Phytochemistry, 1968, Vol. 7, pp. 2117 to 2121. Pergamon Press. Printed in England

THE ALKALOIDS OF *PAPAVER SOMNIFERUM* L.—VIII.

ORGANELLE ACTIVITY OF THE ISOLATED LATEX

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(*Received* 23 *April* 1968)

Abstract—Fractions prepared by differential centrifugation of whole or diluted latex have been examined. High rates of gaseous exchange and of phosphorylation were associated with a fraction expected to contain mitochondria; gaseous exchange possibly due to phenol oxidases was also observed in other fractions. There was some evidence that, in the heavy fractions, radioactive tyrosine was incorporated into certain alkaloids but not into thebaine or morphine. Alkaloids were mainly present in these heavy fractions and not in the supernatants. It is suggested that significant numbers of active mitochondria occur in the expelled latex and that alkaloid biosynthesis is partly associated with heavy particles. After formation, the alkaloids remain attached to the parent particles for further metabolic changes to be effected.

INTRODUCTION

OUR PREVIOUS work[1] has confirmed that the isolated latex of poppy can synthesize morphine, and other alkaloids, from the comparatively simple precursor dihydroxyphenylalanine. It seems probable that this many stage synthesis is carried out in discrete organelles and that expelled latex is therefore cytoplasmic rather than vacuolar. Preliminary electron microscopical studies of poppy latex vessels *in situ* by Sarkany *et al.*[2] (in seedlings), Dickenson[3] (in the young pericarp) and Schulze *et al.*[4] (in the roots) indicate the presence of endoplasmic reticulum, nuclei, mitochondria, Frey-Wyssling particles and other spherical bodies. Isolated latex, however, is the milky juice expelled from the latex vessels when the plant is incised and this expelled latex may be mainly vacuolar sap. In fact Meissner implied this in his study of the (expelled) latices of 29 species of plants whose low protein contents suggest that 'at best they represent extremely diluted cytoplasmic systems'.[5] However, he did find evidence for the presence of ribosomes in poppy latex but was unable to purify them beyond a content of 18 per cent RNA because of phenolase activity. He also reported[6,7] a high rate of endogenous gaseous exchange in poppy latex in comparison with the latices of several *Euphorbia* species and showed that this was not due to the activity of micro-organisms. The isolated latex from the rubber tree (*Hevea brasiliensis*) has been shown to contain many of the enzymes character-

[1] J. W. FAIRBAIRN, M. DJOTE and A. PATERSON, *Phytochem.* **7**, 2111 (1968).
[2] S. SARKANY, L. FRIDVALSKY, B. LOVAS and G. VERZAR-PETRI, *3rd European Regional Conference on Electron Microscopy* (1964).
[3] P. B. DICKENSON, private communication.
[4] CH. SCHULZE, E. SCHNEPF and K. MOTHES, *Flora. Abt. A.* **158**, 458 (1967).
[5] L. MEISSNER, *Flora. Abt. A.* **156**, 634 (1966).
[6] L. MEISSNER, *Flora. Abt. A.* **157**, 1 (1966).
[7] L. MEISSNER and K. MOTHES, *Phytochem.* **3**, 1 (1964).

istic of a tissue homogenate[8] and the metabolism of glucose U-^{14}C indicated the probable presence of both glycolytic and Krebs cycle activity.[9] Electron-microscopical studies of the latex *in situ*[10] showed the presence of mitochondria, lutoids and other cell organelles though relatively few pass into the expelled latex.

The object of this present work was to examine expelled poppy latex cytologically for evidence of the presence of active mitochondria and to determine whether certain organelles are specially connected with alkaloid biosynthesis. The possibility that such organelles can be readily obtained free of cell-debris is an exciting one.

RESULTS

Mitochondrial Activity

(a) *Gaseous exchange in the whole latex.* The latex used in this experiment was stored at 1–4° for 2 hr between collection in the field and use in the laboratory. The rate of oxygen uptake with a mixture of ADP, malate, succinate, glucose and sucrose was measured using standard manometric techniques. Several experiments were carried out using varying volumes of latex and one in which malonate was added to inhibit succinic acid dehydrogenase activity. The results showed that the rate of oxygen uptake was proportional to the amount of latex used and that malonate reduced the rate by about 50 per cent.

(b) *Gaseous exchange and phosphate incorporation in latex fractions.* Differential centrifugation was used to fractionate the latex diluted with of 0·6 M sucrose and 0·05 M tricine (1:3 v/v); in these conditions the latex did not coagulate. Three fractions were obtained: *A*, the precipitate obtained between 0–4000 *g* consisting of nuclei and other debris of similar size, *B*, the precipitate obtained between 4000–40,000 *g* consisting mainly of mitochondria and lysosomes and finally *C*, the supernatant above 40,000 *g* which probably contains ribosomes and soluble enzymes. The oxygen uptake of each fraction was measured as before, and the metabolism of inorganic phosphate determined by adding $KH_2^{32}PO_4$ to the reaction mixture and then measuring the uptake of inorganic phosphate using reversed phase chromatography. The values of oxygen uptake and phosphate esterified obtained for each fraction are given in Table 1.

TABLE 1. GASEOUS EXCHANGE AND PHOSPHORYLATION ACTIVITIES OF THREE FRACTIONS OBTAINED FROM ISOLATED LATEX

	O_2 uptake μAO_2/hr/ml latex	Pi consumed μmoles/hr/ml latex	P/O ratio
Whole latex	26·7	—	—
Fraction A	11·1	6·3	0·57
Fraction B	10·3	24·8	2·38
Fraction C	5·7	1·9	0·33

Alkaloid Biosynthesis

Samples of *whole* latex spun at 100,000 *g* formed six zones (A–F) resembling approximately those reported by Moir for rubber latex.[11] High concentrations of alkaloids were only

[8] B. L. ARCHER and B. G. AUDLEY, *Advan. Enzymol.* **29**, 221 (1967).
[9] J. D'AUZAC, *Rev. Gen. Caoutchouc Plastique* **41**, 1831 (1964).
[10] P. B. DICKENSON, *Proc. Nat. Rubber Prod. Res. Ass.* (edited by L. MULLINGS) p. 52, Maclaren, London (1965).
[11] G. MOIR, *Nature, Lond.* **184**, 1626 (1959).

present in the two heaviest zones (E and F); quite surprisingly the clear serum near the top (zone B) contained no detectable alkaloid. (In the corresponding zone in *Hevea* latex enzymes are present.) Each zone was incubated with radioactive tyrosine and kept at room temperature for 24 hr. The alkaloids were then extracted and purified by partition between immiscible solvents and by paper chromatography.[12] In no instance was morphine, codeine or thebaine significantly radioactive. However, with zone E, a phenolic alkaloid of R_f value greater than that of morphine contained about 1 per cent of the fed radioactivity against 0·3 per cent in a control experiment in which acid methanol had been added as quickly as possible (1min) after the start of the reaction. A much smaller, though significant, increase in radioactivity in this alkaloid also occurred using zone F.

Work with *diluted* latex was only successful when the whole latex was deliberately coagulated by mixing it with sucrose solution of final concentration less than 0·3 M. The coagulated latex was re-homogenized and centrifuged at 1000 *g*, the coarse pellet removed, and the supernatant liquid centrifuged at 8000 *g* and, after removal of the second pellet, at 100,000 *g*. The second pellet was re-homogenized and centrifuged through sucrose gradients All fractions were incubated separately with radioactive tyrosine and added co-factors for 24 hr but only using the coarse pellet was there evidence of incorporation into alkaloids. The crude alkaloids contained 0·76 per cent of the fed radioactivity against 0·47 per cent in a control mixture. After paper chromatography, no significant incorporation was evident in the spots corresponding to morphine, codeine, narcotine and narceine, but that containing papaverine and thebaine combined showed an activity of 918 dpm (0·042 per cent of fed activity) compared with the corresponding alkaloids from the control mix of 288 dpm (0·013 per cent of fed activity).

Similar work with whole and diluted latex to which radioactive glucose had been added gave no evidence of incorporation into alkaloids.

DISCUSSION

Mitochondrial Activity

The results of the oxygen uptake experiments show there was a considerable rate of uptake (230 μl O_2/ml latex/hr) and that this was proportional to the volume of latex used. Furthermore the addition of malonate, a competitive inhibitor of succinic dehydrogenase, resulted in a 50 per cent reduction in uptake suggesting that at least part of the respiration was mediated by the activity of the Krebs cycle. The remaining gaseous exchange may be due to various phenol oxidases, such as those referred to by Meissner,[5] which would not be effected by the presence of malonate. The possible presence of these oxidases in Fractions A and C (Table 1) would explain their high rate of oxygen uptake. Fraction B shows high rates of uptake and of phosphorylation, yielding a P/O ratio characteristic of mitochondria. The differential centrifugation used to prepare this fraction would be expected to yield mitochondria, so that it seems reasonable to conclude from our data that the isolated latex contains significant amounts of viable mitochondria. The electron micrograph published by Schulze *et al.*[4] is consistent with this suggestion as numerous mitochondria occur in the lumen of the laticifer and would be expected to flow out on incision.

[12] J. W. FAIRBAIRN and G. WASSEL, *J. Pharm. Pharmacol.* **15**, 216T (1963).

Alkaloid Biosynthesis in Latex Fractions

Although it was not possible to establish the biosynthetic activity of the fractions as unequivocally as for the whole latex[1] there is evidence to show that tyrosine in converted by the heavier fractions into papaverine and thebaine, and into an unidentified phenolic alkaloid. The incorporation into the former of 0·042 per cent of fed activity is significantly higher than for the control (0·013 per cent) and for contamination with unchanged tyrosine.[1] There was no evidence for incorporation into morphine, thebaine, narcotine and narceine and it may well be that the total synthesis of these involves more than one organelle. Quite surprisingly only traces of alkaloids occurred in the supernatant layers; the bulk was present in the heavy fractions. After synthesis, therefore, the alkaloids may remain bound to organelles and undergo the further metabolic changes already reported.[13] It is interesting to note that radioactive glucose was not incorporated into any alkaloid-like substance; this agrees with our earlier work on feeding radioactive glucose to the latex both *in vitro* and *in vivo*.

This work, together with our previous paper, show clearly that the expelled latex of the poppy plant is capable of sophisticated synthesis due to the presence of viable mitochondria and other interesting organelles.

MATERIALS AND METHODS

Latex was collected from our Turkish variety of poppy[14] 2–3 weeks after petal fall, as already described,[1] and stored at 0° until used. *Centrifugation* was carried out at 1–4°. For the mitochondrial work the latex was diluted with 3 times its volume of 0·6 M sucrose + 0·05 M tricine (pH 7·2). Fraction A was obtained by centrifuging at 4000 ***g*** for 10 min, the resulting precipitate being suspended in 0·6 M sucrose and 0·05 M tricine for further investigation. The supernatant was centrifuged at 40,000 ***g*** for 5 min and the resulting precipitate suspended in the sucrose/tricine medium to give fraction B. The supernatant resulting from this final centrifugation was fraction C. For the biosynthetic work *whole latex* was spun at 100,000 ***g*** for 45 min, when 6 zones were formed: A, the upper, was a sticky white cream, B a clear yellow serum, C a pinkish yellow band, D a bright yellow band, E a stiff pinkish grey gel, F a stiff gel more orange in colour than E. *Diluted latex* was deliberately coagulated by diluting with 0·5 M sucrose in phosphate buffer at pH 7·2 then gradually adding 0·2 M sucrose to bring the final concentration to 0·25 M. The coagulated latex was re-homogenized and centrifuged at about 4000 ***g*** to produce a coarse pellet. The supernatant was centrifuged at increasing speeds to produce further precipitate. Further fractions were also obtained by using sucrose gradients. Each fraction (from while and diluted latex) was incubated at room temperature with radioactive precursor and added co-factors ($MgCl_2$ 1 mM, Adenosine triphosphate 10 mM, Coenzyme A 1 mM, NAD 1 mM, glutathione 1 mM, sucrose 0·5 M, pH 7·2). After 24 hr the reaction was stopped by adding acid, the alkaloids extracted in the usual way and purified by band chromatography on paper buffered at pH 9·0 (mobile phase, *n*-butanol saturated with water). For each fraction a second identically prepared mix was used as a control by adding acid almost immediately after preparation (1 min or less).

Oxygen uptake was measured at 30° by standard manometric techniques.[15] The main body of the Warburg flask contained 2·4 ml of reaction mixture which consisted of 1200 μmoles of sucrose, 100 μmoles tricine, 2 μmoles of ADP, 100 μmoles malate, 100 μmoles

[13] J. W. FAIRBAIRN and S. EL MASRY, *Phytochem.* **6**, 499 (1967); **7**, 181 (1968).
[14] J. W. FAIRBAIRN and G. WASSEL, *Phytochem.* **3**, 253 (1964).
[15] W. W. UMBREIT, R. H. BURRIS and J. F. STAUFFER, *Manometric Techniques*, Burgess, New York (1964).

succinate (plus 200 μmoles of malonate where appropriate), 50 μmoles glucose, 50 μmoles $MgSO_4$, 50 μmoles KH_2PO_4 and 1 mg hexokinase all adjusted to pH = 7·2. The side arm contained 0·6 ml of pure or diluted latex.

Phosphate esterification was measured by adding 200 μc of $KH_2^{32}PO_4$ to the reaction mixture in the Warburg flasks and then measuring the percentage of ^{32}P esterified by using the method of Hagihara and Lardy.[16]

[16] B. HAGIHARA and H. A. LARDY, *J. Biol. Chem.* **235**, 889 (1960).