15
WITHOUT CELLS		
21 days	vindoline (20 mg)	100
21 days	catharanthine (20 mg)	75

obtained. Results show relative higher alkaloid accumulation in media adjusted to pH 7.0. (*S*)-Adenosyl-methionine did not significantly affect the production of alkaloids. Also, alkaloid spectra showed little variation except for the occurence of dimethyltryptamine (**12**) in cells cultured in the presence of (*S*)-adenosyl-methionine. Vindoline and catharanthine, again, were not detected under these conditions.

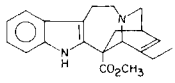
Adding vindoline and catharanthine to cell suspensions which had been growing for 2 weeks and had ceased all mitotic activity resulted in the following observations *(Table 2)*. After 1–3 days of incubation vindoline and catharanthine were almost



1a R = CH_3
1b R = CHO



2a R = $COCH_3$
2b R = H



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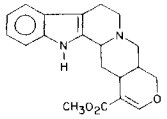

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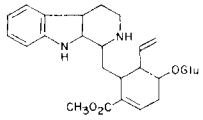

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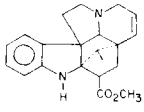

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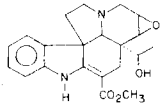

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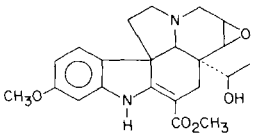

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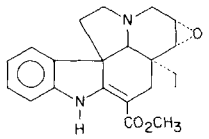

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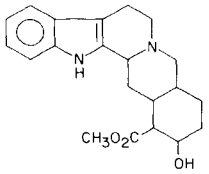

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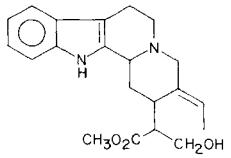

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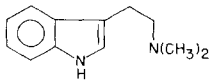

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completely recovered (> 99%). After 21 days of incubation appreciable catabolism (> 85%) of both vindoline and catharanthine had occurred. Control experiments indicated that the medium caused only little degradation (< 5%) of vindoline and some degradation (25%) of catharanthine. Dimeric alkaloids were not detected. In a large scale experiment 200 mg of vindoline and catharanthine were added to a cell suspension which had been grown in a 2 l *Erlenmeyer* flask and shaken on a gyratory shaker (130 rpm) for 14 days. Results indicate that vindoline and desacetyl vindoline (**2b**) were present *i.e.* only 15% of the added vindoline could be readily accounted for. Similarly only 12% of catharanthine was recovered *(Table 2)*. Deacetylation of vindoline has been observed earlier in crowngall [17] and suspension cultures [18] of *Catharanthus roseus*.

In an attempt to determine the products of vindoline and catharanthine catabolism [Ar-^{3}H]-vindoline and [Ar-^{3}]-catharanthine were administered to a two week old cell suspension grown in an *Erlenmeyer* flask on a rotary shaker (130 rpm). After a further two week incubation medium and cells were separated, the cells were thoroughly washed and both fractions freeze-dried. This material was then extracted with methanol and the total radioactivity measured *(Table 3)*. After evaporation of the solvent, the residue was partitioned between water and ethyl acetate. Standard acid/base partition gave three fractions, the residual aqueous, an ethyl acetate fraction containing the neutrals and acids and the ethyl acetate fraction containing bases. The total radioactivity of each was measured. In both instances, the radio-labelled precursor appeared to have been extensively distributed into non-alkaloidal material.

Cell line PRL No. 953 has been analyzed and shown to produce 4 *Aspidosperma*-type alkaloids. Vindoline was not detected. An increase in the pH of the medium while beneficial to total alkaloid production did not stimulate vindoline synthesis. Neither was the presence of (*S*)-adenosyl-methionine supportive of vindoline

Table 3. *Distribution of radioactivity* (dpm) *in periwinkle cells fed with [Ar-^{3}H]-vindoline and [Ar-^{3}H]-catharanthine*

	Total activity added (dpm)	Total activity recovered in MeOH extract	Total activity not recovered	Total activity residual aqueous	Total activity neutral+	Total activity bases
Vindoline	4.4×10^8	1.65×10^8	2.75×10^8	1.25×10^8	0.07×10^8	0.26×10^8
Percent of total activity	100	37.5	62.5	28.4	1.6	5.9
Catharanthine	4.87×10^7	2.4×10^7	2.47×10^7	1.2×10^7	0.7×10^7	0.4×10^7
Percent of total activity	100	50	50	25	14	8.3
Catharanthine	1.06×10^8	5.04×10^7	5.56×10^7	1.90×10^7	2.4×10^7	0.69×10^7
Percent of total activity	100	47.5	52.5	17.9	22.3	6.3

formation. Failure of these experiments to affect vindoline accumulation may be related to compartmentalization of vindoline synthesis and membrane barriers for (*S*)-adenosyl-methionine. Interpretation of the results may be as complex as with cells which show unchanged alkaloid levels despite increased intracellular tryptophane concentrations [14] [15].

The fate of externally supplied vindoline and catharanthine, *i.e.* their degradation to non-alkaloidal substances would have its parallel in similar experiments performed with morphine and poppy plants [6]. Further experiments, however, would have to demonstrate the uptake of vindoline and catharanthine beyond doubt. Apart from finding radioactivity in washed cell preparations after feeding [Ar-^{3}H]-vindoline and [Ar-^{3}H]-catharanthine there is only an indirect indication of uptake of complex alkaloids by periwinkle cells: vinblastine analogues were seen to affect mitosis in a manner reminiscent of colchicine [22].

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