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The control of CTP:choline-phosphate cytidylyltransferase activity in pea (*Pisum sativum* L.)

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Several possible control mechanisms for CTP:choline-phosphate cytidylyltransferase (EC 2.7.7.15) activity in pea (*Pisum sativum* L.) stems were investigated. Indol-3-ylacetic acid (IAA) treatment of the pea stems decreased total cytidylyltransferase activity but did not affect its subcellular distribution. Oleate (2 mM) caused some stimulation of enzyme activity by release of activity from the microsomal fraction into the cytosol, but neither phosphatidylglycerol nor monoacyl phosphatidylethanolamine had an effect on activity or subcellular distribution. A decrease in soluble cytidylyltransferase protein concentrations was found in IAA-treated pea stems, but this was not sufficient to account for all of the decrease in cytidylyltransferase activity. A 50% inhibition of enzyme activity could be obtained with 0.2 mM-CMP, which indicated possible allosteric regulation. Similar inhibition was obtained with 1.5 mM-ATP, but other nucleotides had no effect. The cytidylyltransferase enzyme protein was not directly phosphorylated, and the inhibition with 1.5 mM-ATP occurred with the purified enzyme, thus excluding an obligatory mediation via a modulator protein. The results indicate that the cytosolic form of cytidylyltransferase is the most important in pea stem tissue and that the decrease in cytidylyltransferase activity in IAA-treated material appears to be brought about by several methods.

INTRODUCTION

Phosphatidylcholine is the major phospholipid in the non-photosynthetic membranes of plant tissue (Harwood, 1980), where it is synthesized via the CDP-base or Kennedy pathway (Mudd, 1980). Studies on the effects of indol-3-ylacetic acid (IAA), a plant growth regulator, on the incorporation of ^{14}C-labelled precursors into phospholipids of pea stem explants showed that within 1 h of treatment an inhibition of incorporation of [*Me*-^{14}C]choline into phosphatidylcholine was obtained (Moore *et al.*, 1983). Measurements of pool sizes of phosphatidylcholine precursors *in vivo* and of activities of enzymes in the CDP-base pathway *in vitro* indicated that the inhibition of [*Me*-^{14}C]choline incorporation was due to a decrease in the activity of the CTP:choline-phosphate cytidylyltransferase (EC 2.7.7.15), which suggested that this enzyme was rate-limiting for phosphatidylcholine synthesis in pea stem tissue (Price-Jones & Harwood, 1983).

The cytidylyltransferase enzyme has been extensively studied in mammalian tissue, where it has also been found to catalyse the rate-limiting step for phosphatidylcholine biosynthesis (Vance & Choy, 1979), and several methods have been postulated for the control of the mammalian enzyme. Phospholipids, particularly monoacyl phosphatidylethanolamine and phosphatidylglycerol, have been found to cause aggregation of the enzyme to higher-molecular-mass forms that have greater activity (Choy & Vance, 1978; Feldman *et al.*, 1978). The subcellular distribution of the enzyme has also been implicated in its control, and fatty acids, such as oleate, have been shown to cause translocation of the enzyme from the cytosol to the endoplasmic reticulum, where activity was stimulated by the lipid environment (Pelech *et al.*, 1984). A phosphorylation–dephosphorylation cycle has also been suggested as a method of control in liver tissue, where conditions favouring protein dephosphorylation activate the cytidylyltransferase again by causing translocation to the endoplasmic reticulum (Pelech & Vance, 1984*a*). Similarly in foetal rabbit lung tissue the enzyme is inhibited under conditions favouring protein phosphorylation (Radika & Possmayer, 1985). However, in neither the liver tissue nor the foetal rabbit lung has direct phosphorylation of the enzyme protein been demonstrated, which suggests the possibility of phosphorylation of a modulator protein (Radika & Possmayer, 1985).

We have tested these putative control mechanisms for cytidylyltransferase activity in pea stem tissue, and also the possibility of regulation by alteration in enzyme protein concentrations and allosteric regulation by nucleotides. We now report the findings of these studies.

EXPERIMENTAL

Materials

Pea (*Pisum sativum* L. cultivar Feltham First) seeds were supplied by Asmer Seeds, Leicester, U.K. Phospho[*Me*-^{14}C]choline (ammonium salt; sp. radioactivity 2.18 GBq/mmol), [^{32}P]orthophosphate (initially 37 MBq/ml), PCS scintillant and Amplify fluorographic reagent were purchased from Amersham International, Amersham, Bucks., U.K. Miracloth was obtained from C.P. Laboratories, Bishops Stortford, Herts., U.K., 3MM chromatography paper was from Whatman, Maidstone, Kent, U.K., and absolute ethanol (A.R.) from James Burrough Fine Alcohols Division, London S.E.11, U.K. All other chemicals, which were of

Abbreviation used: IAA, indol-3-ylacetic acid.

the best obtainable grades, were from Sigma Chemical Co., Poole, Dorset, U.K., or BDH Chemicals, Poole, Dorset, U.K.

Methods

Tissue preparation. Pea seeds were germinated in moist vermiculite for 10 days in an incubator at 20 °C with approx. 210 μE/s per m^2 illumination (12 h light/12 h dark cycle). IAA treatment of stem tissue was carried out as described previously (Moore *et al.*, 1983). Homogenates were prepared at 4 °C in 0.32 M-sucrose/2 mM-Tris/HCl buffer, pH 7.4, in a mortar and pestle, filtered through two layers of Miracloth and fractionated by a modified method of Harwood & Stumpf (1972) (cf. Price-Jones & Harwood, 1985).

Cytidylyltransferase assays. Cytidylyltransferase (EC 2.7.7.15) activity was assayed and the products were separated chromatographically by the method of Infante & Kinsella (1978), modified as described previously (Price-Jones & Harwood, 1985).

Preparation of oleate. Potassium oleate was prepared from oleic acid by the method of Pelech *et al.* (1984). Oleic acid was dissolved in sufficient 0.12 M-KOH in 95% (v/v) ethanol to give a 100 mM stock solution of fatty acid. The ethanol was removed by evaporation under N_2 and the residue was resuspended in 0.32 M-sucrose/2 mM-Tris/HCl buffer, pH 7.4, at the required concentration.

Preparation of phospholipids. Suspensions of phosphatidylglycerol and monoacyl phosphatidylethanolamine from soya bean were prepared by the method of Feldman *et al.* (1978) modified as follows: chloroform solutions of the lipids were dried under N_2, and 100 mM-Tris/HCl buffer, pH 7.4, was added to give the required lipid concentration. The mixture was sonicated in a bath sonicator at 4 °C until a clear suspension was obtained.

Measurement of enzyme protein concentrations. Soluble cytidylyltransferase enzyme protein concentrations were measured by using a competitive-inhibition enzyme-linked immunosorbent assay method (Voller *et al.*, 1976). The soluble cytidylyltransferase was purified (Price-Jones & Harwood, 1985) and a polyclonal antibody was raised to it in rabbit. The wells of the assay plate were coated with the purified cytidylyltransferase, and the enzyme-linked immunosorbent 'sandwich' consisted of rabbit anti-cytidylyltransferase IgG mixed with different dilutions of the unknown antigen (105000 *g* supernatant from pea stem tissue), followed by a goat anti-(rabbit IgG) antibody–horseradish peroxidase conjugate. A colorimetric reaction was obtained with a substrate containing *o*-phenylenediamine and H_2O_2. The peroxidase reaction was stopped with 2.5 M-H_2SO_4, and the absorbance was read at 492 nm with a Titertek Multiskan plate reader (Flow Laboratories, Irvine, Ayrshire, U.K.)

Phosphorylation of proteins. [γ-^{32}P]ATP was produced by the method of Glynn & Chappell (1964), as modified by Walsh *et al.* (1970). Soluble proteins were prepared from pea stem tissue and phosphorylated by the method of Veluthambi & Poovaiah (1984). The reaction mixture contained 0.4 mg of protein, 50 mM-Mes/NaOH buffer, pH 7.0, 5 mM-$MgCl_2$, 0.1 mM-dithiothreitol, 0.2 mM-EGTA and about 222 kBq of [γ-^{32}P]ATP, in a final volume of 100 μl. The reaction mixture was pre-incubated for 1 min at 30 °C before addition of the [γ-^{32}P]ATP, which mixture was then incubated for 5 min at 30 °C. The reaction was stopped by the addition of an equal volume of electrophoresis sample buffer (Laemmli, 1970). SDS/polyacrylamide-gel electrophoresis was performed on slab gels by the method of Laemmli (1970) with 10% resolving gels and 3% stacking gels. Gels were fluorographed for 24 h at −70 °C.

Protein determination. Protein was measured by the method of Bradford (1976), with bovine serum albumin as standard.

ATP determination. ATP was determined with a luciferin/luciferase system by the method of Hellstern (1986). Pea stem tissue was homogenized in 2 M-$HClO_4$ in 40% (v/v) ethanol, and the homogenate was centrifuged at 600 *g* for 20 min at 0 °C, the supernatant was collected and the pellet was resuspended in 2 M-$HClO_4$ in 40% (v/v) ethanol before being centrifuged again. The supernatants were pooled and neutralized with 2 M-KOH in 0.2 M-Mops. The precipitate that formed was removed by centrifugation at 600 *g* for 20 min at 0 °C, and the supernatant was assayed for ATP.

The assay was carried out with a Perkin–Elmer LS-5 luminescence spectrometer, programmed to monitor phosphorescence. All solutions used in the assay were filtered through 0.22 μm-pore-size filters before use. A 1 ml portion of assay buffer (made up from 8 vol. of water, 5 vol. of 0.1 M-Na_2HAsO_4/40 mM-$MgSO_4$ adjusted to pH 7.4 with H_2SO_4 and 2 vol. of 50 mM-NaH_2PO_4/10 mM-$MgSO_4$ adjusted to pH 7.4 with NaOH) was added to the cuvette with a 50 μl sample containing ATP followed by 20 μl of luciferin/luciferase (10 mg/ml in water). A standard curve was prepared with ATP made up in water in the range 0–30 μM.

RESULTS

Effect of IAA on subcellular distribution of cytidylyltransferase

The results of examining the subcellular distribution of cytidylyltransferase activity in the IAA-treated pea stems are shown in Table 1. There was an increase in the proportion of activity recovered in the mitochondrial fraction of treated material, but the soluble fraction still contained considerably more activity, and there was no statistically significant decrease in that fraction. The small increase in the proportion of cytidylyltransferase activity associated with the microsomal fraction was not significant and was accompanied by an IAA-induced decrease in overall cytidylyltransferase activity. This differed from the situation found in mammalian tissue, where translocation of the enzyme to the microsomal fraction was accompanied by an overall increase in cytidylyltransferase activity (Pelech & Vance, 1984*a*). These differences may, however, reflect the much greater amounts of soluble cytidylyltransferase activity in pea stem tissue.

Table 1. Effect of IAA on the subcellular distribution of cytidylyltransferase activity

Peas were germinated and stem explants treated as described in the Experimental section. Homogenates were prepared from 40 stem sections in each case and fractionated, before assay of the fractions for cytidylyltransferase activity as described in the Experimental section. IAA treatment was for 1 h. All experiments were performed in triplicate and the results are means ± S.E.M. Statistical significance was estimated by using Student's *t* test for paired samples.

	Cytidylytransferase activity (% of total homogenate activity)							
	Control				IAA-treated			
Fraction	Expt. 1	Expt. 2	Expt. 3	Mean	Expt. 1	Expt. 2	Expt. 3	Mean
10000 *g* pellet	2.2±0.4	0.7±0.1	1.4±0.6	1.4±0.4	5.3±1.1	1.0±0.1	4.9±1.3	3.7±1.4 ($P < 0.05$)
105000 *g* pellet	3.2±0.3	1.4±0.4	1.0±0.1	1.9±0.7	7.6±3.0	1.6±0.8	1.5±0.3	3.6±2.0
105000 *g* supernatant	84.5±3.9	86.4±3.7	101.0±3.6	90.6±5.2	86.8±4.8	64.4±0.2	65.3±12.5	72.2±7.3
Total homogenate activity (nmol/min)	14.9±3.6	22.0±2.7	15.6±4.6	17.5±2.3	7.6±0.8	12.3±3.3	12.4±3.4	10.8±1.6 ($P < 0.025$)
Stem length (mm)	7.30±0.02	7.15±0.02	7.19±0.02	7.16±0.02	7.37±0.03	7.47±0.03	7.56±0.03	7.46±0.05 ($P < 0.02$)

Table 2. Effect of oleate on cytidylyltransferase activity in pea stems

Peas were germinated, the stems were homogenized and assays were carried out with the 10000 *g* (post-mitochondrial) supernatant as described in the Experimental section. Either samples were pre-incubated for 1 h at 25 °C with oleate before assay or oleate was added to the assay mixture. All experiments were done in triplicate and the results are means ± S.E.M. Significance was estimated by using Student's *t*-test for paired samples: *significantly different at the 10% level.

	Cytidylyltransferase activity (nmol/min per mg of protein)									
Concn. of oleate (mM)	Pre-incubated samples					Assay + oleate				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Mean	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Mean
0	1.64±0.06	1.22±0.07	0.41±0.08	0.59±0.08	0.97±0.28	0.76±0.23	0.41±0.10	0.39±0.08	0.11±0.01	0.42±0.13*
0.5	3.04±0.66	0.61±0.07	—	0.47±0.07	1.37±0.84	1.32±0.31	0.07±0.01	—	0.29±0.07	0.56±0.39
1.0	0.76±0.06	3.55±0.47	0.55±0.32	0.41±0.16	1.32±0.75	1.92±0.96	2.30±0.87	0.69±0.19	0.15±0.04	1.27±0.51
2.0	2.11±0.71	5.16±1.03	1.30±0.26	0.60±0.04	2.29±1.00	1.27±0.10	1.56±0.03	1.08±0.14	0.18±0.04	1.02±0.30*

Table 3. Effect of oleate on subcellular distribution of cytidylyltransferase activity

The 10000 *g* (post-mitochondrial supernatant) was prepared from pea stems as described in the Experimental section. The supernatant was incubated for 1 h at 25 °C with oleate and fractionated for 1 h at 105000 *g* to give microsomal and soluble fractions. Assays were performed as described in the Experimental section. Results are means ± S.E.M. ($n = 3$) and values in parentheses are percentages of the original 10000 *g* supernatant activity that was recovered for each oleate concentration. [Mean cytidylyltransferase activity in 10000 *g* supernatant: no oleate, 0.40 ± 0.01 nmol/min per mg of protein; 1 mM-oleate, 0.74 ± 0.15 nmol/min per mg of protein ($P < 0.10$); 2 mM-oleate, 0.62 ± 0.01 nmol/min per mg of protein ($P < 0.001$)]. Statistical significance was estimated by using Student's *t* test.

Fraction	Cytidylyltransferase activity (nmol/min per mg of protein)		
	No oleate	1 mM-Oleate	2 mM-Oleate
105000 *g* pellet	0.33 ± 0.07 (11.5%)	0.65 ± 0.25 (10.1%)	0.11 ± 0.07 (1.4%)
105000 *g* supernatant	0.43 ± 0.23 (100%)	0.61 ± 0.04 (77.9%)	0.96 ± 0.20 (152.7%) ($P < 0.10$)

Table 4. Concentrations of soluble cytidylyltransferase protein in IAA-treated and control pea stem tissue

Peas were germinated and stem explants treated as described in the Experimental section. The 105000 *g* supernatants were prepared from 30 stem sections in each case and cytidylyltransferase protein concentrations were measured by using a competitive-inhibition enzyme-linked immunosorbent assay method. Values are for treated samples expressed as percentages of average control for each experiment and are means ± S.E.M. ($n = 6$). (Mean control values: stem length, 7.26 ± 0.02 mm; cytidylyltransferase activity, 0.85 ± 0.12 nmol/min per mg of protein; cytidylyltransferase protein, 0.37 ± 0.03 μg/mg of total protein). Statistical significance was estimated by using Student's *t* test for paired samples (cf. Price-Jones & Harwood, 1986).

Expt. no.	Stem length (% of control value)	Cytidylyltransferase activity (% of control value)	Cytidylyltransferase protein (% of control value)
1	101.9 ± 0.5	79.3 ± 1.7	96.4 ± 3.6
2	102.1 ± 0.4	26.2 ± 9.2	95.7 ± 0.5
3	104.4 ± 0.6	47.5 ± 9.9	94.4 ± 2.8
Mean	102.8 ± 0.8 ($P < 0.10$)	51.0 ± 15.4 ($P < 0.05$)	95.5 ± 0.6 ($P < 0.05$)

Effect of oleate

Oleate had some stimulatory effect on cytidylyltransferase activity in the post-mitochondrial supernatant, as can be seen from the values given in Table 2.

When the post-mitochondrial supernatant was exposed to oleate and then fractionated further (Table 3), cytidylyltransferase activity was significantly increased in the post-mitochondrial supernatant in the presence of 1 mM- and 2 mM-oleate. This was associated with a significant increase in the soluble activity in the presence of 2 mM-oleate. Again this was the opposite situation to mammalian tissue, where stimulation of activity obtained with oleate was due to translocation of the enzyme to the endoplasmic reticulum. The results suggested that 2 mM-oleate may have had a detergent effect on the microsomal fraction, causing enzyme associated with microsomal membranes to be released into the soluble fraction. However, when 1 mM-oleate was added to cytidylyltransferase assays from IAA-treated material it only stimulated the enzyme to the same degree as it did for non-treated tissue. It did not, therefore, reverse the overall inhibitory effect of IAA (results not shown).

Effect of phospholipids

The effect of the addition of monoacyl phosphatidylethanolamine or phosphatidylglycerol (0.25 mM final concentration) to an assay system containing the post-mitochondrial supernatant was tested. No change in enzyme activity was found in two separate experiments (results not shown).

Alteration of enzyme protein concentrations

It can be seen from the results in Table 4 that a small, but statistically significant, decrease in soluble cytidylyltransferase enzyme protein was found in the IAA-treated pea stems. However, a decrease of 4.5% in soluble enzyme protein was not sufficient to account for the 49% decrease in soluble cytidylyltransferase activity, which suggested that the inhibitory effect of IAA was also mediated by a further mechanism or mechanisms.

Effect of nucleotides

The effect of different nucleotides on the activity of soluble cytidylyltransferase was examined. ATP, ADP, AMP, CDP, CMP or GTP was added to the assay at concentrations of 0–2 mM. It was found that 0.2 mM-CMP or 1.5 mM-ATP caused maximal inhibition of cytidylyltransferase activity, but no effect was obtained with any other nucleotide tried. The effects of 0.2 mM-CMP or 1.5 mM-ATP on the kinetic parameters of the cytidylyltransferase were examined, and the results are shown in Table 5. Both nucleotides, at the

Table 5. Effects of 1.5 mM-ATP and of 0.2 mM-CMP on the activity of cytidylyltransferase from pea stems

Peas were germinated, the stems were homogenized and assays were carried out with the 105000 *g* supernatant as described in the Experimental section, or with cytidylyltransferase purified to homogeneity as previously described (Price-Jones & Harwood, 1985). Values are means ±S.E.M. ($n = 9$ for supernatant enzyme, $n = 2$ for purified enzyme) calculated by using the method of Wilkinson (1961). Statistical significance was estimated by using Student's t test.

		Phosphocholine substrate		
		Control	+CMP	+ATP
Supernatant	K_m (mM)	1.52±0.26	0.38±0.35	0.50±0.13 ($P < 0.001$)
	$V_{max.}$ (μM/min)	4.60±0.46	0.45±0.07 ($P < 0.001$)	1.05±0.09 ($P < 0.005$)
Enzyme	K_m (mM)	2.97±0.58	0.81±0.12 ($P < 0.05$)	0.42±0.10 ($P < 0.02$)
	$V_{max.}$ (μM/min)	4.14±0.91	0.44±0.79 ($P < 0.05$)	0.30±0.03 ($P < 0.05$)
		CTP substrate		
		Control	+CMP	+ATP
Enzyme	K_m (mM)	0.36±0.09	2.92±1.00 ($P < 0.10$)	1.22±0.60
	$V_{max.}$ (μM/min)	2.80±0.48	2.34±0.65	0.70±0.19 ($P < 0.05$)

concentrations used, were found to have significant effects on the K_m and $V_{max.}$ values for the cytidylyltransferase with either substrate. Data obtained with phosphocholine substrates are shown in Table 5. However, because of the equivocal nature of kinetic parameters obtained with crude subcellular fractions, we decided to purify sufficient enzyme to repeat the experiments with a homogeneous preparation. This was considered to be particularly important for experiments with ATP as an inhibitor because phosphorylation of a modulator protein has already been suggested for the regulation of the cytidylyltransferase of foetal rabbit lung tissue (Radika & Possmayer, 1985).

The results shown in Table 5 (taking due account of the experimental reproducibility) indicate that CMP acts as an uncompetitive inhibitor towards phosphocholine substrate but as a competitive inhibitor towards CTP. ATP, in contrast, although also appearing to act as an uncompetitive inhibitor towards phosphocholine substrate, showed characteristics of a mixed inhibitor with CTP as substrate.

Regulation by phosphorylation

The subunit M_r of the pea stem cytidylyltransferase enzyme is known to be 56 000 (Price-Jones & Harwood, 1985). No radioactivity was found corresponding to this M_r value when gels of phosphorylated soluble proteins were fluorographed, although other radiolabelled bands corresponding to M_r values of approx. 68 000 and 98 000 were detected. This indicated that there was no direct phosphorylation of the enzyme under the experimental conditions used.

DISCUSSION

The cytidylyltransferase enzyme from pea stem tissue has been found to be similar to that from onion stem (Morre *et al.*, 1970) and mammalian tissue (Pelech & Vance, 1984*a*) in that it occurred in both particulate and soluble fractions (Price-Jones & Harwood, 1983). However, the translocation of enzyme from the cytosol to the endoplasmic reticulum and stimulation by the lipid environment did not appear to be a regulatory mechanism in pea stem tissue, and in IAA-treated material any increase in the amount of activity associated with the microsomal fraction was accompanied by a decrease in overall cytidylyltransferase activity (Table 1). In mammalian tissue translocation to the microsomal fraction was accompanied by an increase in overall activity (Pelech & Vance, 1984*a*).

In addition, although oleate gave some increase in cytidylyltransferase activity in the post-mitochondrial supernatant (Tables 2 and 3), this was accompanied by an increase in soluble, not microsomal, activity (Table 3). The increase in cytidylyltransferase activity found with 2 mM-oleate in the pea stem tissue may have been due to release of membrane-associated enzyme caused by a detergent effect of the oleate. By contrast, in HeLa cells, 1 mM-oleate, added exogenously, caused almost complete translocation of the enzyme to the microsomal fraction, resulting in an 11-fold increase in cytidylyltransferase activity. It also caused a 22-fold stimulation of enzyme activity at a concentration of 50 μM when incubated with microsomal fractions of HeLa cells, which showed that an intact cellular structure was not required for oleate to exert a stimulatory effect (Pelech *et al.*, 1984).

Oleate or exogenous phospholipid was also found to reverse the inhibitory effects of trifluoperazine and chlorpromazine on the cytidylyltransferase activity of HeLa cells (Pelech & Vance, 1984*b*). However, 2 mM-oleate was found to have no significant effect on the lowered activity of the pea stem cytidylyltransferase in IAA-treated material (results not shown).

Exogenous phospholipids had no stimulatory effect on the pea stem enzyme, whereas 0.25 mM-phosphatidylglycerol was sufficient to cause a 16-fold increase in soluble cytidylyltransferase activity in foetal rat lung (Feldman *et al.*, 1978) and a 2 mg/ml concentration of monoacyl phosphatidylethanolamine gave a 10-fold stimulation of soluble cytidylyltransferase activity in rat liver (Choy & Vance, 1978). The activation in these tissues was thought to be brought about by aggregation of the enzyme to higher-molecular-mass forms that had greater activity than the 'dormant' low-molecular-mass form that was present in the cytosol (Feldman *et al.*, 1978). The small stimulatory effect obtained with oleate and the lack of effect of exogenous phospholipid suggested that in the pea stem tissue the cytosolic form of the enzyme was not 'dormant' but was the more important.

It was possible, therefore, that a decrease in the amount of cytidylyltransferase enzyme protein in the cytosol may have been responsible for the decrease in enzyme activity in IAA-treated material. A small decrease in enzyme protein was found (Table 4), but this was not sufficient to account for all of the decrease in activity. It seemed that IAA may have been responsible for a diminished synthesis or increased breakdown of enzyme protein, but was also involved in a further regulatory mechanism.

Regulation by nucleotides was considered to be a strong possibility, at least in the case of CMP. The rationale behind this was that the next step in the synthetic pathway, the CDP-choline : diacylglycerol cholinephosphotransferase (EC 2.7.8.2) step, generates CMP and was found to be stimulated by IAA treatment (Price-Jones & Harwood, 1983). The increased generation of CMP could have had a negative-feedback effect, since this nucleotide was found to inhibit the enzyme activity severely (Table 5). For a two-substrate reaction showing an ordered mechanism, the type of inhibition apparently shown by CMP towards phosphocholine and CTP respectively is consistent with the proposal that phosphocholine binds to the enzyme first (Dixon & Webb, 1979). This would explain why the inhibition appeared to be uncompetitive towards phosphocholine but competitive towards CTP (when phosphocholine was present at 4 mM), and therefore one effectively can consider the reactions to be:

$$EA + I \rightleftharpoons EA \cdot I$$

$$EA + B \rightleftharpoons EA \cdot B$$

where EA is the enzyme–phosphocholine complex, I is CMP and B is CTP.

The uncompetitive inhibition by ATP towards phosphocholine but mixed inhibition towards CTP substrate can be explained if one assumes that ATP is able to bind to both the EA and the EAB complexes. However, further kinetic analysis would be necessary to conclude these mechanisms with certainty.

The direct action of ATP on the kinetic parameters of the purified enzyme negates the need to assume that its effect is via phosphorylation of a modulator protein as suggested for foetal rabbit lung tissue (Radika & Possmayer, 1985). However, it is possible that such a protein may also play a role in regulation of the pea enzyme *in vivo*. Certainly, our failure to demonstrate any significant radiolabelling from [^{32}P]ATP seems to argue against direct phosphorylation as a mechanism for control. However, although ATP had an effect on cytidylyltransferase *in vitro*, its maximum inhibition was at 1.5 mM. Measurements of the endogenous concentrations of ATP in the soluble fraction from the pea stem tissue showed them to be 2.15 ± 0.10 μM, thus placing a doubt on the physiological relevance of the observations made *in vitro*.

The decrease in cytidylyltransferase activity in IAA-treated pea stems appears, therefore, to be controlled by several factors, including a decrease in enzyme protein and possible modulation by CMP. Mechanisms such as subcellular enzyme translocation, which have been implicated in mammalian tissues, do not appear to play any role in pea stems.

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REFERENCES

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Choy, P. C. & Vance, D. E. (1978) J. Biol. Chem. **253**, 5163–5167
Dixon, M. & Webb, E. C. (1979) Enzymes, 3rd edn., pp. 332–380, Longman, London
Feldman, D. A., Kovac, C. R., Dranginis, P. L. & Weinhold, P. A. (1978) J. Biol. Chem. **253**, 4980–4986
Glynn, I. M. & Chappell, J. B. (1964) Biochem. J. **90**, 147–149
Harwood, J. L. (1980) in Biochemistry of Plants (Stumpf, P. K. & Conn, E. E., eds.), vol. 4, pp. 1–55, Academic Press, New York
Harwood, J. L. & Stumpf, P. K. (1972) Lipids **7**, 8–19
Hellstern, K. H. (1986) Ph.D. Thesis, University of Wales
Infante, J. P. & Kinsella, J. E. (1978) Biochim. Biophys. Acta **526**, 440–449
Laemmli, U. K. (1970) Nature (London), **227**, 680–685
Moore, T. S., Price-Jones, M. J. & Harwood, J. L. (1983) Phytochemistry **22**, 2421–2425
Morre, D. J., Nyquist, S. & Rivera, E. (1970) Plant Physiol. **45**, 800–804
Mudd, J. B. (1980) in Biochemistry of Plants (Stumpf, P. K. & Conn, E. E., eds.), vol. 4, pp. 250–282, Academic Press, New York
Pelech, S. L. & Vance, D. E. (1984*a*) Biochim. Biophys. Acta **779**, 217–251
Pelech, S. L. & Vance, D. E. (1984*b*) Biochim. Biophys. Acta **795**, 441–446
Pelech, S. L., Cook, H. W., Paddon, H. B. & Vance, D. E. (1984) Biochim. Biophys. Acta **795**, 433–440
Price-Jones, M. J. & Harwood, J. L. (1983) Biochem. J. **216**, 627–631
Price-Jones, M. J. & Harwood, J. L. (1985) Phytochemistry **24**, 2523–2527
Price-Jones, M. J. & Harwood, J. L. (1986) Phytochemistry **25**, in the press
Radika, K. & Possmayer, F. (1985) Biochem. J. **232**, 833–840
Vance, D. E. & Choy, P. C. (1979) Trends Biochem. Sci. **4**, 145–148
Veluthambi, K. & Poovaiah, B. W. (1984) Plant Physiol. **76**, 359–365
Voller, A., Bidwell, D. A. & Bartlett, A. (1976) Bull. W.H.O. **53**, 55–65
Walsh, D. A., Perkins, J. P., Broshrom, C. O., Ho, E. S. & Krebs, E. G. (1970) J. Biol. Chem. **246**, 1968–1976
Wilkinson, G. N. (1961) Biochem. J. **80**, 324–332

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