Formation of Benzophenanthridine Alkaloids by Suspension Cultures of *Eschscholtzia californica*

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Eschscholtzia californica, Suspension Cultures, Benzophenanthridine Alkaloids, Phenolics, Media Effects

Suspension cultures of *Eschscholtzia californica* accumulate the dihydro forms of the benzophenanthridine alkaloids sanguinarine, chelirubine, macarpine and chelerythrine, all of which are known to be constituents of the *Eschscholtzia* plant. Under most experimental conditions dihydrochelirubine was found to be the main constituent of the cultured cells. The specific yields of alkaloids varied from zero to 1.7% on a dry weight basis depending on the media conditions. The highest specific yield was 1.5 mg/g dry weight or 13 mg/l with the growth medium B5. After transfer of the cells into the induction medium IM2 the alkaloid accumulation increased to 17 mg/g dry weight and 146 mg/l. The induction medium contained increased levels of sucrose, decreased levels of phosphate and was devoid of phytohormones. The effect of the various media conditions on the biosynthesis of phenolics was quite different to those found for the alkaloids.

Introduction

The family of *Papaveraceae* is known to contain a great number of isoquinoline alkaloids. Some of these alkaloids, such as the benzophenanthridines, are found in many species of this family. These common alkaloids have also been reported to be formed in cell cultures of this family [1–3], while the plant species specific compounds are very rarely synthesized by such cultures [4]. The Papaveraceae *Eschscholtzia* contains the common benzophenanthridine alkaloids and also, as minor components, species specific alkaloids [5, 6]. Thus, it was expected that cell cultures of *Eschscholtzia californica* would accumulate isoquinoline alkaloids. It was noted that the levels of these alkaloids were easily altered. Therefore, this culture seemed to be a good system for studying the biochemical and biotechnological aspects of alkaloid formation.

Here we report the structural identification of the alkaloids (Fig. 1), a revised structure of macarpine [5, 6] and we compare the effect of medium constituents on two independent secondary pathways by following the accumulation patterns of the alkaloids and phenolics.

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Materials and Methods

Plant material

Cultures of *Eschscholtzia californica* were established from seedlings grown under sterile conditions on B5 medium [7] containing 5×10^{-6} M 2,4-dichlorophenoxyacetic acid. The cultures have been grown in suspension for more than 2 years on B5-medium. Every 10 d 2 g fresh weight were transferred to 70 ml of fresh medium. For a chemical comparison of intact plants and cultured cells, plants were analyzed which were grown for 3 months in the garden.

Structural identification

For the identification of the alkaloids freeze dried cells (5 g) were extracted with 80% MeOH, and evaporated to dryness at room temperature. The residue was shaken with a solution of hexane: ether (6:1), filtered and concentrated under nitrogen to a small volume which was then chromatographed on a half-preparative Si-60-10 µ-column with a precolumn with hexane:ether (6:1) as eluent.

^{1}H HMR spectra (400.14 MHz) were recorded at ambient temperature on a Bruker WM 400 spectrometer operating in the Fourier transform mode and locked to the main deuterium resonance of the CD_3OD solvent. Shifts are reported in ppm relative

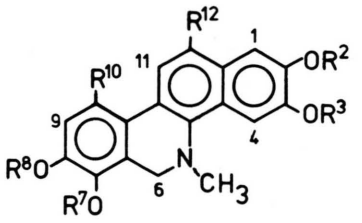


Fig. 1. Benzophenanthridine alkaloids isolated from suspension cultures of *Eschscholtzia californica.*

I $R^2 + R^3 = CH_2$; $R^7 = R^8 = CH_3$; $R^{10} = R^{12} = H$ — Dihydrochelerythrine

II $R^2 + R^3 = R^7 + R^8 = CH_2$; $R^{10} = R^{12} = H$ — Dihydrosanguinarine

III $R^2 + R^3 = R^7 + R^8 = CH_2$; $R^{10} = OCH_3$; $R^{12} = H$ — Dihydrochelirubine

IV $R^2 + R^3 = R^7 + R^8 = CH_2$; $R^{10} = R^{12} = OCH_3$ — 12-Methoxydihydrochelirubine (Dihydromacarpine see text)

V $R^2 + R^3 = CH_2$; $R^7 = R^8 = CH_3$ — Dihydrochelilutine

or $R^2 = R^3 = CH_3$; $R^7 + R^8 = CH_2$ — Dihydrosanguirubine

; $R^{10} = OCH_3$; $R^{12} = H$

to TMS and were measured relative to the residual proton methyl signal of the solvent at 3.35 ppm. Nuclear Overhauser enhancement ^1H difference spectra were recorded using the standard Bruker software package. Mass spectra were recorded on an AEI MS 902S spectrometer operating at 70 and 16 eV.

Dihydrochelirubine (**III**)

NMR: $^1H(CD_3OD)$, $\delta = 8.313$ (d, 1H, H-11, J 8.7), 7.657 (s, 1H, H-4), 7.474 (d, 1H, H-12, J 8.7), 7.149 (s, 1H, H-1), 6.764 (s, 1H, H-9), 6.069 (s, 2H, OCH_2O-7, 8 or -2, 3), 6.033 (s, 2H, OCH_2O-2, 3 or -7, 8), 4.105 (s, 2H, CH_2-6) 3.900 (s, 3H, OCH_3-10), 2.571 (s, 3H, NCH_3-5). Nuclear Overhauser enhancements were observed upon irradiaton of: NCH_3-5 for the signals at 7.657 (st, strong, H-4) and 4.105 (w, weak, CH_2-6), OCH_3-10 for the signal at 6.764 (st, H-9). MS: $M^+ = m/z$ 363, $C_{21}H_{17}NO_5$ requires $M^+ = m/z$ 363.

12-Methoxydihydrochelirubine (**IV**)

NMR: $^1H(CD_3OD)$, $\delta = 7.882$ (s, 1H, H-11), 7.629 (s, 1H, H-4), 7.473 (s, 1H, H-1), 6.771 (s, 1H, H-9), 6.067 (s, 2H, OCH_2O-7, 8 or -2, 3), 6.033 (s, 2H, OCH_2O-2, 3 or -7, 8), 4.105 (s, 2H, CH_2-6), 4.020 (s, 3H, OCH_3-12), 3.927 (s, 3H, OCH_3-10), 2.514 (s, 3H, NCH_3-5). Nuclear Overhauser enhancements were observed upon irradiation of: NCH_3-5 for the signals at 7.629 (st, H-4) and 4.105 (w, CH_2-6), OCH_3-10 for the signals at 6.771 (st, H-10) and 7.882 (w, H-11), OCH_3-12 for the signal at 7.882 (st, H-11). MS: $M^+ = m/z$ 393, $C_{22}H_{19}NO_6$ requires $M^+ = m/z$ 393.

Media variation experiments

Two grams of 10 day-old cultures were transferred to the specified medium. When the effects of various concentrations of a single constituent were measured all cultures were harvested on the 10th day. In all other experiments cultures were harvested after 3, 6, 9, 11, and 14 days. To realize the effects of the various phytohormones the cultures were kept for a second culture period on the same phytohormone composition.

Extraction and quantification of alkaloids and phenolics

The cells were harvested by vacuum filtration. After determination of the fresh weight the cells were freeze dried. These cells (100 mg) were extracted twice with 5 ml of 80% MeOH. All extracts were brought to 10 ml and were filtered. Five hundred µl of the solution was used for the measurement of total phenolics by the Folin-method as described previously [8]. Total phenolics are expressed as µg chlorogenic acid/mg dry weight.

Benzophenanthridine alkaloids occur naturally as dihydro compounds which may easily oxidize during the preparation in an unforeseeable ratio.

Thus, the alkaloids were also determined in the oxidized form. Measurement of the oxidized form: The methanolic solution was oxidized with a small amount of CrO_3 and then 1 to 10 µl were chromatographed on silica-plates with concentration zones with toluene/methanol 50:10 as solvent. The absorbance of the alkaloids was measured at 285 nm with a Shimadzu TLC-scanner and the concentrations were calculated from calibration curves of sanguinarine or chelerythrine. Measurement of the dihydro forms by HPLC: A methanolic solution (4 ml) was evaporated under reduced pressure, taken up in 2 ml H_2O at pH 10 and extracted with 2 ml of $CHCl_3$. The $CHCl_3$-phase was dried over Na_2SO_4 and filtered. The sample (10 µl) was chromatographed on a Licrosorb Si-60-10 µ-column with a precolumn Si-60-prep. with hexane:ether:dichloromethane (75:15:1) as eluent. The data of the two methods of quantification agreed very well.

Results an Discussion

Structual elucidation

The hexane:ether soluble compounds of a crude cell extract, purified by HPLC, were identified by their fluorescence, absorbance, mass and NMR spectral data. The structures of compounds **I** and **II** followed directly from the mass spectral data, fluorescence and from comparison of the HPLC/TLC behaviour in several solvent systems with that of authentic material. Fluorescence data, TLC retention time and mass spectral data ($M^+ = m/z$ 379, $C_{22}H_{21}NO_5$ requires $M^+ = m/z$ 379) for **V** did not allow the distinction between dihydrosanguirubine and dihydrochelilutine (for recent structure clarification of these compounds see ref. 9). Sufficient material was not available for a NMR study.

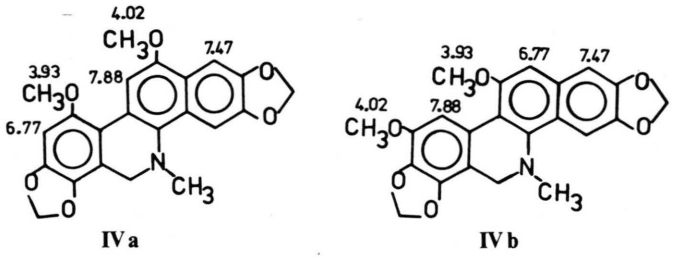
The structures of **III** and **IV** followed from mass spectral and ^{1}H NMR data. These data indicate the presence of the benzo-[*c*]-phenanthridine skeleton with one NCH_3 and two methylene-dioxy substituents together with one methoxyl group for **III** and two for **IV**. Although the early literature (10–12) concerning the structure and NMR data for this type of compound appears confusing, more recently the establishment of the unambiguous structure of the key compound chelirubine [9] allowed the reassignment of its ^{1}H NMR spectrum together with that of several related compounds [9, 13]. The structure of **III** follows directly from the close correspondence of the ^{1}H chemical shift and nuclear Overhauser enhancement (NOE) data found here with the data originally reported for dihydrochelirubine (dihydrobocconine) [11] after reinterpretation [9, 13].

For **IV** the similarity of the ^{1}H NOE's of H-4 and CH_2-6 observed upon irradiation of the NCH_3 group to those of **III** indicates the presence of one methylenedioxy group at C2 and C3. The fact that one aromatic singlet at 7.882 ppm gives enhancements upon irradiation of both methoxyl groups indicates the close proximity of the methoxyl group to this proton. As another aromatic proton at 6.771 ppm shows strong enhancements upon irradiation of one of these methoxyl groups the position of the second methylenedioxy group is established at C7 and C8, which is in keeping with the other compounds found in our culture. Furthermore, only two structures, **IVa** and **IVb,** are now compatible with the NOE data and the relevant shifts would be assigned as shown (Fig. 2). ^{1}H NMR data for an authentic sample of **I** in deuteriomethanol, for **III** and literature data for similar systems [11] all indicate that the shift of H-1 occurs at ca. 7.1 ppm when C11 and C12 are unsubstituted, thus H-1 of **IV** is ca. 0.3 ppm less shielded. Methoxyl substituent chemical shifts (OCH_3-H) for 1-methoxy [14] and 2-methoxy naphthalene (this work, measured in deuteriomethanol) are + 0.41 and − 0.08 ppm, respectively. Thus, only structure **IVa** is compatible with the shift of H-1.

Compound **IV** and a previously reported dimethoxy alkaloid macarpine [5, 6], found in similar situations to those reported here, strongly suggests that they are related, particularly in the light of the structural revisions mentioned above. Thus, **IV** could be called dihydromacarpine.

Growth and productivity characteristics

As with most cultures of Papaveraceae, suspensions of *Eschscholtzia californica* exist of small friable lumps rather than of fine suspensions. The lumps are white, reddish or brownish depending upon the extent of accumulated alkaloids and phenolics. The growth characteristics on B5-medium are shown in Fig. 3. The highest fresh and dry weights per l were 280 g and 10 g, respectively. All attempts to further increase the biomass production have so far failed.



IV a IV b

Fig. 2. Possible structures of **IV**.

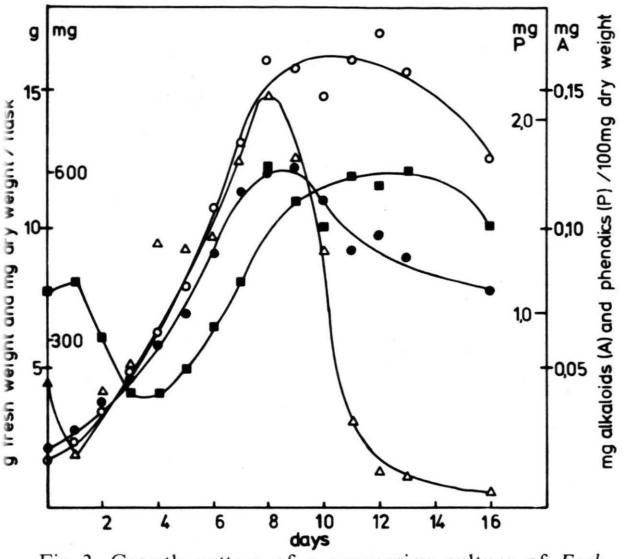


Fig. 3. Growth pattern of a suspension culture of *Eschscholtzia californica* (○—○ fresh weight, ●—● dry weight) and accumulation pattern of alkaloids (△—△) and phenolics (■—■).

On the growth medium the specific yields of alkaloids increased more than 5-fold during the linear growth phase and declined sharply during the stationary phase (Fig. 3). While the alkaloids were evidently further metabolized during the stationary phase, the specific yields of phenolics, representatives of another secondary pathway, remained quite stable during this phase (Fig. 3). On B5-medium the cells accumulate up to 0.15% (benzophenanthridine alkaloids) on a dry mass basis or 13 mg/l. The relative ratio of benzophenanthridine alkaloids was dihydrochelirubine 46%, dihydromacarpine 45%, dihydrosanguinarine 8%. In roots dihydrosanguinarine made up 80% of the total alkaloids (Fig. 4), while in the aerial parts additional alkaloids were found. The roots contained 0.38% total alkaloids and the aerial parts (stem/leaves) 0.12%. Some of the unidentified alkaloidal peaks of the plant extracts were sometimes also found in traces in the cell extracts.

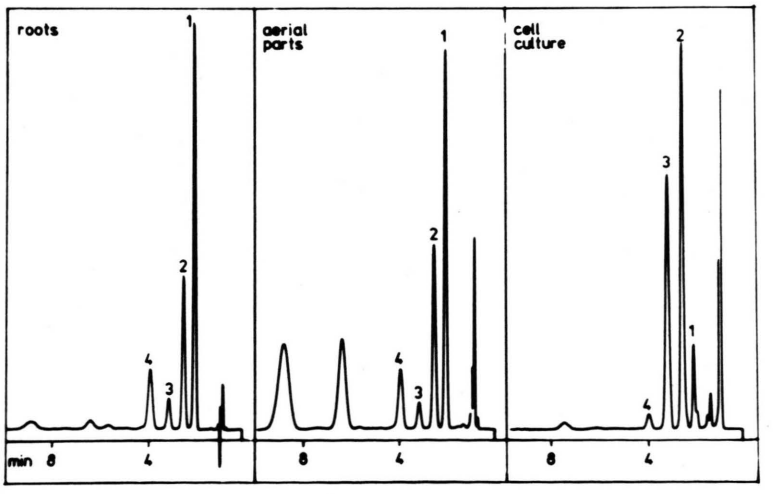


Fig. 4. HPLC-chromatograms of extracts from suspension cultures, roots or aerial parts of *Eschscholtzia californica*. Licrosorb Si-60; hexane:ether:dichloromethane 75:15:1. (1) Dihydrosanguinarine, (2) dihydrochelirubine (3) dihydromarcapine (4) dihydrochelerythrine.

Effects of sucrose

The fresh weight increase was the highest at 2% sucrose which is the concentration of the growth medium (Fig. 5). With increasing sugar concentrations the ratio fresh weight: dry weight changed from 32 to 10. Thus, the biomass yield was quite high in the "growth" inhibited cells. A dramatic increase of specific alkaloid yields was observed with increasing sugar concentrations (Fig. 5). Compared to the highest value ever achieved on B5-medium (2% sucrose) (Fig. 3) there was a 7-fold increase at 8% sucrose. The 2-fold higher increase in this experiment is explained by the fact of a further turnover of benzophenanthridine alkaloids in the growth medium. It should be mentioned that the sharp decline in alkaloid levels (Fig. 3) was only found in the growth medium during the experimental period of 14 days. In longer experiments alkaloid levels declined in growth inhibited cells, too.

Alkaloid levels up to 120 mg/l on B5 medium containing 8% sucrose have been observed. The positive effect of increased sucrose levels on alkaloid biosyntheses has also been reported for cultures of *Catharanthus roseus* [15]. The highest specific yield of phenolics was reached at 4% sucrose. The importance of the C/N-ratio for optimal phenolic biosynthesis has recently been demonstrated for cultures of Pauls Scarlet Rose and soybeans [16]. The different secondary pathways of a cell are evidently independently regulated as can be seen from the different responses of phenolic and alkaloid biosynthesis to the various sugar concentrations. At 8% sucrose the ratio of the dihydro forms of chelirubine: macarpine: sanguinarine was 62:14:22. Traces of the dihydro forms of chelerythrine and chelilutine/sanguirubine were also detected.

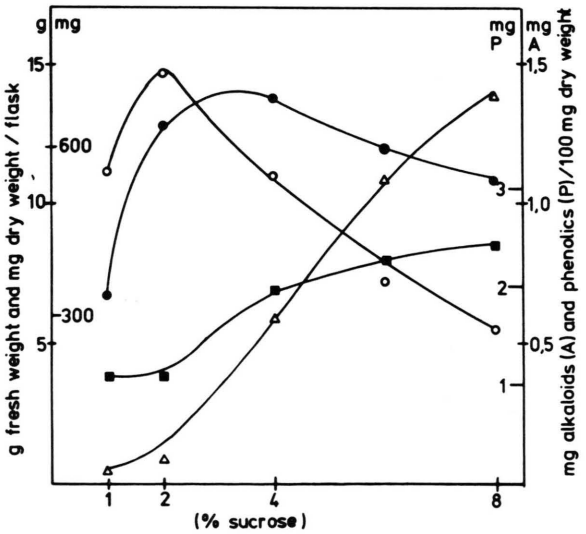


Fig. 5. Effects of various sucrose concentrations on fresh weight (○—○), dry weight (●—●) alkaloids (△—△) and phenolics (■—■). All samples were harvested on the 10th day of the growth cycle.

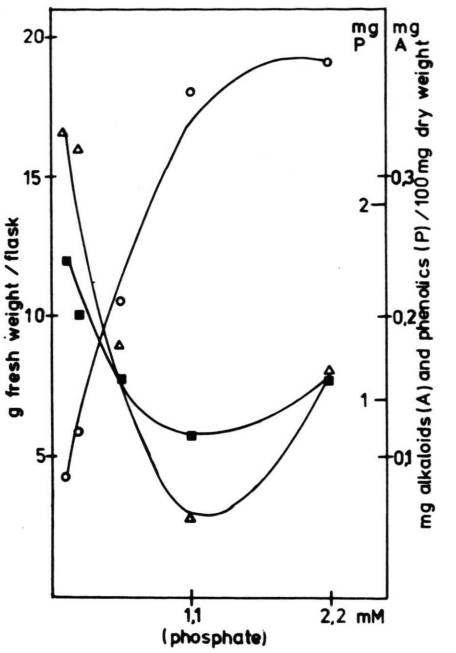


Fig. 6. Effects of various phosphate levels of the culture medium on fresh weight (○—○), alkaloids (△—△) and phenolics (■—■). Samples were harvested on the 10th day of the growth cycle.

Effect of phosphate

It has been shown that phosphate is often a negative effector of secondary metabolism [15, 17]. At the lowest phosphate concentration the lowest growth rate but the highest specific yield were found (Fig. 6). The induction was, however, only 1/3 of that observed at 6% or 8% sucrose. Reduction of the phosphate concentration had similar effects on alkaloid and phenolic biosynthesis. The changes were, however, more dramatic for the alkaloid biosynthetic pathway.

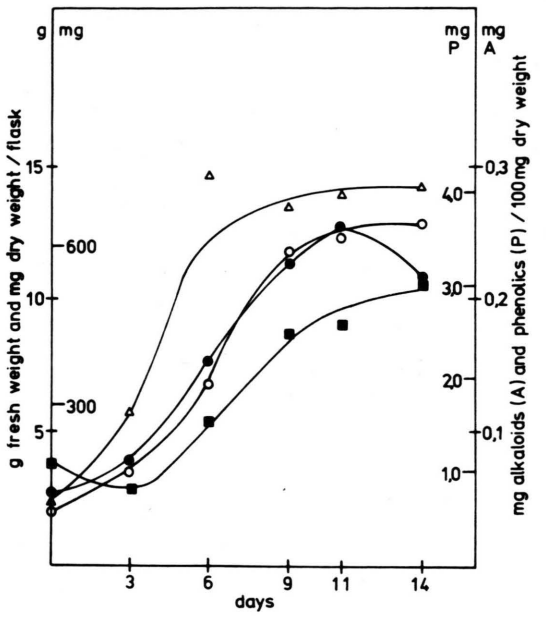


Fig. 7. Effects of deleting the phytohormone 2,4-D from the culture medium on fresh weight (○—○), dry weight (●—●) alkaloids (△—△) and phenolics (■—■).

Effects of phytohormones

Again it is known that the phytohormone 2,4-dichlorophenoxyacetic acid often prevents a good expression of secondary pathways in cultured cells [18, 19]. When cells were grown for the first time on B5-medium free of 2,4-D this caused only a slight reduction in fresh weight and no changes in dry weight (Fig. 7). When these cells were transferred for a second time into 2,4-D free medium growth decreased to give only a doubling of fresh and dry weight. Already, at the end of the second growth cycle sporadic morphological structures were detected. After 4 passages without 2,4-D a great number of root like structures were found.

After the first transfer to the 2,4-D free medium alkaloid levels increased on the average 4-fold while phenolics were 2-fold higher compared to the growth medium. When 2,4-D was replaced by various concentrations ($10^{-6} - 10^{-5}$ M) of naphthyl acetic acid, kinetin, benzylaminopurine (BAP) or indole acetic acid only BAP ($5 \times 10^{-6} - 10^{-5}$ M) increased the levels of alkaloids (100%) and phenolics considerably compared to the hormone free medium. During the first period growth was unaffected with these hormones ($10^{-6} - 10^{-5}$ M). However, after the second transfer to the same hormone composition growth was reduced by 50% under such conditions. The specific alkaloid yields were also reduced and were for the BAP-media only slightly higher than for the phytohormone free medium.

Table I. Composition of the induction medium IM2/liter.

NH_4NO_3	165 mg	Micronutrients	as MS-medium [22]
KNO_3	630 mg	Vitamines and Inositol	as MS
$CaCl_2 \cdot 2H_2O$	440 mg	Phytohormones	none
$MgSO_4 \cdot 2H_2O$	370 mg	Sucrose	80 g
KH_2PO_4	80 mg		

Effects of other medium constituents

When the cells were grown on 4–20 mM ammonium dihydrogen citrate as sole nitrogen source the biomass production was unchanged. However, the cultures contained only traces of alkaloids and 50% of total phenolics. When nitrate was the sole nitrogen source one could reduce the level to 1/5 of the B5-medium without losses in biomass. However, the reduction of nitrogen to 1/5 and less caused the

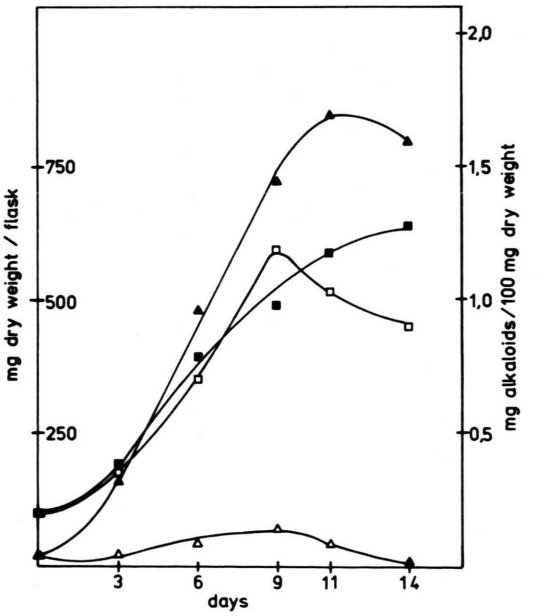


Fig. 8. Comparison of the accumulation patterns of alkaloids on B5-medium (△—△) and the induction medium IM2 (▲—▲). Dry weight per flask on B5-medium (□—□) and IM2 (■—■) are also shown.

depletion of alkaloids and decreased the content of phenolics. In all medium compositions deficient of nitrogen alkaloid biosynthesis ceased completely. The importance of Cu^{2+} for maximal formation of naphthoquinones has recently been demonstrated [20]. The increase or lack of micronutrients and the vitamines, respectively, did not cause significant quantitative changes of alkaloids and phenolics in the *Eschscholtzia* cultures.

Induction and production medium

An induction medium should make the cells produce the highest specific yields while a production medium should lead to highest yields per liter [21]. A rapid induction is especially useful for elucidating the biochemical basis of increased secondary metabolism [21]. The positive effects of increasing the sucrose concentration, of reducing the phosphate level and of deleting the phytohormones led us also to test IM 2, an "induction" medium developed by Knobloch (Table I) for indole alkaloid biosynthesis in *Catharanthus roseus*. This medium, although it had a reduced level of nitrogen (1/3), provided the highest yields of benzophenanthridine alkaloids with 1.7% on a dry weight basis and 146 mg/l. The specific alkaloid levels are 4–8 fold higher than in the plant. The main increase of alkaloid biosynthesis occurred after 3 days (Fig. 8). Therefore, this medium provides a convenient system to study biochemical aspects of the benzophenanthridine biosynthesis. At the moment this medium is the best induction, as well as the best production medium. Whether this medium, however, will remain the best production medium during our attempts to scale up (< 70 l and more) the production of benzophenanthridine alkaloids has to be seen. IM 2 was not the best induction medium for phenolic biosynthesis in *Eschscholtzia californica*. A B5 medium minus 2,4-D and phosphate resulted in higher specific yields.

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