Pergamon

S0031-9422(96)00072-6

Phytochemistry, Vol. 42, No. 4, pp. 985–996, 1996

0031-9422/96 $15.00 + 0.00

COMPARTMENTATION OF CAFFEINE AND RELATED PURINE ALKALOIDS DEPENDS EXCLUSIVELY ON THE PHYSICAL CHEMISTRY OF THEIR VACUOLAR COMPLEX FORMATION WITH CHLOROGENIC ACIDS

SIMONE S. MÖSLI WALDHAUSER and THOMAS W. BAUMANN*

Institute of Plant Biology, University of Zurich, CH-8008 Zürich, Switzerland

(*Received in revised form* 19 *December* 1995)

Key Word Index—*Coffea arabica*; Rubiaceae; caffeine, chlorogenic acid; 5-caffeoylquinic acid; PAL; purine alkaloids; compartmentation; complex formation; tissue culture; vacuole.

Abstract—*Coffea* seeds accumulate purine alkaloids (PuAs) and chlorogenic acids (CGAs) in a correlated manner: a high concentration of PuAs (mostly caffeine) accompanies a considerable accumulation of CGAs (mostly 5-caffeoylquinic acid; 5-CQA) and vice versa. Since caffeine and related PuAs *per se* freely penetrate cell-, tissue-, and organ-related barriers, we suggested that the physico-chemically well-characterized PuA–CGA complex is the crucial mechanism of PuA sequestration in these plant species and therefore the cause of the above-mentioned correlation. Suspension-cultured coffee (*C. arabica*) cells produce the complex partners in readily measurable concentrations, and were therefore an ideal system for investigating the *in situ* significance of complex formation. The partition of caffeine between cells and medium was studied in relation to the concentration of 5-CQA which, like the complex, is confined to the vacuole. Induction of the phenolic pathway was monitored by measuring PAL activity. To create concentration ranges of the complex partners as wide as possible, the cultures were subjected to various conditions such as the addition of a photoperiod or methyljasmonate (both stimulating secondary product formation), 2-aminoindan-2-phosphonic acid (2-AIP) (a most potent inhibitor of PAL), and exogenous caffeine. In all experimental sets, compartmentation of caffeine (and also that of theobromine) was highly correlated to the concentration of 5-CQA. In addition, inhibition of 5-CQA synthesis by 2-AIP consequently led to a reduction of caffeine biosynthesis whereas exogenous caffeine evoked the synthesis of the phenolic counterpart (5-CQA), this indicating a regulatory connection between the complex partners. When the cell cultures were transferred from 25° to 10°, or to 37°, caffeine shifted rapidly, as expected, from the medium into the cells or vice versa. Moreover, a modelling study showed that complex formation almost fully explains the measured degree of compartmentation. Similarly, in tissues and organs of the intact coffee plant the driving force of caffeine compartmentation was also shown to be defined by the physical chemistry of the complex. Finally, all caffeine-containing plants may have evolved basically one common strategy to sequester PuAs, i.e. the vacuolar allocation of high concentrations of one or several complexing phenols.

INTRODUCTION

The seeds of all *Coffea* species analysed show obvious parallels in their levels of purine alkaloids (PuAs) and chlorogenic acids (CGAs) [1, 2]. Thus *Coffea canephora* Pierre ex Froehner originating from Central and West Africa contains very high levels of both secondary compounds i.e., as related to dry wt, 2–3% PuA (almost exclusively caffeine) and 7–9% CGA (predominantly 5-CQA) whereas the East African *C. arabica* L. has about half the caffeine content of the former (1.1 to 1.7%) and accumulates distinctly less CGA (5.6 to 6.8%). Finally, the wild coffee species growing on and in the neighbourhood of Madagascar (section Mascarocoffea) are, with rare exceptions [3], either low in or free of caffeine and other PuAs (altogether 0 to 0.09%) and sequester less than 1% CGA. Furthermore, the outlined coherence exists even among different populations of one single species, e.g. the Madagascan *C. kianjavatenis* belonging to the above-mentioned exceptions: population A213 contains *ca* 0.9% PuA (mostly caffeine) and 3.8% 5-CQA, whereas population A602 accumulates almost 2% PuA (predominantly theacrine [4]) and 7.5% 5-CQA [5, 6].

This consistently correlated allocation of PuAs and CGAs is, as far as investigated, also inherent to coffee leaves [7] and undoubtedly requires a coordinated metabolism to meet the mutual physiological inter-

*Author to whom correspondence should be addressed.

action discussed below. A well-known feature common to the two compound classes is that they interact in a physico-chemical way [8]. In aqueous media caffeine and 5-CQA combine into a 'hydrophobically-bound π-molecular complex' [9], which is most likely the precursor of the well-described crystalline caffeine-potassium chlorogenate 1 : 1 complex [10, 11]. Complex formation between 5-CQA and PuAs other than caffeine (i.e. theobromine, paraxanthine, theophylline, and theacrine) also occur and the pertinent equilibrium constants have been determined [12]. Moreover, since the aromatic part of the molecule participates in the complex [8], CGAs other than 5-CQA will likewise undergo complex formation.

Suspension-cultured cells of coffee readily produce the two complex partners caffeine and 5-CQA in, compared with other cell constituents, very large amounts. The depside 5-CQA and also the complex with caffeine is stored intracellularly in the central vacuole, whereas free caffeine, presumably owing to its dual, hydrophilic and lipophilic character, is found both intra- and extracellularly [13]. However, we always observed that caffeine partition within the culture was correlated with the (intracellular) concentration of 5-CQA or, in other words, increasing 5-CQA concentrations led to enhanced cellular compartmentation of caffeine. Therefore, we wondered whether complex formation *per se* with 5-CQA and other CGAs could fully explain caffeine partition in the tissue culture system and thus may be the key mechanism in all

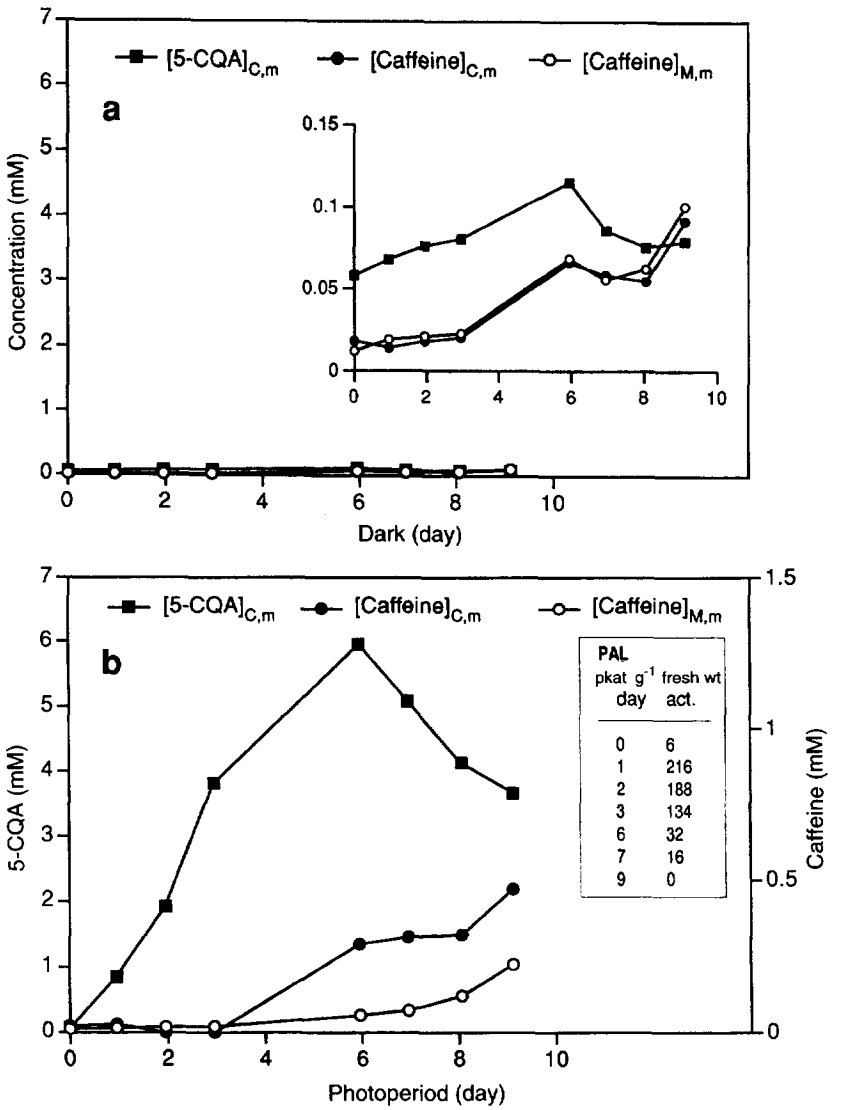

Fig. 1. Caffeine and 5-CQA concentrations in suspension-cultured coffee cells grown either (a) in the dark or (b) with a photoperiod. To facilitate comparison between (a) and (b) the same scale is used. The inset shows in (a) the scaled up details and in (b) the PAL activities. In (a) PAL activity was very low (see text). Cultures were started at a ratio of 3 g cells to 3 g medium and precultured for 1 day in the dark prior to imposition of the experimental conditions. For each time point one culture was harvested and analysed. C, cell; M, medium; m, measured. For further explanations see Experimental (Formulae and definitions).

coffee plant tissues for the accumulation of caffeine within the vacuole against a concentration gradient. In short, does physico-chemical polyphenol complexation render any other transport and trapping systems for PuAs unnecessary? In order to elucidate these aspects we measured and calculated the concentrations of the PuAs theobromine and caffeine, as well as of the CGAs in suspension-cultured cells under various experimental conditions. Finally, the data were used for a modelling study, which showed that complex formation with CGA is most likely the only driving force of PuA compartmentation.

RESULTS AND DISCUSSION

Effect of various parameters on the formation of the complex partners and on caffeine compartmentation

Photoperiod. When cell cultures were grown in the dark (Fig. 1a), all parameters measured, i.e. PAL activity, concentrations of 5-CQA and of intra- and extracellular caffeine, were at a very low level. Within the accuracy of the method (see Experimental) there was no visible concentration difference between extra- and intracellular caffeine reaching a value of 0.1 mM (see inset, Fig. 1a). 5-CQA concentration was in the same range, and PAL activity (not shown) was, as expected, hardly measurable and at *ca* 4 pkat g^{-1} fresh wt. However, in the presence of a photoperiod (Fig. 1b) PAL activity reached, within 24 hr, more than 200 pkat g^{-1} fresh wt, and consequently, the formation of 5-CQA was strongly stimulated, e.g. by a factor of *ca* 50 on day 6 based on the concentration, and was, after a delay of 3 days, followed by an increase in caffeine accumulation to *ca* 3–5 times the level found in the dark control (Fig. 1a). Caffeine concentration was distinctly higher in the cell than in the medium. This uneven distribution is the result of caffeine compartmentation. The apparent compartmentation factor (CF_{app}; see Experimental, Formulas and Definitions) reached a value of 5.1 (Fig. 1b) on day 6, i.e. at the maximum 5-CQA concentration. The effective compartmentation factor of caffeine (CF_{eff}) takes account of the considerable free-space and was calculated by the use of equation 14 (see Experimental) to be 19.7 for day 6. Similar experiments under a photoperiod with various coffee cell-lines always resulted in a typical sequence of events: (a) a maximum induction of PAL after 24–48 hr; (b) a rapid onset of 5-CQA synthesis within the same period with a maximum after 5–7 days; (c) after a lag of 3 days an increase in caffeine accumulation not reaching its peak during the time of observation, but always being clearly higher intracellularly than in the medium. Finally, in a separate experiment with data points (not shown) taken at shorter intervals (2.5 hr), PAL induction was found to start 5 hr after illumination and was paralleled by 5-CQA formation without noticeable (at this resolution) delay.

Methyljasmonate (MeJa). In the dark, MeJa treatment (Fig. 2) resulted, compared with the control (Fig. 1a), in a remarkable stimulation of all three parameters patterned similarly to that found in the photoperiod study (Fig. 1b). However, the onset of 5-CQA formation was delayed by one day and the maximum of PAL activity (64% of that in photoperiod) by two days and, astonishingly enough, caffeine synthesis was induced readily and coincided with the formation of 5-CQA. Again, caffeine was intracellularly accumulated with a CF_{app} of *ca* 2 on day 6. The combination of photoperiod and MeJa (not shown) led to a biphasic induction of PAL with a small activity peak after one day (*ca*

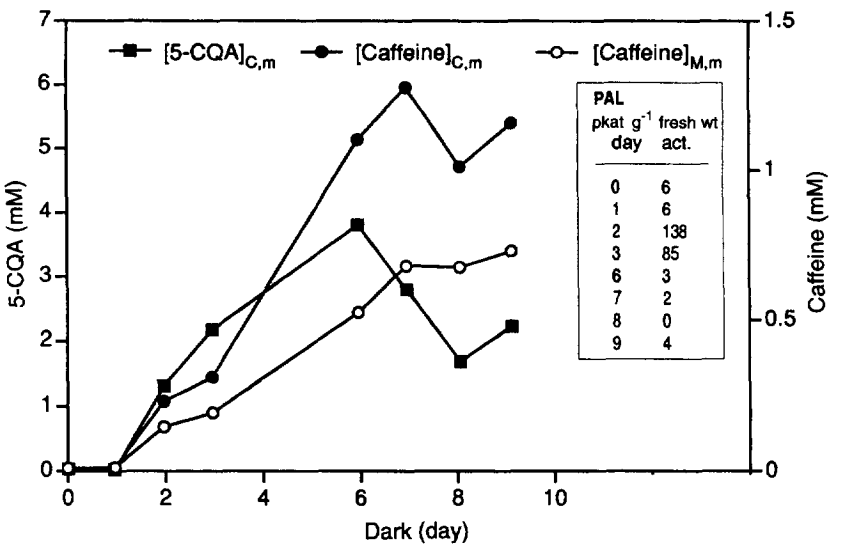

Fig. 2. Effect of MeJa (500 ppm) on the caffeine and 5-CQA concentrations in suspension-cultured coffee cells grown in the dark. The inset shows the corresponding PAL activities. For further parameters and conditions see Fig. 1.

20 pkat g^{-1} fresh wt) followed by a large peak (260 pkat g^{-1} fresh wt) on day 3. Most likely, because of delayed and overlaying induction, synthesis of the complex partners began simultaneously on day 2 and at a very low rate until day 6, and only thereafter showed the stimulated increase. Therefore, in the combination of MeJa with photoperiod, both caffeine and 5-CQA were always distinctly lower than in the dark with MeJa (Fig. 2) or in the photoperiod control (Fig. 1b). A noticeable caffeine compartmentation occurred only after triggering the accumulation of 5-CQA.

The exposure to MeJa (in the dark) caused an almost linear accumulation of the concentration of (cellular) caffeine and 5-CQA for at least 6 days with a stimulation factor of 17 and 34, respectively (Figs 2 and 1a). This is in full agreement with an investigation on the MeJa-mediated (10 to 250 μM) response of several taxonomically distantly related cell cultures: in each case, the unique major compound was produced in amounts greater by a factor of 9 to 30 compared with the control [13]. Unfortunately, owing to a calculation error we used a MeJa concentration of *ca* 2 mM. Since the effective concentration of the physiologically active principle in the preparation was not known, no attempt was made to investigate maximum induction of caffeine and 5-CQA. Finally, the formation of theobromine, the precursor of caffeine [14], was induced by a factor of 10 on day 8 as based on total amounts.

2-Aminoindan-2-phosphonic acid (2-AIP). This compound is the most powerful competitive PAL inhibitor known today, and completely inhibits formation of anthocyanins in buckwheat hypocotyls at 10–30 μM concentrations [15]. Interference with protein synthesis can be ruled out because phenylalanyl tRNA synthetase is not inhibited by 2-AIP. The rationale of the experiment outlined below was to block selectively the PAL reaction by 2-AIP and subsequently the formation of (*E*)-cinnamic acid, which is a precursor of 5-CQA. Eventually, the reduced formation of 5-CQA in the presence of 2-AIP should, if compartmentation is

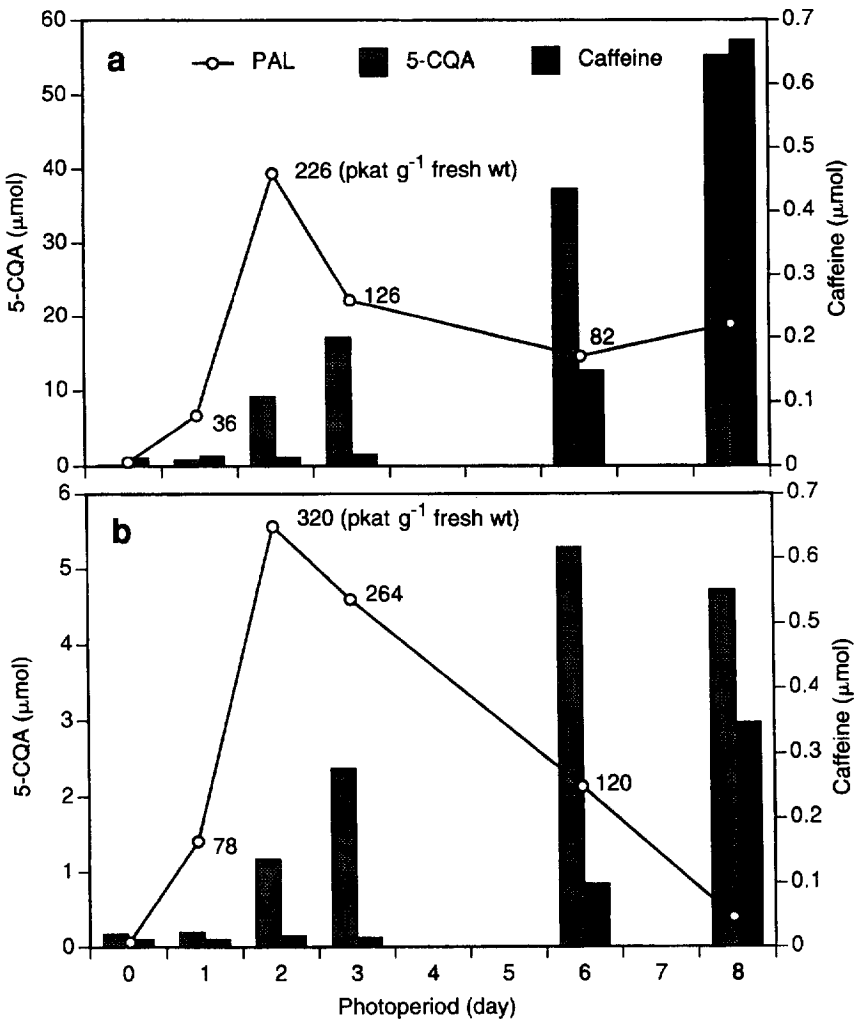

Fig. 3. Influence of 2-AIP (10 μM) on overall caffeine and 5-CQA formation and on PAL activity in suspension-cultured coffee cells grown with a photoperiod. (a) Control, (b) with 2-AIP. The amounts of caffeine found in both medium and cells were summed. Note the different scales for 5-CQA in (a) and (b). For further parameters and conditions see Fig. 1.

governed by the complex, lower the intracellular caffeine accumulation and perhaps also influence PuA synthesis. Indeed, the synthesis of both 5-CQA and (overall) caffeine was reduced on day 8 by 85% and 48%, respectively, (Fig. 3a and 3b). There was a remarkable superinduction of PAL activity by 2-AIP, as has been observed in gherkin hypocotyls with AOPP as inhibitor [16].

Table 1 summarizes some of the parameters measured or calculated during this inhibition experiment, and at the same time provides an insight into data processing (see also Experimental). In the control, the (free space-uncorrected) concentration of 5-CQA [5-CQA]$_{C,m}$ increased rapidly one day after exposure to photoperiod and reached a value of almost 18 mM on day 8. At this point, the compartmentation factor CF_{eff} for caffeine was 10.2, and 79.2% was complexed by 5-CQA. Conversely, in the presence of 2-AIP (and simultaneously under photoperiod), the concentration of 5-CQA remained below 2 mM, CF_{eff} was maximally 2.2, and at that time only 27.6% of the caffeine was in a complexed form. In conclusion, the experimentally reduced chlorogenic acid concentration not only lowered compartmentation but also downregulated caffeine biosynthesis in an unknown way. An indication of how this may operate is given by the caffeine concentrations in the medium ([Caffeine]$_{M,m}$), which, throughout the whole experiment, were strikingly similar to each other (control against 2-AIP). The term [Caffeine]$_{M,m}$ corresponds to the concentrations of free, uncomplexed caffeine in the symplast and consequently signifies the caffeine concentration in the cytoplasm. Thus, no matter how much caffeine was totally synthesized under either condition, the cytoplasmic caffeine concentration was the same. Therefore, we assume a growth-dependent threshold concentration of cytoplasmic caffeine by which regulation perhaps of both caffeine and 5-CQA is controlled.

Addition of caffeine. Studies with caffeine exogenously added to the culture was expected to provide additional evidence for our hypothesis of caffeine partition effected by 5-CQA, and to give some insights into the regulatory supply of the latter in the presence of high PuA concentrations. In the dark, only the concentration of 10 mM caffeine had a noticeable effect on 5-CQA biosynthesis: PAL activity was weakly induced and reached a peak of 11 pkat g^{-1} fresh wt on day 6 followed by a slight 5-CQA accumulation with a low maximum level of 0.4 mM on day 7 (data not shown). Moreover, due to catabolism the caffeine level was reduced by *ca* 20% at both 1 and 10 mM, whereas at 0.1 mM it remained unaffected. At 10 mM the theobromine level increased compared with the control by a factor of 14. Under the photoperiod, caffeine additions resulted in a complex pattern of PAL induction (Fig. 4): there was an initial increase of activity corresponding to the induction by light as can be seen in the control and in the presence of 0.1 mM caffeine. At 1 and 10 mM caffeine, however, PAL activity persisted much longer. Although too few samples were taken during the critical period from days 3 to 6, the pattern indicates a second, delayed induction of PAL by these high caffeine concentrations. Under all conditions tested (including the control) formation of 5-CQA was not visibly displaced from PAL induction (after 24 hr) and reached a high level (12–15 mM) on day 6 which was maintained until day 8. Thereafter, 5-CQA concentrations dropped in the control to about 8 mM on day 10, whereas in the caffeine-treated cultures a

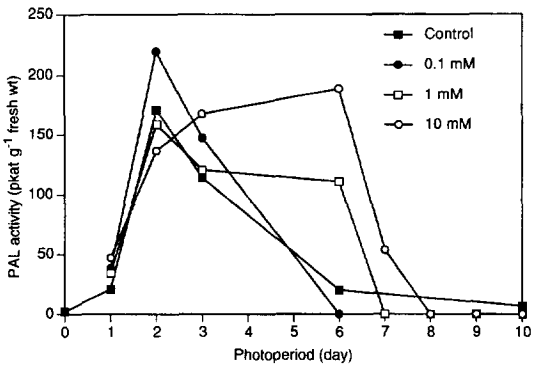

Fig. 4. Time course of PAL activity in suspension-cultured coffee cells exposed to different caffeine concentration. Exogenous caffeine was added after preculture in the dark for 1 day. The concentrations were related to the total volume of the culture (cells and medium). For further parameters and conditions see Fig. 1.

Table 1. Inhibition study with 2-AIP. The parameters listed are as follows: $V_{M,m}$ and $V_{C,m}$ are the measured volumes of the medium and of the cells respectively; [5-CQA]$_{C,m}$ is the measured concentration of 5-CQA (in the cells), whereas [Caffeine]$_{C,m}$ and [Caffeine]$_{M,m}$ are the caffeine concentrations in the cells and medium respectively; $CF_{eff(Caffeine)}$ is the effective compartmentation factor (as calculated by equation 14, see Experimental) and $DC_{(Caffeine)}$ the degree of complexation of caffeine. See also Fig. 3

	$V_{M,m}$ (ml)		$V_{C,m}$ (ml)		[5-CQA]$_{C,m}$ (mM)		[Caffeine]$_{C,m}$ (mM)		[Caffeine]$_{M,m}$ (mM)		$CF_{eff(Caffeine)}$		$DC_{(Caffeine)}$ %	
Day	Control	AIP	Control	AIP	Control	AIP	Control	AIP	Control	AIP	Control	AIP	Control	AIP
0	2.96	2.96	2.47	2.47	0.074	0.074	0.0019	0.0019	0.0029	0.0029	0.66	0.66	1.6	1.6
1	2.76	2.65	2.60	2.79	0.317	0.074	0.0025	0.0015	0.0033	0.0033	0.76	0.45	6.4	1.6
2	2.60	2.71	2.47	2.85	3.744	0.413	0.0023	0.0025	0.0033	0.0040	0.70	0.63	44.6	8.2
3	2.55	2.65	2.65	2.71	6.496	0.878	0.0040	0.0022	0.0031	0.0037	2.32	0.59	58.3	15.9
6	2.30	2.24	2.83	2.96	13.191	1.785	0.0394	0.0209	0.0161	0.0165	7.58	2.21	73.9	27.6
8	1.79	1.44	3.10	3.80	17.829	1.242	0.1812	0.0699	0.0599	0.0562	10.20	2.11	79.2	20.8

second increase was observed (16–19 mM 5-CQA) that did not reach a plateau at the end of observations on day 10. This rise was most likely due to the 'second' PAL induction evoked by high concentrations of caffeine. Under the photoperiod, the caffeine level remained either constant (at 10 mM) or steadily increased (at 1 and 0.1 mM) by the net amount formed in the control. Considerable accumulation of theobromine (*ca* 5 times the control value) occurred at 10 mM caffeine, most likely as a result of feedback inhibition. To test our hypothesis of partition by complex formation, the compartmentation factors (CF_{eff}) for caffeine (Fig. 5) of the culture cycle at 10 mM caffeine were plotted against the (free space-corrected) concentrations of 5-CQA. The correlation is very tight and holds also for theobromine (0.99, data not shown). When the data points (53 from 8 culture cycles and two cell lines) of all experiments (with and without caffeine added, stimulation by MeJa, several culture cycles under photoperiod) were treated in the same manner and assembled into one diagram, correlation factors of 0.81 and 0.88 were obtained for caffeine and theobromine respectively. Unlike MeJa which induced PAL activity in the dark, exogenous caffeine had a significant effect on PAL only in the presence of a photoperiod, where essentially a continuous induction resulted. In conclusion, exogenous caffeine evokes either the formation of a surplus of 5-CQA (in photoperiod) or induces PuA catabolism (in the dark) with the ultimate purpose of downregulation of cytoplasmic caffeine to a physiological level. Based on our results on the mutual interaction between PuAs and CGAs, we are convinced that tailoring the caffeine content of a plant species to meet the need of consumers can be achieved by changing the expression not only, as generally envisaged [17], of the caffeine but also of the 5-CQA pathway.

Temperature. The equilibrium constant of the caffeine–chlorogenate complex is temperature-dependent

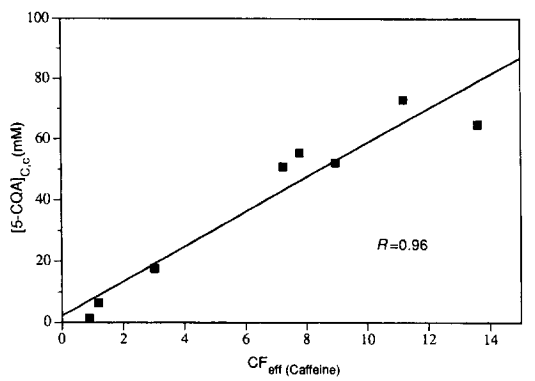

Fig. 5. Correlation of the corrected (cellular) concentration of 5-CQA with the effective compartmentation factor CF_{eff}. The values were taken from the experiment in which exogenous caffeine (10 mM) was added to eight cultures. Each data point represents one culture harvested after a certain cultural period. For further explanations see Experimental (Formulae and definitions).

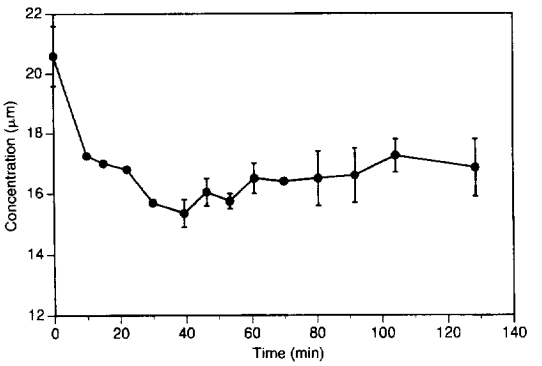

Fig. 6. Time course of caffeine concentration in the medium after lowering the temperature to 10°. Two cultures with a ratio of 6 g cells to 6 g medium were, after a one-day preculture, raised at 25° for 6 days with a photoperiod and then kept at 10°. Samples of the medium (100 μl) were taken at the intervals indicated. Data points (mean of the two cultures) without error bars signify that the two values were identical or too close for resolution.

[12]. It is, for example, higher at 10° (K_c = 109.0 l mol^{-1}) and lower at 37° (K_c = 26.7 l mol^{-1}) than at 25° (K_c = 47.3 l mol^{-1}). Therefore, if a suspension culture grown at 25° is transferred to 10°, a small fraction of the caffeine is expected to shift from the medium into the cells where it will be complexed owing to the increasing 'complexation constant'. Conversely, cells cultured at 25° should release caffeine when transferred to 37°. Figure 6 shows the behaviour of the caffeine concentration in the medium after transfer of the culture to 10°. The asymptotic changed was −5.3 μM and fits into the theoretically calculated range of −5.0 to −5.8 μM. Because temperature was lowered by 15° it is very unlikely that this rapid decrease in concentration results from an active uptake mechanism. The concentration change shortly after transition to lower temperature can only be explained by a physico-chemical reaction, namely by the association of caffeine with CGA, most of it represented by 5-CQA. Alternatively, an increase in caffeine concentration in the medium was observed when cultures were put at 37° (data not shown). However, the rate of caffeine synthesis was accelerated and made mathematical treatment difficult.

Modelling study

In Table 2, the initial values as they were used in the model are listed for a number of cultures kept under various conditions. Cultures subjected to the temperature experiment (see above) were all grown at 25° and harvested after a 4-hr exposure to the new temperature regime (rows 2–4). For a given 5-CQA concentration the degree of caffeine complexation ($DC_{(Caffeine)}$) is markedly lowered by an increase of the temperature and of the caffeine concentration (rows 1–4). Caffeine complexation is also reduced by lowering the 5-CQA concentrations. In the parent leaf tissue the related

Table 2. Initial values of the concentrations of 5-CQA ($[5\text{-CQA}]_0$), caffeine ($[\text{Caffeine}]_0$), the complex ([C]), and of the degree of caffeine complexation ($DC_{(\text{Caffeine})}$) as they were determined after the time indicated, and as used to start the iterations in the modelling study

Culture conditions	$[5\text{-CQA}]_0$ (mM)	$[\text{Caffeine}]_0$ (mM)	[C] (mM)	$DC_{(\text{Caffeine})}$ (%)
MD: 3 C/3 M; 1 PD/9 PP; 10 mM Caffeine	64.877	96.928	45.877	47.3
MD: 6 C/6 M; 1 PD/6 PP; 37°	64.877	0.413	0.282	63.3
MD: 6 C/6 M; 1 PD/6 PP; 25°	62.793	0.485	0.376	77.4
MD: 6 C/6 M; 1 PD/6 PP; 10°	61.794	0.366	0.319	87.0
S: 3 C/3 M; 1 PD/8 PD; MeJa	7.680	6.499	1.475	22.7
S: 3 C/3 M; 1 PD/7 PP; MeJa Control	27.087	1.443	0.800	55.4
S: 3 C/3 M; 1 PD/2 PD; MeJa Control	0.308	0.137	0.002	1.4
MD: 3 C/3 M; 1 PD/8 PP; AIP	8.114	0.152	0.042	27.6
MD: 3 C/3 M; 1 PD/6 PP; AIP Control*	59.960	0.239	0.176	73.9

MD, S = cell lines; 3 C/3 M means that the culture was started with 3 ml cells and 3 ml medium; e.g. 1 PD/9 PP stands for 1 day preculture in permanent darkness followed by 9 days in photoperiod; AIP = 2-aminoindan-2-phosphonic acid; MeJa = methyljasmonate.

*Modelling presented in Fig. 7.

concentration of 5-CQA is estimated to be in the range 50–150 mM, whereas that of caffeine must be distinctly lower [7], this means that the DC of caffeine may fluctuate between 30% and 70%.

In the model, all caffeine is primarily packed into the compartment where the complex is to be formed (see Experimental). After a suitable number of iterations a certain fraction of the total caffeine will have eventually escaped into the medium. The size of this fraction is dependent, among other parameters, on the volume of the medium. A similar situation is present in the caffeine-containing tissues of the coffee plant. The intracellularly synthesized PuA will be allowed, dependent on the extent of complexation and of apoplastic volume, to diffuse into the aqueous surrounding. Thus, the concentration of caffeine in the complex-containing compartment will be lowered whereas its degree of complexation will be increased. Hence, the existence of a large apoplast minimizes the cytoplasmic concentration of free caffeine and thus the risk of autotoxicity.

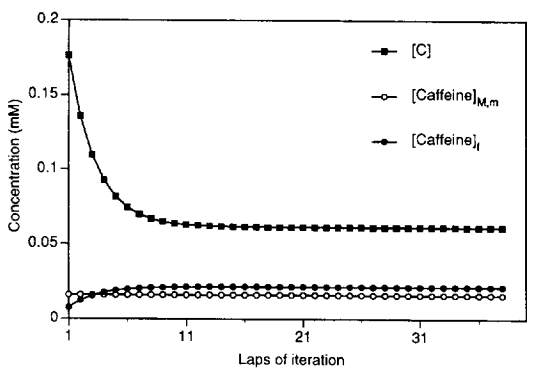

Fig. 7. Modelling of caffeine compartmentation as a function of complexation. The data of day 6 from the control of the '2-AIP experiment' (Fig. 3a) were taken and processed as outlined in the 'Experimental'. The calculation was based on '100% vacuole' and '100% 5-CQA'.

Figure 7 illustrates the modelling of caffeine compartmentation for suspension-cultured coffee cells. In all cultures analysed in the same way, the concentration of the modelled free caffeine at the equilibrium was higher than that measured in the medium. However, the following factors should be considered. (1) It is conceivable that in a 'rapidly producing' cell culture 'processing' of newly formed caffeine, i.e. diffusion from its cytoplasmic site of synthesis into the vacuole, interaction with CGA and finally migration into the medium compartment, will preclude a steady-state condition for this PuA. (2) Our failure to recognize until towards the end of these studies the full impact of temperature on complex formation, may have resulted in medium sampling on the sterile bench being made occasionally at a temperature slightly lower than during the experimental conditions. (3) In reality the vacuole occupies distinctly less than 100% of the symplast. (4) Caffeine is strongly lipophilic; a small fraction of free caffeine might have escaped into lipids of e.g. membranes. (5) The most important correction, however, may be achieved by considering the CGAs other than 5-CQA which also accumulated in these coffee cell lines. Based on the UV signal (spectrum and area) they regularly account for up to *ca* 15% of 5-CQA. One of them was 3,5-diCQA (= *ca* 2/3 of all CGA other than 5-CQA) which supposedly complexes two caffeine molecules but which might have about the same molar extinction as 5-CQA.The others, perhaps mainly di-caffeoylQAs, were not identified. We decided that a value of 25% is representative for the surplus of CGA complexing activity that should be taken into account. Hence, all these factors favour congruence between model and reality but at present only (3) and (5) can be considered and therefore were treated mathematically. Nevertheless, if we calculate the data for '90% vacuole' and '125% 5-CQA', the model comes very close to the cell culture, i.e. modelled caffeine is 1.22 times higher than measured caffeine and is within the tolerances of a biological system!

Compartmentation of caffeine by complexation, does it also operate in the plant?

Originally we discovered this type of compartmentation when we placed coffee leaf disks infiltrated with water into a stirred washout chamber. Depending on the leaf age, 40–65% of the caffeine was released asymptotically within 3 days. CGAs did not appear in the washout liquid. Calculations showed that the amount of washed-out caffeine corresponded exactly to the theoretically uncomplexed caffeine in the middle-aged leaves (5–14 weeks), whereas this correlation was less close for the earlier or later stages [17, 18]. The relatively long period required to reach the washout equilibrium for caffeine signifies that diffusion is strongly impeded by the leaf structures such as the large intercellular space of the mesophyll and the waxy layers at the leaf surface. Similar washout curves were obtained with whole leaves, indicating that most of the caffeine released by the leaf disks did not pass through the cut edge. We do not know whether the coffee leaf contains, in addition to vacuolar CGAs, caffeine-complexing polyphenols that are cell wall-associated. The coffee bean has been reported to possess a physiological barrier on its surface, most likely to CGAs [18], that efficiently hinders caffeine diffusion during imbibition and germination [19]. When we isolated protoplasts from coffee leaves, a considerable fraction of 5-CQA was always lost, as based on chlorophyll as a marker. Initially, this led us to conclude that this portion was cell wall-associated. However, extended studies including the use of suspension-cultured cells revealed that during protoplast isolation cysteamine, the only agent found to effectively prevent browning, was conjugated to 5-CQA. The conjugate, the 2-cysteaminyl derivative of 5-CQA, was formed in a time-dependent manner at the expense of 5-CQA and was readily released by the protoplasts. Thus, purified protoplasts and consequently the vacuoles isolated from them eventually contained, after extended digestion, a comparatively small fraction of the 5-CQA initially present in the suspension-cultured cells. We must assume that symplastic 5-CQA reacted, after enzymatic oxidation to the corresponding quinone, with cysteamine, even though we can only speculate about how this could happen in a symplast with 'intact compartmentation'. In conclusion, we assume that in young, rapidly growing tissues and organs most of the CGAs are assembled in the vacuole, as it was shown for suspension-cultured cells by the indirect method of vital staining the CGAs with methylene blue [20]. In the ripe coffee bean, the allocation of CGAs on the periphery and perhaps in the massive walls of the endosperm cells additionally contribute to the physico-chemical fixation of caffeine. When the bean is cut apart the fragments easily release caffeine, as it was described for guaraná seeds [21] rich in catechins [22]. During germination the concentration of 5-CQA of the coffee seedling decreases and caffeine is released by the primary root [7]. Thus, there is good evidence that in the whole coffee plant caffeine (or PuA) compartmentation operates in the same manner as shown for suspension-cultured cells.

Is the allocation of a strong phenolic principle indispensable to caffeine-containing species?

With regard to the occurrence of polyphenols in PuA-containing plants other than coffee we focus on species consumed by humans because they are well investigated. Indeed, all were found to be rich in one or several phenols with complexing ability, e.g. cocoa [23]. Among them the tea plant, *Camellia sinensis*, is the most spectacular as it accumulates phenols amounting for up to 30% of the dry wt [24]. The major compound in the unfermented tea leaf is the flavanol epi-gallocatechin gallate (9–13% of dry wt). In a study dealing with the mode of interaction between polyphenols and caffeine this compound was found to have a high affinity for caffeine [25]. However, we should mention that recent re-examinations of several green teas from China and Japan showed that epi-gallocatechin is the most prevalent flavanol [26]. It also complexes with caffeine, but has a lower affinity constant than epi-gallocatechin gallate [25]. In young tea leaves this PuA may be accumulated to greater than 7.5% of dry wt [27]. Hence, we suggest that compartmentation of caffeine and related compounds by polyphenol complexation is common to all PuA-containing plants. However, none of the PuA-containing plant species other than coffee has been investigated with respect to the *in situ* participation of individual substances in the related complexation reaction.

Is vacuolar trapping by hydrophobic π-molecular complex formation a feature exclusive to PuA-containing plant species?

If we compare this type of accumulation with others found for secondary compounds, we see that it shares uptake by simple diffusion with a number of lipophilic, weakly basic alkaloids such as e.g. some isoquinoline and indole alkaloids [28–30]. For a comprehensive review of vacuolar uptake and sequestration see ref. [31]. However, in contrast to these, PuAs will not become protonated in the vacuole, and sequestration by an ion trap mechanism [28], as suggested for the above-mentioned compounds, is therefore not possible. Despite this we see similarities between caffeine complexation and the sequestration of isoquinoline alkaloids in the latex vacuoles (vesicles) of *Chelidonium majus* [28, 32]. They were reported to associate (ionic binding) in the reagent tube with tannin, gallate, or chelidonic acid to form precipitates [32]. This behaviour was taken as a possible explanation for the *in vivo* situation. However, in our view a π-molecular complex of chelidonic acid with the related alkaloid(s) is sufficient to explain the situation in *Chelidonium majus*. Preliminary partition studies with sanguinarine and chelidonic acid in a two-phase solvent system [8] showed significant molecular complex formation be-

tween the two species (not published). In the latex vesicle, sanginuarine and chelerythine were reported to accumulate to a larger extent than berberine and coptisine [32]. This may be simply explained by the nature of the π-electron system which in the first pair are conjugated to a higher degree than in the last pair. Recently, it was shown that solubility of berberine is dependent on its paired anion, and that in vacuoles of *Coptis japonica* a supersaturated solution (more than 70 mM) is achieved by the presence of high concentrations (168 mM) of malate [33]. This kind of solubilization may also operate in *Chelidonium*. In conclusion, it is conceivable that the complexation of isoquinoline alkaloids, although much stronger, is similar to that of caffeine in the coffee plant and must be based on a hydrophobic π-molecular association. The principal complex partner is most likely the γ-pyron chelidonic acid, as previously suggested and in latex vesicles stored at a concentration above 600 mM [30]. The hydrophobic interaction is considerably strengthened by the very high solute concentrations achieved by solubilization mechanisms. Since the degree of complexation is dependent on the concentrations of the partners involved, it will become nearly 100% for the corresponding alkaloids. The low percentage of molecules not engaged in the complex will escape only slowly through the tonoplast because of reduced lipophilicity due to the vesicular pH. Hence, basic physico-chemical complexation studies regarding γ-pyrons and isoquinoline alkaloids will contribute much to the general knowledge of vacuolar sequestration.

EXPERIMENTAL

Cell lines. From orthotropic internodes of *C. arabica* var. *catuai* five lines were established [20], which differed in morphology and productivity, but all shared the characteristics of caffeine compartmentation explored in this work. To be concise, we have only presented the results obtained with two cell lines. Line MD attained (in the dark) a lower PuA concentration (*ca* 19 μM; medium) than line S (40 μM) but the stimulation factor for caffeine synthesis by the introduction of a photoperiod was much higher, i.e. 21 versus 7. Both lines were of the small-aggregate type (1–2 mm). It should be noted that during these studies the productivity of PuA dropped as usual to a lower but still well-measurable level as seen e.g. in Fig. 6 (temperature experiment; *ca* 20 μM after stimulation in photoperiod).

Standard conditions. Suspension-cultured cells were grown in commercially available Murashige and Skoog (MS) medium (4.71 g l^{-1}; Flow Laboratories), supplemented with (mg l^{-1}) sucrose (30 000), L-cysteine (10), thiamine (1), 2,4-D (1) and kinetin (0.2). The pH was adjusted from 5.7 to 5.8 with 1 M KOH. Cells (10 g) were usually subcultured at 2-week intervals in 60 ml medium and kept in a 250 ml Erlenmeyer on a gyratory shaker (90 rpm) at 25° in the dark.

Experimental conditions. Cells grown for 14 days under standard conditions were collected on a tea-strainer and transferred at a ratio of 1:1 into fresh medium. After 1–3 days of pre-incubation in the dark at 25°, the cultures were exposed to various conditions, i.e. photoperiod (9 hr, 350 μE m^{-2} s^{-1}) at 25° in an environmental chamber (Weiss): 500 ppm MeJa (Firmenich); 10 μM 2-AIP [15] (kindly provided by Prof. N. Amrhein); 0.1, 1, and 10 mM caffeine (Fluka, Buchs). The concns of the additions are related to the entire culture vol. (cells plus medium). For the temp. experiment, suspension cultures grown under photoperiod at 25° for 6 days were wrapped in black plastic foil and incubated, with shaking, at 10, 25 or 37°. At intervals, samples (100 to 170 μl) of the medium were taken and stored at −20° until HPLC analysis. At the end of incubation the whole culture was harvested and the related parameters determined.

Determination of various parameters. Volume of cells and medium. At each time point, one culture was poured through a tea strainer into a tared beaker. The vol. of both cells and medium was determined by weighing and by assuming that their specific weight was close to 1. The values were corrected for the loss due to handling as measured in separate experiments. It amounted to 6.4% and did not affect the cell–medium ratio. It should be noted that of this 'wet' cell vol. only 22% was symplast vol. as determined by [^{14}C]mannitol and [^{14}C]inulin [34].

PAL. Cells (0.5 g wet wt) were frozen in liquid N_2, homogenized in a tissue grinder glass tube with a glass piston with 2 ml borate buffer (0.1 M borate, 5 mM cysteine, pH 8.8) and 0.25 g PVPP. The homogenate was transferred into a centrifuge tube, and 0.5 g Dowex 1, ×2, 50–100 mesh added. After vigorous shaking the sample was centrifuged at 15 000 *g* at 4° for 20 min. The vol. of the crude extract was determined and an aliquot (400 μl) desalted on Sephadex G-25 by centrifugation [35]. The assay contained 500 μl borate buffer, 200 μl 0.1 M L-phenylalanine in borate buffer, 300 μl desalted enzyme extract, and was incubated at 35° (H_2O bath). Absorption at 290 nm was measured after 30 min, 1 hr, and 2–3 hr. The reference consisted of 700 μl borate buffer and 300 μl enzyme extract.

PuA and CGA. Cells (0.5 g wet wt) were mixed with 1 ml H_2O and 50 μl concd HCl, and sonicated at 30° for 30 min. After centrifugation (microfuge) for 5 min the supernatant was analysed by HPLC either directly (CGA) or after purification (PuA): 500 μl extract were applied to a Pasteur pipette filled with *ca* 1.6 cm^3 Extrelut (Merck) and, after 5 min, eluted with 5 ml CH_2Cl_2 [21]. The solvent was removed by a stream of N_2 at 45°, the residue dissolved in 500 μl and subjected to isocratic HPLC.

HPLC separation. Hypersil ODS column (5 μm; 2.1 × 100 mm; pre column 2.1 × 20 mm; Hewlett Packard) with 1% THF in 0.025 M citric acid monohydrate pH *ca* 2.5 (A) and 1% THF in MeOH (B) at a total flow rate of 0.4 ml min^{-1} to give the following gradient (% B over A): 0–5 min (0); 5–15 min (0–20); 15–

17 min (20–35); 17–22 min (35–50). Parameters were controlled by a Gynkotek liquid chromatograph (Munich) equipped with a diode array detector set at 254 (PuA) and 325 nm (CGA). Injection volume was 20 μl. Peak identification was achieved by comparing the UV spectrum and R_t with those of authentic standards (library established under separating conditions). The R_ts (min) were as follows: 7-methylxanthine (2.4), theobromine (4.0), caffeine (12.5), 5-CQA (18.8).

The isocratic system for separation of PuA extracted from cells (Extrelut method) consisted of the same column but the mobile phase was 25% MeOH with 1% THF. The R_ts (min) were 3.0 and 6.0 for theobromine and caffeine, respectively. The variation of the PuA content between individual cultures was *ca* 3–5%, the detection limit was 1–2 μM.

Formulae and definitions. Complex formation. All PuAs undergo complex formation with 5-CQA [12]. However, for quantity and concentration, caffeine was the most important one to focus on in this study. Therefore, mathematical treatment was related to caffeine, but is essentially valid for other methylated xanthines or uric acids. Similarly, all CGA might form complexes with PuA, but since among the CGA in coffee 5-CQA is dominant and served for investigation of the complex nature [8], we relate the formulas to 5-CQA.

The complex is present in solution and the components associate in a ratio of 1:1. Therefore we can formulate

$$\text{5-CQA} + \text{Caffeine} \Leftrightarrow \text{C} \tag{1}$$

and by applying the mass law, we obtain

$$K_c = \frac{[\text{C}]}{([\text{5-CQA}]_0 - [\text{C}])([\text{Caffeine}]_0 - [\text{C}])} \tag{2}$$

where,

K_c = equilibrium constant,
$[5\text{–CQA}]_0$ = initial concentration of chlorogenic acid,
$[\text{Caffeine}]_0$ = initial concentration of caffeine,
[C] = concentration of the complex in equilibrium.

By rearrangement of (2) we obtain

$$[\text{C}]^2 \cdot K_c + [\text{C}] \cdot (-K_c \cdot ([5 - \text{CQA}]_0 + [\text{Caffeine}]_0) - 1) + K_c \cdot [\text{5-CQA}]_0 \cdot [\text{Caffeine}]_0 = 0 \tag{3}$$

which can be solved for [C], where only the smaller value is valid (meaningful):

$$[\text{C}]_{1,2} = \frac{K_c \cdot ([\text{5-CQA}]_0 + [\text{Caffeine}]_0) + 1 \pm \sqrt{(-K_c \cdot ([\text{5-CQA}]_0 + [\text{Caffeine}]_0) - 1)^2 - 4K_c^2 \cdot [\text{5-CQA}]_0 \cdot [\text{Caffeine}]_0}}{2K_c} \tag{4}$$

In our cell culture system the following parameters were measured (raw data): total wet cell vol., $V_{C,m}$; vol. of the medium, $V_{M,m}$; caffeine concentration in the medium, $[\text{Caffeine}]_{M,m}$; caffeine concn in the wet cell probe, $[\text{Caffeine}]_{C,m}$; 5-CQA concn in the wet cell probe, $[\text{5-CQA}]_{C,m}$. The equilibrium constant K_c for caffeine at 25° is 47.3 l mol^{-1} as determined by [12]. In order to apply the formulae together with the raw data to the *in vivo* situation, the following assumptions and adjustments were necessary:

(a) Complexation takes place in the central vacuole and both 5-CQA and the complex are restricted to this compartment, the vol. of which corresponds in a first approximation to that of the symplast. In the following this compartment is named 'cell'.
(b) Free space was determined and was found to be 78% of the wet cell vol. [33] as outlined above.
(c) The caffeine concentration in the free space (including the apoplast) is equal to that in the medium.

The raw data were therefore corrected as follows:

$$V_{C,c} = V_{C,m} \cdot 0.22 \tag{5}$$

$$V_{fs} = V_{C,m} \cdot 0.78 \tag{6}$$

$$[\text{5-CQA}]_{C,c} = [\text{5-CQA}]_0 = \frac{100}{22} \cdot [\text{5-CQA}]_{C,m} \tag{7}$$

where $V_{C,c}$ is the corrected cell vol, V_{fs} the fr. (of the measured cell vol.) occupied by the free space, and $[\text{5-CQA}]_{C,c}$ the corrected concn within the cell.

The corrected cellular caffeine concn ($[\text{Caffeine}]_{C,c}$) is the difference between the amount of caffeine (mols) in the measured cell vol. and that in the free space divided by the corrected cell vol. and is by the use of (5) and (6) expressed as:

$$[\text{Caffeine}]_{C,c} = \frac{[\text{Caffeine}]_{C,m} \cdot V_{C,m} - [\text{Caffeine}]_{M,m} \cdot V_{C,m} \cdot 0.78}{V_{C,m} \cdot 0.22} \tag{8}$$

and converted to:

$$[\text{Caffeine}]_{C,c} = \frac{[\text{Caffeine}]_{C,m} - 0.78 \cdot [\text{Caffeine}]_{M,m}}{0.22} \tag{9}$$

Modelling of caffeine complexation. Since the complex between 5-CQA and caffeine is formed rapidly, we may assume that not only the stationary but also the actively producing and growing culture is at any time point of observation in equilibrium and contains, together with intracellular 5-CQA both intra- and extracellular caffeine. Also the latter was once located intracellularly for a short time after its synthesis. So,

the actual distribution of caffeine at each culture stage is, if we believe that physical chemistry is the driving force of compartmentation, nothing other than the result of the complexing ability of 5-CQA restricted to the cell. In the model, we put in as the first step the total of caffeine (of medium and cell) into the cell and calculate the corresponding initial value (the initial value of 5-CQA is not affected by this procedure and corresponds to $[5\text{-CQA}]_0$ as determined by eq. (7)):

$$[\text{Caffeine}]_0 = \frac{[\text{Caffeine}]_{C,m} \cdot V_{C,m} + [\text{Caffeine}]_{M,m} \cdot V_{M,m}}{0.22 \cdot V_{C,m}} \quad (10)$$

The concentration of the complex [C] within the cell is then calculated by the use of eq. (4). Mathematically, uncomplexed, free caffeine $[\text{Caffeine}]_f$ has thereafter to be distributed evenly between cell and medium and consequently results in a decrease in the theoretical concentration of caffeine available for complexation (in the cell) and leads to a new initial condition:

$$[\text{Caffeine}]_{0_{x+1}} = [C]_x + [\text{Caffeine}]_{f_x} \quad (11)$$

where both K_c and $[5\text{-CQA}]_0$ remain unchanged, and the concn of free caffeine in the total vol. (cell and medium) is as follows:

$$[\text{Caffeine}]_f = \frac{[\text{Caffeine}]_{C,m} \cdot V_{C,m} + [\text{Caffeine}]_{M,m} \cdot V_{M,m} - [C] \cdot V_{C,m} \cdot 0.22}{V_{C,m} + V_{M,m}} \quad (12)$$

The calculation is repeated until both $[C]_n - [C]_{n+1}$ and $[\text{Caffeine}]_n - [\text{Caffeine}]_{n+1}$ are smaller than 10^{-5} mM, signifying that a theoretical equilibrium is achieved. In the ideal system the level of free caffeine at the equilibrium as obtained by these iterations should be equal to the measured caffeine concn of the medium $[\text{Caffeine}]_{M,m}$.

Degree of complexation (DC). The quotient of [C] over $[5\text{-CQA}]_0$, and $[\text{Caffeine}]_0$, multiplied by 100 gives the degree of complexation (DC) in % for CGA and caffeine, respectively. Since this term was used in the modelling studies with the cellular compartment as target, it denotes in this context the corresponding values for the cellular compartment. For example, a DC of 75% for caffeine means that three quarters of this PuA present in the cell (and not in the entire culture) are engaged in a complex with 5-CQA.

Compartmentation factor (CF). We distinguish between the apparent and the effective compartmentation factor. CF_{app} is obtained directly from the caffeine concentrations measured in the cell and medium probe:

$$CF_{app} = \frac{[\text{Caffeine}]_{C,m}}{[\text{Caffeine}]_{M,m}} \quad (13)$$

CF_{eff}, however, considers the free-space and reflects the actual degree of compartmentation being much larger than CF_{app}:

$$CF_{eff} = \frac{[\text{Caffeine}]_{C,m} - 0.78 \cdot [\text{Caffeine}]_{M,m}}{0.22 \cdot [\text{Caffeine}]_{M,m}} \quad (14)$$

Acknowledgements—We are grateful to Professor Niklaus Amrhein, Institute of Plant Sciences, Swiss Federal Institute of Technology, Zurich, Switzerland, and to Professor M. N. Clifford, School of Biological Sciences, University of Surrey, U.K., for generous gifts of 2-AIP and 3,5-diCQA, respectively. Part of this work has been supported by the Swiss National Science Foundation, Grant No. 31-39588.93.

REFERENCES

1. Carelli, M. L. C., Lopes, C. R. and Monaco, L. C. (1974) *Turrialba* **24**, 398.
2. Anthony, F., Clifford, M. N. and Noirot, M. (1993) *Res. Crop Evol.* **40**, 61.
3. Rakotomalala, J.-J. R., Cros, E., Clifford, M. N. and Charrier, A. (1992) *Phytochemistry* **31**, 1271.
4. Petermann, J. B. and Baumann, T. W. (1983) *Plant Physiol.* **73**, 961.
5. Clifford, M. N., Gibson, C. L., Rakotomalala, J.-J. R., Cros, E. and Charrier, A. (1991) *Phytochemistry* **30**, 4039.
6. Rakotomalala, J.-J. R. (1993) *Diversité Biochimique des Caféiers*. Institut Français de Recherche Scientifique pour le Développment en Coopération, Collection: Travaux et Documents Microédités N° 99, ORSTOM, Paris.
7. Aerts, R. J. and Baumann, T. W. (1994) *J. Exp. Botany* **45**, 497.
8. Sondheimer, E., Covitz, F. and Marquisee, M. J. (1961) *Arch. Biochem. Biophys.* **93**, 63.
9. Horman, I. and Viani, R. (1972) *J. Food Sci.* **37**, 925.
10. Martin, R., Lilley, T. H., Bailey, N. A., Falshaw, P., Haslam, E., Magnolato, D. and Begley, M. J. (1986) *J. Chem. Soc., Chem. Commun.* 105.
11. Martin, R., Lilley, T. H., Falshaw, P., Haslam, E., Begley, M. J. and Magnolato, D. (1987) *Phytochemistry* **26**, 273.
12. Kappeler, A. W., Baumann, T. W. and Greutert, H. (1987) In *Douzième Colloque Internationale sur le Café*. Montreux, ASIC, Paris, p. 247.
13. Gundlach, H., Müller, M. J., Kutchan, T. M. and Zenk, M. H. (1992) *PNAS* **89**, 2389.
14. Looser, E., Baumann, T. W. and Wanner, H. (1974) *Phytochemistry* **13**, 2515.
15. Zoń, J. and Amrhein, N. (1992) *Liebigs Ann. Chem.*, 625.
16. Amrhein, N. and Gerhardt, J. (1979) *Biochim. Biophys. Acta* **583**, 434.
17. Baumann, T. W. and Frischknecht, P. M. (1988) in *Medicinal and Aromatic Plants I* (Bajaj, Y. P. S. ed.), Vol. 4, p. 264. Springer-Verlag, Berlin.
18. Dentan, E. (1985) in *Coffee: Botany, Biochemistry, and Production of Beans and Beverage*. (Clifford, M. N. and Willson, K. C. eds), p. 284. Croom Helm, London.

19. Baumann, T. W. and Gabriel, H. (1984) *Plant Cell Physiol.* **25**, 1431.
20. Baumann, T. W. and Röhrig, L. (1989) *Phytochemistry* **28**, 2667.
21. Baumann, T. W., Schulthess, B. H. and Hänni, K. (1995) *Phytochemistry* **39**, 1063.
22. Marx, F. (1990) *Z. Lebensm. Unters. Forsch.* **190**, 429.
23. Griffiths, L. A. (1958) *Biochem. J.* **70**, 120.
24. Roberts, E. A. H. (1962) in *The Chemistry of Flavonoid Compounds* (Geissman, T. A., ed.), p. 468. Pergamon, Oxford.
25. Spencer, C. M., Cai, Y., Martin, R., Gaffney, S. H., Goulding, P. N., Magnolato, D., Lilley, T. H. and Haslam, E. (1988) *Phytochemistry* **27**, 2397.
26. Price, E. W. and Spitzer, J. C. (1993) *Food Chem.* **47**, 271.
27. Liang, Y. R., Liu, Z. S., Xu, Y. R. and Hu, Y. L. (1990) *J. Sci. Food Agric.* **53**, 541.
28. Matile, P., Jans, B. and Rickenbacher, R. (1970) *Biochem. Physiol. Pflanzen* **161**, 447.
29. Renaudin, J. P. and Guern, J. (1988) *Biochem. Soc. Trans.* **16**, 63.
30. Hauser, M.-T. and Wink, M. (1990) *Zeitschrift Naturforsch.* **45c**, 949.
31. Wink, M. (1993) *J. Exp. Botany* **44** (Suppl.), 231.
32. Matile, P. (1976) *Nova Acta Leopoldina, Suppl.* **7**, 139.
33. Sato, H., Taguchi, G., Fukui, H. and Tabata, M. (1992) *Phytochemistry* **31**, 3451.
34. Baumann, T. W., Schulthess, B. H., Linden, A. and Rüedi, P. (1994) *Phytochemistry* **36**, 537.
35. Helmerhorst, E. and Stokes, G. B. (1980) *Analyt. Biochem.* **104**, 130.