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

BIOSYNTHETIC ACTIVITY OF THE ISOLATED LATEX

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Abstract—Earlier work on the biosynthesis of alkaloids by poppy latex has been criticised on the grounds that the final products owed their radioactivity to contamination with the precursor. In the present work, not only has the extent of contamination been studied, but radioactive 3,4-dihydroxyphenylalanine has been found to be much more efficiently incorporated into the alkaloids than is tyrosine or glucose. It was therefore possible to isolate substantial quantities of radioactive morphine whose purity was established by radioactive measurements after re-crystallisation, formation of diacetyl derivative and picrate, and by radioisotope dilution techniques. Four other alkaloids were also shown to be radioactive though not with such certainty. The fact that such sophisticated syntheses take place in isolated latex indicates that the latter is cytoplasmic with adequate organelle activity.

SEVERAL workers[1-4] have claimed that poppy latex, when isolated from the plant, is still capable of synthesising alkaloids from simple precursors such as radioactive tyrosine and protein from radioactive glycine. Recently this work has been criticised on the grounds that the final products owed their radioactivity to contamination with the precursor[5] or to irreversible adsorption of the precursor by the protein.[6] Although such contamination may also occur in rubber latex,[7] there is nevertheless good evidence that acetate,[8] mevalonate and isopentenyl pyrophosphate[9] is converted into rubber in the latex of *Hevea brasiliensis*. Similarly acetate has been shown to be incorporated into triterpenes in the latex of certain *Euphorbia* species.[10] However, it does not necessarily follow that all latices are capable of such independent synthesis. In fact the work of Meissner[11] indicates there is considerable variation in the physiological activity of latices isolated from different plants. We decided, therefore, to investigate whether the original claims for the biosynthesis of morphine in isolated poppy latex from simple precursors such as tyrosine were in error due to contamination or not. Other simple precursors were also investigated with a view to producing more highly radioactive morphine so that its purity could be established unequivocally. If such a sophisticated synthesis[12] involving many enzyme systems does take place in the latex, it

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may well be carried out at particulate sites. The necessary organelles should therefore be present in the isolated latex and this could lead to a number of interesting developments.

RESULTS

Contamination of the Separated Alkaloids with Radioactive Tyrosine

Our method of isolating and estimating the alkaloids from the poppy latex[13] involves a preliminary fractionation between immiscible solvents, at which stage tyrosine would be expected to remain in the aqueous alkaline layer and the alkaloids pass into the organic solvent layer. Experiments with a mixture of the alkaloids present in poppy latex and radioactive tyrosine, however, showed that 0·34 per cent of the radioactivity was extracted into the organic solvent layer. When radioactive tyrosine was mixed with killed latex (15,000–90,000 dps/100 mg latex) and the extraction repeated, 0·25 to 0·54 per cent of radioactivity was present in the organic solvent. Subsequent chromatography,[13] however, greatly reduced this contamination. The eluted morphine contained 0·008–0·016 per cent of the original radioactivity; codeine (0·006 per cent) and thebaine (0·001 per cent) were less contaminated as they have R_f values which show greater differences from that of tyrosine than does morphine. Autoradiography also confirmed that contamination was extremely low. Similar experiments with radioactive glucose and killed latex also showed contamination rates as low as 0·014 per cent (morphine), 0·020 per cent (codeine) and 0·014 per cent (thebaine). Further details of this work have been published by Paterson.[14]

Relative Incorporation of Radioactive 3,4-*dihydroxyphenylalanine (DOPA), Glucose and Tyrosine*

To avoid osmotic or other damaging effects to the latex, the precursors were not added in solution. Instead, freshly drawn latex was immediately transferred to stoppered tubes containing a thin film of the solid precursor; the tubes were gently rotated and allowed to stand at room temperature for 24 hr. The alkaloids were then separated by distribution between aqueous and organic solvent layers and the radioactivity of the latter determined ("Total alkaloid fraction", Table 1). This was further fractionated into phenolic and non-phenolic alkaloids and the corresponding radioactivities determined. A control was also run by transferring the latex to a tube containing radioactive DOPA plus 0·1 N HCl which immediately coagulated and killed the latex. After 24 hr the contents were treated exactly as above. The results are given in Table 1

Radioactivity of the Morphine from the DOPA Fed Latex

The "phenolic alkaloids" fraction from the first three DOPA fed samples (Table 1), which represented 5·6 g latex, were combined and crude morphine precipitated from them. This was purified by preparative TLC and recrystallised twice from acid solution by the cautious addition of sodium bicarbonate solution. The identity and purity of the crystals were checked by melting point, u.v. spectrum and co-chromatography with authentic morphine in four solvent systems. The diacetyl derivative was prepared and recrystallised from methanol. Finally the picrate was formed and recrystallised till its melting point and u.v. spectrum were identical with those of authentic morphine picrate. To 5 mg of this picrate

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TABLE 1. RADIOACTIVITIES OF ALKALOIDAL FRACTIONS AFTER FEEDING D-GLUCOSE-^{14}C(U), L-TYROSINE-^{14}C(U) HYDROCHLORIDE AND DL-β(3,4-DIHYDROXYPHENYL)ALANINE-α-^{14}C (DOPA) TO ISOLATED LATEX

Precursor	Radioactivity fed	Radioactivity of fractions after 24 hr*		
		Total alkaloid	Phenolic alkaloids	Non-phenolic alkaloids
Glucose	2,754	Nil	n.d.	n.d.
Tyrosine	2,403	Nil	n.d.	n.d.
DOPA 1	2,834	70·5 (2·5%)	17·2 (0·6%)	11·0 (0·4%)
DOPA 2	2,804	161·0 (5·7%)	62·4 (2·2%)	49·9 (1·8%)
DOPA 3	7,144	333·4 (4·7%)	97·6 (1·4%)	77·4 (1·1%)
DOPA 4	14,100	1,340 (8·1%)	263·0 (1·8%)	270·2 (1·9%)
Control†	3,835	Nil	n.d.	n.d.

* All radioactivities calculated as dps/100 mg latex; percentage incorporations in brackets. (n.d. = not done)
† DOPA and 0·1 N HCl added at start.

TABLE 2. DETAILS OF RADIOACTIVE MORPHINE SEPARATED FROM THE DOPA FED LATEX*

	M.p.	$\lambda^{max\ and\ min}$ (in MeOH)	Radioactivity (dpm/mM × 10^{-2})
Morphine (1)	247° (decomp.)	max. 287 nm min. 264 nm	138
Morphine (2)	247° (decomp.)	max. 287 nm min. 264 nm	129
Diacetyl morphine	†170°	max. 282 nm min. 252 nm	153
Morphine picrate	‡160–162°	max. 255, 245 nm	166
Diluted picrate (dilution ½) (1)	‡161–163°	max. 255, 245 nm	55·3
(dilution ½) (2)	‡161–163°	max. 255, 245 nm	51·9

* See Table 1.
† Literature 169°–170°.
‡ Literature 161°.

10 mg of non-radioactive morphine picrate was added and the combined picrates recrystallized. Details of the specific activities of all these substances are given in Table 2.

Radioactivity of the Non-Phenolic Alkaloids

The non-phenolic alkaloids were separated from the DOPA fraction 4 (Table 1) by band chromatography on TLC. Although the quantities were too small for recrystallizations and isotope dilution techniques the u.v. curves were very similar to those of the pure alkaloids. The following were all radioactive: codeine, thebaine, papaverine and narcotine with activities ranging from 89 to 160 × 10^2 dpm/mM.

In Vivo *Feeding of Radioactive Precursors*

Parallel experiments were carried out with the same radioactive precursors fed to living plants *via* the phloem region of the pedicel.[15] The proportion of radioactive substances in the capsule latex from the tyrosine-fed plants rose to a maximum of about 8 per cent in agreement with earlier findings.[16] The maximum incorporation from the DOPA-fed plants was 0·5 per cent and that from the glucose-fed plants 0·34 per cent. However, in the DOPA-fed plants the percentage of radioactive substances reaching the capsule latex which was converted into morphine was about twice that of the tyrosine-fed plants. No radioactive alkaloids were produced as a result of glucose feeding.

DISCUSSION

This work has shown that in *in vitro* experiments contamination of poppy alkaloids with added precursor can take place even though a fairly elaborate analytical procedure for separating the two fractions is carried out. The percentage contamination of morphine, codeine and thebaine with radioactive tyrosine was from 0·001 to 0·016 per cent so that incorporation rates of this order are ruled out. Earlier work gave figures from 0·07 per cent[1] to 0·14 per cent (calculated from the highest figure[4]) so that it was possible that genuine incorporation had taken place. The data from the contamination experiments, however, refer to unchanged precursor; a more serious problem would arise if contamination was due to partially transformed precursors whose physical properties more closely resemble those of the alkaloids. For example it has been shown[17] that radioactive tyrosine fed to peyote is readily converted into tyramine and *N*-methyltyramine. Such phenolic amines would have similar solubility characteristics to those of the alkaloids, especially morphine, and therefore represent a more serious contamination hazard. Using killed latex (Control; Table 1), no detectable radioactivity passed into the "total alkaloids". With the live latex, however, significant amounts of radioactivity were found in the alkaloid fraction. For instance, DOPA samples 1, 2 and 3 (Table 1) represented 5·6 g of latex, the radioactivity of "phenolic alkaloids" in which was actually 3296 dps. Assuming that 5 per cent morphine was present in the original latex, this fraction should contain 280 mg morphine of radioactivity 166×10^2 dpm/mM (Table 2). This radioactivity (representing a total of 272 cps) is only 8 per cent of the total radioactivity of this phenolic alkaloid fraction. Examination showed that a small amount of the remaining 92 per cent was due to other phenolic alkaloids e.g. reticuline, but the major part was due to unknown phenolic substances with solubility properties similar to those of morphine. These facts underline the necessity of carefully purifying the morphine, preparing crystalline derivatives and testing these by isotope dilution techniques. The results in Table 2 show clearly that the morphine is radioactive, and that the specific activity is retained after recrystallization and formation of the picrate or the diacetyl derivative, with a slight increase in specific activity in the latter two compounds. The radioisotope dilution technique confirms that the morphine is genuinely radioactive. The minor alkaloids, codeine, thebaine, narcotine and papaverine, were not completely purified, but were separated chromatographically and had u.v. spectra characteristic of authentic samples. All were significantly radioactive and this confirms that a biosynthesis of alkaloids takes place in the isolated latex.

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[16] J. W. FAIRBAIRN and S. EL-MASRY, *Phytochem.* **6**, 499 (1967).
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Relative Incorporation of DOPA, Glucose and Tyrosine

Table 1 shows clearly that DOPA is much more readily metabolised into alkaloid-like substances than is tyrosine or glucose. In previous experiments with tyrosine[4] about 90,000 cps/100 mg latex were used and some incorporation into morphine took place. In the present experiments only about 1/40th of this activity was used and, not surprisingly, no incorporation was detectable. With DOPA of similar low concentrations, however, quite significant incorporation into morphine took place. Since DOPA is one biosynthetic step nearer morphine than is tyrosine[11] the poor incorporation of the latter may be due to low phenolase (tyrosine oxidase) activity in the latex. Jindra *et al.*[18] have shown that phenolase activity is very low in seedlings, roots and leaves of poppy using tyrosine, although with DOPA as substrate activity is usually well marked. Meissner[6] implies that phenolases are present in the latex but gives no evidence for tyrosine oxidase activity. Our *in vivo* experiments suggest that DOPA is less readily translocated from the pedicel phloem region to the capsule latex than is tyrosine, although its incorporation rate into morphine in the latex is superior to that of tyrosine. Glucose does not seem to be incorporated into alkaloids in the isolated latex; the results we report in Table 1 have been confirmed using much larger concentrations of radioactive glucose (90,000 dps/100 mg latex)[14] but no trace of radioactivity was found in the alkaloids. This again suggests that the enzymes necessary for conversion of glucose to tyrosine or DOPA are absent from the latex.

Similarly in isolated rubber latex glucose, fructose and sucrose are very poorly incorporated into rubber, if at all.[7]

EXPERIMENTAL

Collection of the Latex

Latex was withdrawn from the Halle variety of poppy[19] 2–3 weeks after petal fall by decapitating the capsules and pipetting the expelled latex from the cut ends of the capsule and the stem. The latex was rapidly transferred to stoppered tubes packed in ice and containing the radioactive precursor distributed as a thin film of solid near the bottom of the tube. (This was achieved by measuring into the tube the required volume of precursor solution and evaporating to dryness while slowly rotating the tube.) The tubes were gently rotated and stored at room temperature (20–24°) for 24 hr.

Extraction of the Alkaloids

To each tube, 10 ml 0·1 N HCl was added, vigorously shaken, filtered and washed with a further 10 ml of acid. The combined filtrate was adjusted to pH 8 to 8·5 with NH_3 solution and extracted with several portions of $CHCl_3$/isopropanol (3 : 1); the combined organic solvent extracts were dried (Na_2SO_4), filtered and made up to volume. This was the "total alkaloid" fraction of Table 1. This fraction was evaporated to dryness *in vacuo*, redissolved in 25 ml 0·1 N HCl, the pH adjusted to 12 with 2 N NaOH and the non-phenolic alkaloids extracted with several portions of $CHCl_3$. The combined $CHCl_3$ extracts were washed with 0·1 N NaOH, dried (Na_2SO_4), evaporated to dryness and redissolved in ethanol to volume, to give the "non-phenolic alkaloids" (Table 1). The alkaline aqueous layer was adjusted to pH 8–8·5 by addition of phosphoric acid, extracted with $CHCl_3$/isopropanol and treated as before to produce the "phenolic alkaloid fraction".

Radioactivities of the Morphine and the Other Alkaloids

The combined ethanolic solutions of the phenolic alkaloids fraction of DOPA-fed samples 1–3 (Table 1) was evaporated to dryness, redissolved in 0·1 N HCl and 5% $NaHCO_3$ solution cautiously added until a slight turbidity occurred. The solution was then stored in the refrigerator overnight when about 130 mg of crude morphine crystals separated out. A suitable portion was further purified by TLC band chromatography (System I[19]) and recrystallised as above. The identity and purity of these crystals were checked by melting point (Koffler), u.v. spectrum and co-chromatography with authentic morphine in four TLC systems (Systems

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[19] J. W. FAIRBAIRN and S. EL-MASRY, *Phytochem.* **7**, 181 (1968).

I II[19] and silica gel G with xylene/ethyl methyl ketone/methanol/diethylamine (20:20:3:1) and silica gel G with xylene/acetone/methanol/0·880 ammonia (20:20:3:1)). This sample was recrystallized once more in the same manner as before, the two batches are Morphine (1) and (2) of Table 2.

The *picrate* was formed by dissolving 20 mg of the purified morphine in warm ethanol and adding to this saturated solution of picric acid, dropwise, till a turbidity appeared. After standing in the refrigerator overnight 17 mg of crystals were formed; these were recrystallized from ethanol, dried, their melting point and u.v. spectrum determined, and were found to agree with those of authentic morphine picrate.

Diacetyl morphine was prepared by refluxing 20 mg of the purified morphine and 20 mg fused sodium acetate in 1 ml acetic anhydride for 30 min on a water bath. The acetic anhydride was evaporated off *in vacuo* and the residue washed out with dilute $NaHCO_3$ solution and the insoluble acetyl morphine filtered off and washed. This product was recrystallized from methanol and yielded 16 mg of crystals.

The non-phenolic alkaloids were separated from the appropriate fraction of DOPA sample 4 (Table 1) by band chromatography (System II[18]). The eluted alkaloids were dissolved in 0·1 N HCl and their u.v. spectra determined. These were practically identical with those of the pure alkaloids, as has already been shown for papaverine and narcotine when chromatographed in similar circumstances.[19] The specific activities were: codeine 160×10^2 dpm/mM, thebaine 89×10^2, narcotine 130×10^2 and papaverine 160×10^2.

All radioactivities were determined with a Nuclear Chicago Gas Flow Counter Model 1105 Spectro Shield (background count, less than 2 per min), as scintillation counting was not convenient with the picrates because of intense quenching effects.

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