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BOUND FORMS OF ALKALOIDS IN *PAPAVER SOMNIFERUM* AND *P. BRACTEATUM*

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Key Word Index—*Papaver somniferum*; *P. bracteatum*; Papaveraceae; poppy capsule; bound alkaloids; morphine metabolism; polysaccharides.

Abstract—The polysaccharide fraction of the pericarp and seed of *Papaver somniferum* were shown to contain bound forms of morphine which were derived from radioactive morphine fed to living plants. Bound forms of codeine, thebaine and some unidentified alkaloid-like compounds were also detected in the pericarp and bound thebaine occurred in the pericarp of *Papaver bracteatum*. The complexity and molecular weight of the bound alkaloids seemed to increase during ripening, and it is suggested that these substances represent transitional forms in the metabolism and translocation of morphine from latex to seed.

INTRODUCTION

It is now well established that morphine in *Papaver somniferum* is not an 'end-product' substance, but at certain stages of development is rapidly metabolized, especially in the capsule [1–4]. The problem arising, therefore, is, what are the metabolites and have they a function in the plant? Morphine *N*-oxide [5] and normorphine [3] have both been shown to be metabolites of morphine and Miller *et al.* [3] have therefore suggested that the known sequence in the plant, thebaine to codeine, codeine to morphine and morphine to normorphine, which involves successive demethylation, indicates that these alkaloids act as methylating agents. However, a series of papers by Fairbairn and co-workers indicate that morphine is built up into complex 'bound' forms in the latex; these pass into other tissues of the pericarp and some are translocated to the developing ovules, in which no free morphine could be detected by normal chemical methods. Thus in work involving 69 plants over two seasons, in which radioactive morphine was fed to the stem below developing capsules, of the radioactivity in the methanol extracts of the capsules, 18% was in the latex, 58% in the pericarp and 24% in the ovules [4]. During development of the ovules into ripe seeds there seems to be a change from methanol-soluble to methanol-insoluble, but water-soluble, radioactive metabolites [6]. Vigorous hydrolysis, or germination of the seeds, led to the production of radioactive alkaloid-like compounds, one of which was said to be codeine. There was also some evidence that these alkaloid metabolites were necessary for the early stages of germination [7]. Later, Wold and co-workers [8] showed that ripe capsules contained morphine, codeine and other Dragendorff-positive substances bound to polysaccharides. Two types of binding occurred; one was easily hydrolysed (NH_4Cl) whereas the other required vigorous hydrolysis (HCl). As these substances might well represent part of the metabolic story from the morphine and other alkaloids in the latex to the metabolites in the seed, we decided to investigate their occurrence in the unripe capsule as well as in the ripe and in the seeds. Wold was not very successful using unripe capsules. Furthermore, he dialysed his aqueous extract and examined the retenate only: we decided to look also at the smaller molecules in the dialysate. In addition, since it cannot be decided from the earlier work whether the alkaloids are synthesized before being attached to the polysaccharides or whether some precursor is attached and later converted into alkaloid, we have used radioactive alkaloids in our investigation.

RESULTS

Unripe pericarp

Tritiated morphine (1110×10^6 dpm) was fed to the stems [2] about 4 cm below the developing capsules of 96 plants, 4 days after petal opening. Three days later, the capsules were severed from the stems and the latex allowed to drain away. Ovules were separated from the pericarp which was dried and powdered. The dry weight of the pericarp was 128 g.

The powder was successively extracted in a Soxhlet apparatus with petrol (bp 60–80°), chloroform and methanol, thus removing lipids, free alkaloids and the methanol-soluble bound forms referred to earlier. The residue was dialysed against water and the retenate (R, 6.6 g) and dialysate (D, 5.6 g) were freeze-dried. Each fraction was hydrolysed further under mild conditions using M NH_4Cl, basified and extracted with $CHCl_3$–isopropanol (3:1) to produce two organic solutions R_1 and D_1 from the retenate solids and the dialysate solids, respectively. The aqueous solutions were acidified to 2 M HCl and vigorously hydrolysed, basified, extracted with $CHCl_3$–isopropanol (3:1) to produce two further organic solutions R_2 and D_2. The radioactivity in each fraction is shown in Table 1.

Table 1. Radioactivities (dpm × 10^{-3}) of the alkaloid fractions of the retenate (R_1 weakly bound, R_2 strongly bound) and the dialysate (D_1 weakly bound, D_2 strongly bound) after feeding 1110×10^6 dpm [^{3}H]-morphine via the stem below the capsules, 4 days after petal opening

(a) Unripe pericarp			
R_1	26.88	D_1	377.23
R_2	3.37	D_2	400.23
$R_1 + R_2$	30.25	$D_1 + D_2$	777.77
(b) Ripe pericarp			
R_1	365.65	D_1	1758.82
R_2	802.29	D_2	508.57
$R_1 + R_2$	1167.94	$D_1 + D_2$	2267.39
(c) Seeds			
R_1	6.1	D_1	11.9
R_2	6.3	D_2	2.3
$R_1 + R_2$	12.4	$D_1 + D_2$	14.2

(a) Unripe pericarp, collected 3 days later. (b) Ripe pericarp and (c) seeds, collected 21 days later.

Ripe pericarp

Triturated morphine (1110×10^6 dpm) was fed to 92 plants 4 days after petal opening and the capsules were collected when ripe 21 days later. The seeds (232 g) were separated from the pericarps which were dried and powdered (dry weight 139 g). The latter was treated in exactly the same way as the unripe pericarp and the results are also recorded in Table 1. Retenate, 5.4 g; dialysate 14.0 g.

Seeds

Before working on the 232 g of seed from the radioactively fed capsules, we first checked whether the method used by Wold and us in the pericarp would yield meaningful results on the seeds. Accordingly 111 g of 'cold' seeds were treated exactly as already described. The various hydrolysates were examined by TLC for the presence of alkaloids; morphine (0.3 mg) was identified only in the D_1 hydrolysate. A further five substances, each giving a positive reaction to Dragendorff's and to Ninhydrin reagents, were found in the other fractions.

The 232 g of seeds from the [^{3}H]-morphine fed plants were then fractionated as already described: the amount of radioactivity in each fraction was considerably less than that found in the pericarp (Table 1). Retenate 2.3 g; dialysate 3.9 g.

Papaver bracteatum *pericarp.* A sample of dried pericarp (96.7 g) was extracted as already described and the dialysate and retenate were hydrolysed with NH_4Cl solution. The dialysate yielded 5.8 mg thebaine and the retenate 0.93 mg; its identity was established by TLC and mass spectral characters. It was not possible to test for strongly bound forms as thebaine rapidly decomposes when boiled in strong acids.

Separation of the alkaloids. Each of the $CHCl_3$–isopropanol solutions was made up to a known volume and the amounts of morphine, codeine and thebaine present determined. The remainder was evaporated to a small volume and the alkaloids separated by preparative TLC (system 1, see methods). The radioactivities of the eluates were determined and as morphine alone was radioactive it was crystallized from aqueous MeOH to constant radioactivity (Table 2). Other Dragendorff-positive substances were eluted and those which were radioactive were further purified by using successively two additional TLC systems.

DISCUSSION

We have confirmed the presence of the water-soluble, polysaccharide bound forms of morphine and codeine first reported by Wold, and have also shown that similar

Table 2. Alkaloids found in the hydrolysates R_1, R_2, D_1 and D_2 referred to in Table 1.

Fraction	Alkaloid	Quant.* (mg)	Radioactivity† (dmp × 10^{-3}) Specific (per mg)	Radioactivity† (dmp × 10^{-3}) Total	Mass spectral data *m/e* of important ions
Unripe pericarp					
R_1	morphine	4.6	5.82	26.7	285 (100%), 256, 162
D_1	morphine	14.6	6.70	97.8	285 (100%), 256, 162
	codeine	4.9	0		299 (100%), 284, 270, 162
	thebaine	3.4	0		311 (100%), 310, 255, 254
Ripe pericarp					
R_1	morphine	2.12	17.10	36.3	285 (100%), 256, 162
R_2	morphine	0.23	47.90	11.0	285 (100%), 256, 162
D_1	morphine	2.27	83.46	189.5	285 (100%), 256, 162
	codeine	0.1	0		299 (100%), 284, 270, 162
D_2	morphine	0.66	120.00	79.2	285 (100%), 256, 162

* Corrected for losses during hydrolysis; see Experimental.
† Crystallized to constant activity; details in Experimental.

complexes with thebaine occur. Like Wold, we dialysed the aqueous extracts containing these polysaccharides but examined the dialysate as well as the retenate. The dialysate behaved exactly as the retenate in releasing alkaloids after mild treatment for the weakly bound, and vigorous treatment for the strongly bound alkaloids. It is reasonable to conclude, therefore, that it contains polysaccharides or oligosaccharides of MW < 12000 similar to the larger polysaccharides retained by the dialysis membrane. Of more importance, we found that in the unripe pericarp a much higher amount of bound morphine occurred in the dialysate (14.6 mg) than in the retenate (4.6 mg) so that the total bound morphine in the unripe pericarp (19.2 mg) was more than that in the ripe (5.3 mg) (Table 2). This is in contrast to Wold's results, which were based on retenate only, and we shall comment on this later.

It seems clear that these complexes are directly formed from radioactive morphine fed to the pedicel which, on the basis of previous work [4, 6], would be translocated to latex and then exported from it. The complexes are therefore true metabolites formed *after* morphine has been synthesized in the latex, and are not themselves sites of synthesis from alkaloid precursors.

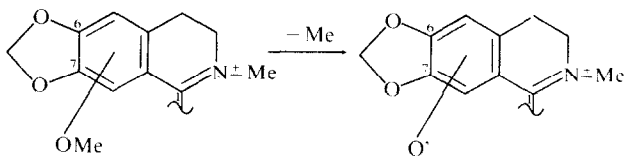
Previous reports on the presence of bound forms in the seeds have also been confirmed. In the progression from morphine in the latex to bound forms in the seed, there seems to be an increase in molecular complexity and size. Thus, in the unripe pericarp only weakly bound forms occur, whereas in the ripe pericarp and seeds strongly bound forms also occur (Tables 1 and 2). In addition, an increasing proportion of the radioactivity occurs in the retenate (MW > 12,000) during ripening. Thus it can be calculated from the results given in Table 1 that the ratio of the radioactivities in the retenate ($R_1 + R_2$) to that in the dialysate ($D_1 + D_2$) increases from the unripe pericarp 0.04, to the ripe pericarp 0.52, to the seeds 0.87. This progression towards larger molecules during ripening might indicate that in the seeds even larger molecules occur which are insoluble in water and would explain our comparatively low yield of radioactive substances from the seed. In fact Grove *et al.* [9] failed to release any alkaloid from the water-soluble fraction of the seed after vigorous hydrolysis, but they did detect bound morphine and codeine in the seed residue after exhausting with solvent and with water. Their conclusion was that 'any bound alkaloids present in the seed are not in a water-soluble form'.

The situation is therefore complicated! This present paper is deliberately concerned with the methanol-insoluble, water-soluble fraction only. Previous work on the methanol-soluble metabolites [6] showed that there was a marked decrease in these as the capsule ripens. These presumably smaller molecules are converted into the water-soluble (methanol-insoluble) bound forms and later they seem to be converted into very strongly bound forms then to water-insoluble forms in the seeds. These, methanol-insoluble, water-soluble forms are readily extracted, and therefore not membrane-bound and may well represent mobile transitional metabolites which will decrease during the ripening process. Thus the absolute amount of the bound morphine in the unripe pericarp (19.2 mg in 128 g) is about four times that in the ripe pericarp (5.3 mg in 138 g) (Table 2). During this time, the specific activity rises from *ca* 7×10^3 dpm per mg in the unripe to an average of more than 100×10^3 dpm per mg in the ripe capsule. This also supports the above hypothesis since, in the unripe capsule 3 days after feeding, the newly formed complexes are well diluted with unlabelled material; as time proceeds and the complexes themselves are further metabolized and replaced by further newly formed radioactive ones the dilution factor will be less and the specific activity will rise.

Besides bound morphine, weakly bound forms of codeine and thebaine were found (Table 2). They were not radioactive as would be expected from the fact that the plant does not convert morphine back into these alkaloids [10]. It also indicates that during the manipulative programme there has been no transfer of ^3H from morphine to the other closely related alkaloids. We have also shown that weakly bound forms of thebaine occur in the polysaccharide fraction of *P. bracteatum* pericarp. These are almost certainly the same as the bound forms referred to by Fairbairn and Helliwell [11], who showed that significant amounts occur in the young pericarps but decrease as the capsule ripens. Since thebaine, unlike codeine and morphine, has no hydroxy groups the bond with the polysaccharide cannot be glycosidic; it is also unlikely to be a –C–C bond. All these alkaloids may therefore have an ionic bond between the basic nitrogen and the glucuronic acid groups of the polysaccharide, as suggested by Wold [5].

However, the weakly and firmly bound morphine in the unripe pericarp (124.5×10^3 dpm) only represents *ca* 15.4% of the radioactivity in the retenate and dialysate (808×10^3 dpm, Tables 1 and 2) and only 9.2% in the ripe pericarp. Some of the remaining radioactivity may be in the bound alkaloid-like substances (see below) but we found that they represented only a small proportion of the total radioactivity. The bulk of the water-soluble radioactive substances are probably too polar to be extracted into $CHCl_3$ by our methods. The entire water-soluble fraction itself only represents *ca* 0.07% and 0.31% of the fed radioactivities in the unripe and ripe capsules, respectively. Obviously a large amount of the radioactive morphine fed to the pedicel will be lost due to lack of absorption, transfer to sites other than the capsule, etc. and we have found over many seasons that *ca* 15–20% only reaches the capsule. Our water-soluble radioactive substances may therefore represent up to *ca* 1.5% of the radioactivity in the capsule. This is still much less than the values of 33–37% found in the methanol-soluble fractions a few days after petal opening [4, 6] and 11% about 3 weeks later [6], thus giving further support to the suggestion that they are transitional forms.

A number of alkaloid-like substances bound in similar manner to the normal alkaloids were also found. They had similar solubility properties to the alkaloids, were Dragendorff- and Ninhydrin-positive, and were radioactive and therefore derived ultimately from the fed morphine. Similar substances have been found previously in the latex, pericarp and ovules [6, 7]; they may represent metabolites of the morphine which are bound in transitional water-soluble forms like the unchanged alkaloids. However, both the retenate and the dialysate of the unripe pericarp each contained a weakly bound form which were identical in three TLC systems. They were purified by three successive runs on TLC and yielded 0.2 and 0.25 mg with specific activities of 10.05 and 8.2 $\times 10^3$ dpm per mg, respectively. The MS showed peaks at *m/e* 220, 205, 122 (100%). In the Papaveraceae alkaloids, *m/e* 220 is often associated with ion **1** (Scheme 1) which loses a methyl group to produce ion **2** at *m/e* 205. Ions **1** and



1 *m/e* 220 **2** *m/e* 205

Scheme 1

2 are characteristic of the fragmentation pattern of some 6,7-methylenedioxyphthalideisoquinolines (e.g. narcotine) and 6,7-methylenedioxy-1-benzyltetrahydroisoquinolines (e.g. macrantoline, macrantoridine), in which they arise from rupture of the C-1–C-9 band. These compounds give poor molecular ions in electron impact MS [12]. The UV spectra in MeOH showed maxima at 262 and 255 nm and these values were unaffected by the addition of NaOH indicating the absence of phenolic groups. There was no evidence for the presence of a lactone group, nor were the absorption maxima consistent with literature values for macrantoline or macrantoridine [13]. Thus the Dragendorff-positive substance seems clearly related to certain Papaveraceous alkaloids.

This present work fits in well with the general picture of the translocation of morphine metabolites, in particular, from latex to seed. The polysaccharide bound forms may represent a transitional stage during which there is an increase in complexity and molecular size. Probably quite complex forms eventually reach the seeds and we have earlier produced some evidence that here they exert an effect on seed viability [7]. Hitherto unpublished work adds some further support to this idea. Very occasionally we have found young capsules which showed no evidence of latex ('non-bleeders'). One of these was allowed to grow to maturity and, as expected, only small amounts of alkaloid (mainly thebaine) were present. On testing, only 9% of the seeds germinated in contrast to 94% in controls. Similar work on a Soma SV 74204 [14] strain of poppy, which produces only traces of alkaloid in the capsules, showed that 38% of seeds germinated against 94% in controls [15].

EXPERIMENTAL

Feeding procedure. Healthy outdoor plants of *P. somniferum* L. var. Halle 4/2 [7] and *P. bracteatum* Lindley var. Arya II were fed [1-*n*-^{3}H]-morphine hydrochloride (Radiochemical Centre, Amersham) by the cup method [2] to the stems just below developing capsules which were all at the same stage of development [1]. At appropriate intervals the capsules were harvested by severing from the stems and allowing the exuded latex to drain away. After slicing, they were dried at 50° in an oven with a forced draught and the ovules or seeds removed, leaving only the pericarps.

Extraction. Dried pericarp or seeds were ground and Soxhlet-extracted successively with petrol (bp 60–80°), $CHCl_3$ and MeOH, 8 hr each. The residue was dried at 50° and stirred with H_2O (2 × 2 l.) at 50° for 24 hr. After filtering, the filtrate was concd to *ca* 200 ml *in vacuo* and dialysed against H_2O (2 × 2 l.) for 24 hr. (Medicell International dialysis tubing, size 5–24/32″, claimed to retain molecules of MW > 12 000). The dialysate and retenate were freeze-dried separately and used for further work.

Hydrolysis of the bound forms. (a) Mild hydrolysis; refluxed in M NH_4Cl for 1 hr at 100°. (b) Vigorous hydrolysis; refluxed in 2M HCl for 1 hr at 100°. Since it was likely that morphine would decompose and that the tritium might exchange with hydrogen the effect of these conditions on [^{3}H]-morphine was determined. The mild hydrolysis conditions led to 15% loss of morphine and 17.5% loss of radioactivity; these two values are within experimental error so that we assumed in our calculations a 15% loss of morphine with no exchange of tritium. The vigorous hydrolysis conditions led to a loss of 49% morphine and 65% of radioactivity indicating some tritium exchange. Our calculations, therefore, took these two figures into account separately. No similar corrections were made for codeine and thebaine as no arguments have been based on the quantities produced; neither alkaloids were radioactive.

TLC. Adsorbents: system 1, Si gel F254; systems 2 and 3, cellulose F254. Solvents: system 1, Me_2CO–toluene–EtOH–25% NH_3 (40:40:12:2.5); system 2, *n*-BuOH–HOAC–H_2O (35:3:10); system 3, PrOH–EtOAc–H_2O (4:3:2). R_f in system 1, morphine 0.25, codeine, 0.47, thebaine 0.67.

Quantitative determination of morphine, codeine and thebaine was based on the HPLC method of the U.N. Narcotics Laboratory [16]. Column: spherisorb silica 5 μm. Mobile phase; *n*-hexane (290 ml) ($MeOH$–$CHCl_3$–Et_2NH, 30:10:1) (37 ml).

Mass spectrometry. Probe 70 eV at 200°. Lit. [17, 18]. Morphine 285 (100%), 256, 162; codeine 299 (100%), 284, 270, 162; thebaine 311 (100%), 310, 255, 254.

Specific activity of morphine (see Table 2). Recrystallized from aq. MeOH; sp. activities in dpm × 10^3 per mg; (a) Unripe pericarp R_1 6.36, 5.82. D_1 7.43, 6.70. (b) Ripe pericarp: R_1 21.74, 18.09, 17.10. R_2 58.36, 48.15, 47.90. D_1 105.60, 83.46. D_2 161.67, 120.00.

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