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POLYPHENOLASES IN THE 1000*g* FRACTION OF *PAPAVER SOMNIFERUM* LATEX*

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Abstract—A polyphenolase complex was isolated from the 1000-*g* organelle fraction of the latex of the opium poppy. This enzyme oxidized a variety of phenolic substrates, including *p*-cresol, catechol, *p*-coumaric acid, hydroquinone and tyrosine, but would not oxidize the opium alkaloid intermediates reticuline and salutaridinol. The activity of the enzyme complex was enhanced by techniques which involved the mechanical damage of the organelles and by solubilization of the organelle membrane with the detergent Triton X-100. The enzyme activity was inhibited by KCN and DIECA.

INTRODUCTION

PREVIOUS work by Fairbairn *et al.*[1,2] on the biosynthesis of morphine in poppy latex has shown that morphine can be synthesized by the latex from tyrosine and dihydroxyphenylalanine (DOPA) and that this synthesis is associated with a group of organelles which sediment when latex in mannitol phosphate buffer is centrifuged at 1000 *g* for 30 min.

Dickenson's[3] preliminary electron microscope studies of the 1000-*g* fraction of poppy latex indicated the presence of organelles which physically resembled the lysosomal particles found by de Duve[4] in rat liver homogenates and the lutoids found by Pujarniscle[5] in *Hevea braziliensis* latex.

Preliminary experiments in this laboratory and Meissner's[6,7] reports of the occurrence of polyphenolase in latex, together with the fact that various workers[8] have considered this type of enzyme complex as responsible for the oxidative coupling reactions involved in the biosynthesis of the alkaloids of *Papaver somniferum*, have resulted in the present work, in which a preliminary study has been made of polyphenolase of poppy latex and its possible association with alkaloid biogenesis.

RESULTS

Polyphenolase and the 1000 *g Fraction of Poppy Latex*

In preliminary work, browning of latex samples allowed to stand for a time was clearly indicative of an active polyphenolase, and since the brown colour on initial development appeared to be associated with the particulate fraction of the latex, which sedimented on

* Part I in a projected series "Enzymic studies with *Papaver somniferum*".

[1] J. W. FAIRBAIRN, J. M. PALMER and A. PATERSON, *Phytochem.* **7**, 2117 (1968).
[2] J. W. FAIRBAIRN and M. DJOTE, *Phytochem.* **9**, 739 (1970).
[3] P. B. DICKENSON, Unpublished work.
[4] C. DE DUVE, in *Subcellular Particles* (edited by T. HAYASHI), pp. 128–159, Ronald Press, New York (1959).
[5] A. PUJARNISCLE, *Physiol. Veg.* **6**, 27 (1968).
[6] L. MEISSNER, *Flora, Abt. A.*, *Bd.* **157**, 1 (1966).
[7] L. MEISSNER, *Flora, Abt. A.*, *Bd.* **156**, 634 (1966).
[8] D. H. R. BARTON and T. COHEN, *Festschrift A. Stoll, Basel* 117 (1957).

standing, it was considered likely that this enzyme was particulate in nature and possibly associated with the 1000-*g* organelles concerned in morphine biogenesis.

The latex collected in an equal volume of 0·5 M mannitol/phosphate buffer pH 7·0 was separated into an (1000 *g*) organelle fraction, a mitochondrial fraction (11,000 *g*) and the supernatant. In these experiments both the 1000-*g* and the 11,000-*g* fraction were washed twice and resuspended. Using standard manometric techniques, the results given in Table 1 were obtained.

TABLE 1. THE ASSOCIATION OF POLYPHENOLASE WITH THE 1000 *g* ORGANELLE FRACTION OF *P. somniferum*

Latex fractions	Oxygen absorption μM/ml latex			
	minus catechol 1×10^{-2} M		plus catechol 1×10^{-2} M	
	30 min	60 min	30 min	60 min
Whole latex	1·0	2·5	n.d.	n.d.
1000 *g* organelles	3·0	5·0	16·0	28·0
11,000 *g* fraction	0·0	0·5	0 0	0·7
Supernatant	0·0	0·5	0·0	0 6

The 1000-*g* organelles and the 11,000-*g* fraction were resuspended in a volume of 0·5 M mannitol/phosphate buffer pH 7·0 equivalent to that of the original latex. n.d. indicates not done.

The results in Table 1 show the high levels of oxygen absorption with the 1000 *g* fraction of the latex, and similar low oxygen absorption values for the 11,000-*g* and supernatant fractions presumably because of damage to the mitochondria during processing. The experiments indicate that the enzymes responsible for the oxidation of catechol principally reside in the 1000 *g* fraction, and when 11,000-*g* fractions and supernatant were washed free of 1000 *g* organelles, no oxygen absorption attributable to the oxidation of catechol was observed. The fact that the oxygen absorption of the 1000-*g* organelles minus catechol was greater than that for a similar whole latex sample would suggest that some organelle damage has occurred during centrifuging and resuspension in buffer solution. In subsequent experiments, it was found adequate to wash and resuspend the organelles once only.

The Effect of Disintegration of the 1000-*g Organelles on Oxygen Absorption. Freeze/Thawing, Sonication and the Use of the Detergent Triton X*-100

In further experiments (Table 2) treatments were designed to rupture the membranes of the 1000-*g* organelles so that the effects of these treatments on polyphenolase could also be observed.

The results show that the organelles treated over 16 hr with 0·1% Triton X-100 or freeze/thawed absorb slightly more oxygen than untreated organelles, and that when 0·1% Triton is added at the commencement of the experiment, or when sonication is used, a much larger oxygen absorption is observed. This would suggest the occurrence of another oxygen requiring enzyme which is inhibited by the products of polyphenolase, or that the phenolic substrates present in these organelle suspensions may have been used prior to the commencement of the experiment. The latter suggestion appears acceptable in the light of

TABLE 2. THE EFFECT OF TRITON X-100, SONICATION AND FREEZE/THAW TECHNIQUES ON THE POLYPHENOLASE ACTIVITY OF THE ORGANELLE FRACTION OF *P. somniferum*

Treatment of organelles	μM/ml latex/30 min	
	Organelles	Organelles plus catechol 10^{-2} M
Triton X-100 treated for 16 hr prior to experiment	1·0	62
Triton X-100 treatment at commencement of experiment	6·5	9·5
Sonication for 2 min	3·6	36
Freeze/thawing of organelles (×6)	1·0	14
No treatment	0·6	7·1

Latex samples were collected into an equal volume of mannitol/phosphate buffer pH 7·0, centrifuged and the 1000-*g* organelles, after one washing, were resuspended in a volume of buffer equivalent to the original volume of latex. Portions of the organelle preparation were freeze/thawed ×6 prior to the experiment; treated with 0·1% Triton X-100 16 hr or 1 hr prior to the commencement of the experiment. A further portion was sonicated at 20° for 2 min prior to the commencement of the experiment The original organelle preparation was left at 2°.

the experiments in which catechol 1×10^{-2} M was added to the variously treated organelle suspensions since, with the addition of a phenolic substrate, the sonicated and 16 hr Triton treated suspensions showed large increases in oxygen absorption. Catechol added to organelle suspensions freeze/thawed or Triton treated 1 hr prior to the experiment, however, gave somewhat lower levels of oxygen absorption.

TABLE 3. THE EFFECT OF THE POLYPHENOLASE INHIBITORS ON THE OXYGEN ABSORPTION OF THE ORGANELLES OF *P. somniferum*

	Absorption of oxygen μMO_2/ml latex			
	Minus catechol 10^{-2} M		Plus catechol 10^{-2} M	
	30 min	60 min	30 min	60 min
Organelles untreated	4·6	5·6	16·5	34·0
Organelles + *KCN 10^{-4} M	2·6	4·3	7·1	25·0
+ *KCN 10^{-3} M	2·2	4·0	7·6	12·3
Organelles + *φDIECA 10^{-4} M	0·5	2·1	7·1	12·3
*DIECA 10^{-3} M	0	0	0·5	5·7

The inhibitor and the catechol were placed in the side arm of the flask, the organelles in 0·5 M mannitol/phosphate buffer in the main compartment of the Warburg flask. Both inhibitors and catechol solutions were made up in mannitol buffer.

* These were final concentrations of the substances within the reaction media.
φDIECA—diethyldithiocarbamate.

The Effect of Potassium Cyanide and Diethyldithiocarbamate on Latex Polyphenolase

These substances have an inhibitory effect on the activity of the 1000-*g* organelles (Table 3) and the inhibition of oxygen absorption of this fraction in the presence of catechol is of the order of 20% with KCN 10^{-4} M and 40% with KCN $^{-3}$ M, and somewhat greater with similar concentrations of diethyldithiocarbamate. These results indicate that the polyphenolase activity of the 1000-*g* fraction of the latex is not so sensitive to KCN, a factor which has been previously reported for some polyphenolase systems.[9]

Substrates Oxidized by the Latex 1000 *g Organelle Fraction Polyphenolase*

Experiments in which organelle preparations were adjusted to a series of pH after sonication and dialysis at pH 5·6, showed that polyphenolase had maximum activity in a broad peak pH 8·0–10·0. Since some of the substrates used were less stable above pH 8·5, dialysed organelle samples were adjusted to pH 8·0, and used in subsequent experiments. If the solid material was removed from the suspension, preliminary experiments indicated that up to 50% of the polyphenolase activity was lost and treatment with Triton X-100 in place of sonication did not greatly improve this situation. It would appear, therefore, that part of the polyphenolase within the organelles is soluble and the remainder insoluble to the extent that attempted solubilization with Triton X-100 would not completely bring this part of the enzyme complex into solution.

TABLE 4. SUBSTRATES OXIDIZED BY POLYPHENOLASE IN THE 1000 *g* ORGANELLES OF *P. somniferum* LATEX

Substrates at a total conc. 1×10^{-2} M	Oxygen absorption μMO_2/ml latex/60 min
Caffeic acid[4]	61
Catechol[1,2]	115
p-Coumaric acid[4]	44
p-Cresol[1,2]	195
DOPA[2]	35
Ferulic acid[4]	0
Guaiacol[1]	0
p-Hydroxybenzoic acid[4]	0
Hydroquinone[1]	14
Hydroxytyramine[2]	24
2,6-Dimethoxyphenol[1]	0
(±)-Reticuline[3,4]	0
Salutaridinol[3,4]	0
Salutaridinol + ATP (0·05 M)	0
Tyrosine[2–4]	26
Vanillic acid[1,4]	0
Organelles (sonicated and dialysed) no additives	0

[1] Substrates for laccase. [2] For tyrosinase type polyphenolase. [3] Intermediates in morphine biogenesis. [4] Recorded as occurring in poppy. The protein content of the latex used in this experiment (estimated using Folin's method) was 9812 μg/ml organelle suspension.

[9] W. O. JAMES, E. A. H. ROBERTS, H. BEEVERS and P. C. DE KOCK, *Biochem. J.* **43**, 626 (1948).

Using the dialysed organelle fraction at pH 8·0, it was possible to investigate some of the common substrates frequently oxidized by this enzyme system. The results of these preliminary experiments are given in Table 4. *p*-Cresol and catechol were readily oxidized, though the oxidation of catechol appeared to be adversely affected if the dialysis period was more than 24 hr.

Somewhat surprisingly, hydroquinone was not readily oxidized as suggested by Meissner,[6] and contrary to his report, the experiments indicate that tyrosine is oxidized by this enzyme system. The results indicate that the enzyme complex shows characteristics of catechol oxidase (E.C.1.10.3.1) and appears to show both laccase and tyrosinase activities. No oxidation of either (±)-reticuline or salutaridinol was observed, and though this result could have been due to the removal of other essential substrates by dialysis; experiments in which latex had not been treated in any way other than the separation into a 1000-*g* fraction and supernatant or to which ATP was added gave similar results.

DISCUSSION

Methods of centrifugation would suggest that the organelles of the 1000 *g* fraction of poppy latex are heavier and more sensitive to mechnical damage than the particles found by de Duve[4] in rat liver cells and by Pujarniscle[5] in *Hevea braziliensis* latex and the fact that to date[10] polyphenolase is the only enzyme detected in the poppy latex organelles would further suggest that they are not lysosomal in nature, nor could they be considered similar to the peroxisomes and glyoxosomes found by Tolbert[11] and Beevers[12] in the broad bean.

The experiments described indicate that the whole of the polyphenolase activity resides in the 1000-*g* organelle fraction and the fact that browning is observed only when the organelles are damaged suggests that though the organelles contain both the enzyme and substrate, these only come into contact as a result of damage, thus indicating some compartmentalization within the organelle. However, the addition of a phenolic substrate to a suspension of organelles in buffer solution does produce increases in oxygen absorption with eventual browning, but oxygen absorption is low compared with that observed with damaged particles. This would suggest that with undamaged organelles, the substrate does not readily reach the enzyme site.

The association of polyphenolase with the 1000-*g* organelles which have been observed by Fairbairn[2] to convert ^{14}C labelled tyrosine and DOPA into labelled morphine, is of considerable interest since Barton[8] suggests that this enzyme system could theoretically be responsible for the series of oxidative coupling reactions required for conversion of the intermediate reticuline to morphine. Jindra[13] also showed that KCN and DIECA, known inhibitors of polyphenolase, produced a reduction in the levels of morphine content when fed to poppy seedlings. However, experiments to date have given no indication that this enzyme complex will oxidize either reticuline or salutaridinol, and whilst it may be suggested that a peroxidase system could produce a similar series of reactions, this enzyme has not been found to occur in poppy latex despite rigorous tests[10] and the fact that it is a very active enzyme in the seedlings.[13] Further experiments with these unlabelled intermediates of morphine biogenesis has been difficult, since it has proved almost impossible

[10] M. F. Roberts, to be published.
[11] N. E. Tolbert, A. Oeser, T. Kisaki, R. H. Hageman and R. K. Yamazaki, *J. Biol. Chem.* **243**, 519 (1968).
[12] T. G. Cooper and H. Beevers, *J. Biol. Chem.* **244**, 3507 (1969).
[13] A. Jindra, *Acta. Facult. Pharm. Bohrmoslov.* **13**, 51 (1967).

to remove all the alkaloids from latex enzyme preparations using present methods with acid dialysis (pH 5·6), and this may indicate some protein binding of the alkaloids.

The nature of the polyphenolase system of poppy latex appears to be complex and deserves a more detailed study, since certain interesting features were observed as a result of this enzyme's response to the treatment of the particles to mechanical damage or solubilization. If it is assumed that the particles become more fragmented as a result of mechanical damage, (a) by freeze/thaw techniques, and (b) by sonication, then increased fragmentation produces increased enzyme activity, and solubilization of the organelle membrane with Triton X-100 (16 hr prior to the experiment) supports this view, since this treatment gives the greatest increase in oxygen absorption. However, experiments where organelle fractions were treated with Triton X-100 1 hr prior to the experiment give rather anomalous results, and it is suggested that Triton X-100 may have some inhibitory effect on the polyphenolase system, a view which will be considered in more detail elsewhere.

The phenolic compounds oxidized by poppy latex polyphenolase suggest the occurrence of both laccase and tyrosinase activities.[14] Unlike mushroom laccase, this enzyme will not oxidize phenols containing methoxy groups in the *ortho* position such as guaiacol and 2,6-dimethoxyphenol, and it is perhaps not surprising, therefore, that neither (±)-reticuline nor salutaridinol are oxidized despite the addition of ATP to the reaction media containing salutaridinol plus organelles. Leete[15] in a recent review article has suggested that salutaridinol would have to be activated at the C-7 hydroxyl group prior to displacement of this group, and since Rapoport[16] has indicated a requirement for ATP in the conversion of reticuline to thebaine by young 5-day-old seedlings, Leete postulates the initial formation of the 7-phosphate and its subsequent displacement by an enzyme functional group such as –SH. Should this theory prove correct, it would seem unlikely that polyphenolase is the participating enzyme.

EXPERIMENTAL

Preparation of fresh latex. For all experiments, the opium poppy *Papaver somniferum* var. Halle[17] was used. Latex was collected fresh into tubes at 0° containing a 0·5 M mannitol/0·1 M phosphate buffer pH 7·0 such that the final concentration in each tube was latex/mannitol–phosphate buffer (1 : 1). This was used as the enzyme in all experiments involving whole latex

Preparation of the 1000-*g organelle fraction.* Made by centrifuging for 30 min at 1000 *g* since this caused less damage to the organelles than using 3000 *g* for 5 min.[1] The pellet obtained was once re-suspended in mannitol buffer and re-centrifuged at 1000 *g* for 30 min. The resultant pellet was re-suspended in 0·5 M mannitol/phosphate buffer pH 7·0 such that the concentration of the organelles in buffer was similar to that in the original latex/buffer solution. This solution was used as the enzyme preparation in experiments involving the 1000-*g* organelle fraction.

Dialysed samples of the 1000-*g fraction.* A preparation of the 1000-*g* fraction of the latex was made and re-suspended in mannitol/phosphate buffer containing 5 mM sodium metabisulphite. Samples were sonicated for 2 min and then dialysed for 12 hr against three changes of 3 l. of 0·05 M phosphate buffer pH 5·6 containing 5 mM metabisulphite. This was then followed by a further three changes of buffer without the addition of sodium metabisulphite. Samples were adjusted to pH 8 0 with 0·5 M NaOH prior to use.

Manometric estimations. A conventional Warburg apparatus was used for the determination of oxygen absorption by poppy latex. In all experiments, 0·2 ml of a 1% solution of the substrate was placed in the side arm of the manometric flasks and 0·2 ml of the latex enzyme solution in the main compartment (except

[14] B. R. BROWN, in *Oxidative Coupling of Phenols* (edited by W. I. TAYLOR and A. R. BATTERSBY), p. 119, Arnold Press, London; Dekker, New York.
[15] E. LEETE, *Adv in Enzymology* **32**, 373 (1969).
[16] H. RAPOPORT, *International Symposium on the Biochemistry and Physiology of Alkaloids*, 3rd Halle (Saale) 24–27 June 1965. Abhandl.Deut.Akad., Wiss, Berlin 3, (1966).
[17] J. W. FAIRBAIRN and S. EL-MASRY, *Phytochem.* **7**, 181 (1968).

in experiments where organelle breaking treatments were used, and in these experiments the latex was placed in the side arm). The contents of the flasks were made up to a total volume of 2 ml with buffer solution. Any CO_2 produced during the reaction was absorbed by 2 N KOH in the centre walls of the manometric flasks. All other additives, KCN 10^{-3} M and 10^{-4} diethyldithiocarbamate 10^{-3} M and 10^{-4} M, 0·1 % Triton X-100 and 0·05 M ATP were added as volumes of 0·1 ml. The inhibitors KCN and diethyldithiocarbamate were put in the side arm of the manometric flasks with the substrate; the other substances were put in the main compartment. In some experiments, latex enzyme solutions were pre-treated with Triton X-100 at a concentration of 0·1 % 16 hr prior to the commencement of the experiment, and in other experiments samples were sonicated for 2 min prior to use. Where freeze/thaw techniques were used, latex samples were frozen six times at −20° followed by thawing.

In experiments where fresh latex was used, all solutions of substrates, inhibitors and detergents were made up in the mannitol/phosphate buffer to ensure that there was no variation in the osmotic pressure of the total solution in the manometric vessel.

Reagents. Buffer solutions: mannitol/phosphate buffer consisted of a 0·1 M NaH_2PO_4/NaOH solution at pH 7·0 to which mannitol was added to give a solution of 0·5 M with respect to mannitol. Salutaridine was kindly supplied by Prof. G. W. Kirby of the University of Technology, Loughborough, and from this salutaridinol was prepared using the method of Barton.[18] (±)-Reticuline was kindly supplied by Dr. S. Teitel of the Chemical Research Department, Hoffman-La Roche Inc., New Jersey, U.S.A.

[18] D. H. R. Barton, G. W. Kirby, M. Steglich, G. M. Thomas, A. R. Battersby, T. A. Dobson and H. Ramnz, *J. Chem. Soc.* 2431 (1965).