Phytochemistry, 1973, Vol. 12, pp. 597 to 603. Pergamon Press. Printed in England.

MORPHINE METABOLISM AND NORMORPHINE IN *PAPAVER SOMNIFERUM*

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(*Received* 15 *September* 1972. *Accepted* 5 *October* 1972)

Key Word Index—*Papaver somniferum*; (Papaveraceae); morphine metabolism; normorphine; alkaloid function.

Abstract—Normorphine has been established as an active metabolite of morphine in *P. somniferum*. This was done by, (a) demonstrating the presence of normorphine throughout the life cycle of the plant, (b) finding normorphine-^{14}C after feeding morphine-^{14}C via the roots, and (c) exposing opium poppies to $^{14}CO_2$ under steady state conditions which led to morphine and normorphine of the same specific activity. Feeding normorphine-^{14}C showed that the *N*-demethylation step is irreversible. A sensitive procedure was developed for the detection of normorphine in the presence of large amounts of morphine; using this procedure, normorphine was found in raw opium. These results indicate that the major, if not the sole, morphine degradative pathway involves an initial demethylation to normorphine, which is subsequently degraded to non-alkaloidal metabolites. The high rates of turnover observed led to the conclusion that the morphine alkaloids do play an active metabolic role, perhaps as specific methylating agents.

INTRODUCTION

A SIGNIFICANT amount of evidence has been presented within the past decade which suggests that plant alkaloids play an active role in plant metabolic processes. In most cases, this evidence is still limited to a demonstration of general alkaloid turnover. Such is the case for morphine in the opium poppy,[1-3] nicotine in *Nicotiana Rustica*,[4] and the alkaloids of the hemlock. In one case, however, ricinine in *Ricinus Communis*, specific metabolites have been isolated, including *O*- and *N*-demethylricinine, and more extensive metabolism has been indicated.[5]

Our early biosynthetic studies with *P. somniferum* suggested that both thebaine and codeine are dynamic intermediates in the plant's metabolism.[6] Results from several $^{14}CO_2$ exposures indicated that the rate of synthesis of these morphine precursors required that they be metabolically active, since the concentration of neither increased to any extent. More recent evidence for morphine turnover includes marked daily variations in the concentrations of the morphine alkaloids,[1] and morphine-^{14}C feedings which suggest that morphine is broken down further to non-alkaloidal metabolites.[2,3]

Our present experiments were directed to the question of morphine metabolism and to the specifics of the actual degradative sequence. In particular, an attempt was made to study the possible role that normorphine might play in the metabolism of morphine. If, indeed, normorphine is present in the plant, and is involved as an active metabolite, one possible role for the morphine alkaloids might be as active methylating agents.

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[2] J. W. FAIRBAIRN, A. PATERSON and G. WASSEL, *Phytochem.* **3**, 577 (1964).
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[5] H. J. LEE and G. R. WALLER, *Phytochem.* **11**, 965 (1972).
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RESULTS

Detection of Normorphine in Opium Poppy Plants

It was recognized early in our study that the more traiditional methods of alkaloid extraction and analysis were inadequate to detect trace quantities of normorphine, especially in the presence of larger amounts of morphine. Due to its more polar nature, normorphine is more difficult to extract from aqueous solution than the normally encountered opium alkaloids. As the free base, it is also relatively unstable in TLC and GLC, making purification and detection of small quantities more difficult. When labeled normorphine was extracted in the presence of plant material with a butanol–benzene organic phase and a slightly alkaline aqeuous phase, only 50% was recovered in the organic layer. The same procedure gives a nearly quantitative yield of morphine and codeine. If the plant material was extracted with an aqueous acid phase, however, it was possible to isolate nearly all the normorphine in the aqueous extract. Recovery from this aqueous phase could then be accomplished in 75% yield, by extracting with six portions of $CHCl_3$–isoPrOH (3:1) at pH 8·6.

The problem of normorphine analysis was solved by the use of acetyl derivatives. The best GLC separation obtained for morphine and normorphine as free bases was about 1 min. The acetyl derivatives, on the other hand, had a separation of 24 min at a column temp. of 240° (diacetylmorphine: R_t 8:20; triacetylnormorphine: R_t 32:00). In addition, the resolution of normorphine was also enhanced, the lower limit of detection decreasing from about 200–50 ng. The acetyl derivatives were also easily separable by TLC; using C_2H_5OH–C_6H_6 (4:1), diacetylmorphine has R_f 0·27, while triacetylnormorphine has R_f 0·53. Using direct acetylation of the crude plant alkaloid extract, GLC analysis can detect a normorphine–morphine mass ratio as low as 0·1%.

Extraction Procedure; Control Experiments

The results concerning the presence of normorphine in opium poppy plants depend on proof that normorphine is not an artifact of the isolation procedure or analysis. Two controls were run on the total extraction and analytical procedure, using 1 N HCl as the initial aqueous phase. One was done with inactive morphine, without plant material, while the other was done with morphine-^{14}C, in the presence of plant material. Inactive carrier normorphine was added to the latter to pick up any activity residing in normorphine. Both these controls produced significant amounts of normorphine (2–2·5%). Further experiments implicated the combination of strong acid, heat and air (the latter two a result of the blending process) as the responsible factor. When the aqueous phase was changed to buffered H_3PO_4 (pH 2·5, 1 M), and the temperature kept below 40°, no aberrant normorphine production was observed.

In the process of establishing the analytical procedure for normorphine, it was discovered that some commercial morphine contained a normorphine impurity (~1·5%, as seen by GLC both before and after acetylation). Precautions were necessary, therefore, to assure that the morphine being used for these studies, both as standards and for feeding, was free from normorphine. Purification could most easily be accomplished by a combination of TLC and sublimation at 180°/0·1 mm.

Normorphine Presence in Plants and Opium

A series of plants of various ages was extracted using the aqueous acid/butanol–benzene extraction procedure, and the ratio of normorphine to morphine at each age determined by GLC analysis of the acetylated extracts (Fig. 1). At the same time, the absolute morphine

concentration was determined for each age (Fig. 2). The result for 20-day-old plants is an approximation, since only trace quantities of normorphine and morphine were present, codeine and thebaine being the most abundant at that age. The plants used here were flowering at approximately day 100.

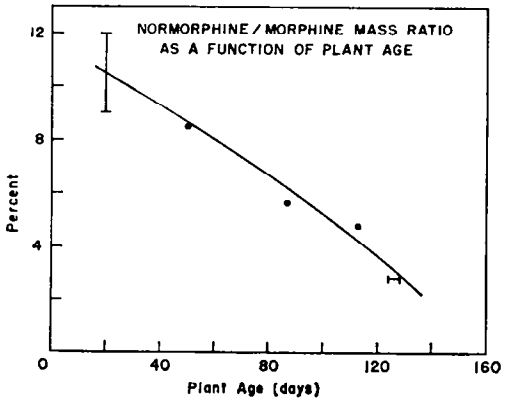

FIG. 1. RATIO OF NORMORPHINE TO MORPHINE AS A FUNCTION OF PLANT AGE.
The vertical and horizontal lines designate the limits of error at the points shown.

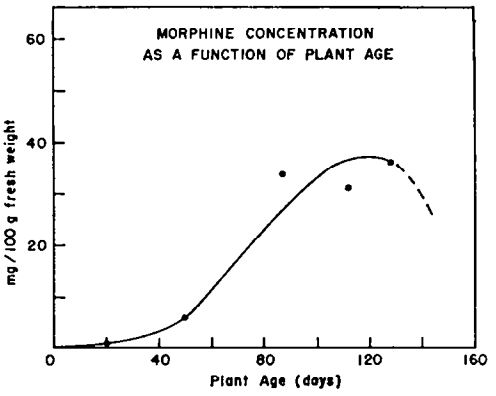

FIG. 2. MORPHINE CONCENTRATION AS A FUNCTION OF PLANT AGE.

In addition to the plants, two samples of raw opium were also examined for normorphine. A sample of Turkish opium showed a normorphine–morphine mass ratio of 3·1%, with morphine comprising 12·5% of the raw wt. Indian opium, on the other hand, had less morphine (7·0%), but a significantly higher normorphine–morphine mass ratio, 8·0%.

Labeled Morphine Feedings

Two morphine-^{14}C feedings were performed to examine whether morphine is the biosynthetic precursor of normorphine. The morphine used was obtained from a 4-day $^{14}CO_2$ exposure of 80- to 90-day-old plants. It was necessary to replace the labeled N–Me carbon with an unlabeled one in order to limit the study to the fate of the carbons comprising the ring structure of morphine. Demethylation of the morphine via cyanogen bromide to normorphine,[7] followed by remethylation with ethyl chloroformate and $LiAlH_4$ reduction back to morphine,[8] afforded nuclear labeled morphine ($3{\cdot}14 \times 10^7$ dpm/mmol).

The first feeding employed a dilute root feeding technique, with aeration of the nutrient solution. Three 72-day-old plants were fed hydroponically a solution containing about 10 mg of labeled morphine in 300 ml of nutrient solution. The level of nutrient was maintained at 300 ml for 22 hr, at which time the plant roots were rinsed with dil. H_3PO_4 to remove any unincorporated morphine. The plants were then killed by freezing in liquid N_2. After adding 10 mg of inactive carrier normorphine, the plants were subjected to the isolation procedure for morphine and normorphine. The activity remaining in the residual nutrient solution and the root washes was present as morphine and indicated that 65% of the fed activity had been incorporated by the plants. Both the morphine and normorphine (as their

[7] J. VON BRAUN, *Chem. Ber.* **47**, 2312 (1914).
[8] C. ELISON, H. W. ELLIOTT, M. LOOK and H. RAPOPORT, *J. Med. Chem.* **6**, 237 (1963).

acetyl derivatives) were purified by TLC to constant specific activity. Since the native pool of normorphine in these plants amounts to less than 0·5 mg, the total activity due to normorphine could be satisfactorily determined on the basis of the total carrier added.

The second feeding employed a 'semi-starvation' root feeding technique. Three 70-day-old plants were fed a total of 6·8 mg of labeled morphine, dissolved in a minimal amount of nutrient solution (about 2 ml/plant). The amount of nutrient was kept to a minimum for 6 hr, at which point the roots were rinsed in dilute acid and the unincorporated morphine recovered. The plants were then allowed to metabolize the incorporated morphine for an additional 18 hr, in a normal amount of nutrient solution, and the plants were then harvested and extracted, after adding 14·7 mg of inactive carrier normorphine. It should be noted that no activity was found in the last residual nutrient solution, indicating no outward flow of alkaloids or metabolites from the roots. The morphine and normorphine were purified as before, to determine the total activity residing in each.

TABLE 1. ^{14}C-NUCLEAR LABELED MORPHINE FEEDING EXPERIMENTS

Feeding experiment	1	2
Method of root feeding	Dilute solution, with aeration	'Semi-Starvation'
Feeding time (hr)	} 24	6
Metabolic time (hr)		18
Morphine fed: activity (dpm)	1 170 000	751 000
amount (mg)	10	6·8
Incorporated: activity (dpm)	764 000	281 000
(%)	65	37
% of incorporated activity in:		
Codeine	0	0
Morphine	40	37
Normorphine	5	1·5
Non-alkaloidal Metabolites	~50	~40
% of metabolized activity in:		
Normorphine	8	2·5

The results of these feeding experiments are summarized in Table 1. In both feedings, no other alkaloidal metabolites could be detected. A majority of the activity resides in non-basic, non-alkaloidal metabolites. It should be mentioned that other preliminary labeled morphine feedings showed these latter metabolites to be of a diverse nature, especially with regard to their ionic character. A good portion were acidic in nature, while others were amphoteric.

$^{14}CO_2$ *Biosynthesis*

In order to further determine the biosynthetic relationship of morphine and normorphine, a $^{14}CO_2$ 'steady-state' exposure experiment was performed.[9] By maintaining the specific activity of the labeled CO_2 at a constant level for the entire exposure, the specific biosynthetic sequence will be reflected in the specific activities of the alkaloids in question,

[9] H. I. PARKER, G. BLASCHKE and H. RAPOPORT, *J. Am. Chem. Soc.* **94**, 1276 (1972).

i.e. assuming pool equilibration and depending on relative pool sizes, a product will have a lower specific activity than its precursor. Four opium poppy plants, 90 days old, took up 50 mCi of $^{14}CO_2$ over a 4-hr period, under steady-state conditions, after which the alkaloids were isolated using the aq. acid/butanol–benzene procedure. The specific activities of the alkaloids in question were determined, as their acetyl derivatives, after successive purification by TLC and GLC. To determine the activity residing in those carbon atoms common to both morphine and normorphine, a portion of the morphine was demethylated to normorphine. The results of this experiment are summarized in Table 2. The relative specific activities of the codeine and morphine demonstrated that this was a valid short term 'steady-state' exposure,[9] since the last step in the biosynthesis of morphine is the irreversible *O*-demethylation of codeine, which is present in smaller pool size than morphine.

TABLE 2. $^{14}CO_2$-STEADY STATE EXPERIMENT

Compound	Specific activity (dpm/nmole)	Total alkaloid (mg/100 g fr. wt.)	Compound	Specific activity (dpm/nmole)	Total alkaloid (mg/100 g fr. wt.)
Codeine	440	4	Morphine (minus N-CH_3)	26	—
Morphine	38	26	Normorphine	27	~1

Labeled Normorphine Feeding

The steps leading from thebaine via codeine to morphine have all been shown to be irreversible.[10] It was of interest, therefore, to determine whether the conversion to normorphine was also irreversible. For this purpose, two 60-day-old plants were fed 6·5 mg of normorphine-^{14}C (110 000 dpm), which had been obtained by demethylating biosynthetic morphine-^{14}C. The semi-starvation root feeding technique was used, and the total feeding time was 24 hr. About 50% of the normorphine was incorporated by the plant and the rest was recovered from the nutrient solution and washes. After adding 100 mg of inactive morphine to frozen plant material, it was extracted as before. The purified morphine was inactive. Of the incorporated activity, about half was recovered as unchanged normorphine, while the other half resided in non-alkaloidal metabolites.

DISCUSSION

The results presented here suggest several significant conclusions regarding morphine metabolism and function. They not only establish the existence of normorphine as a natural plant product, but also indicate it is the direct mediator of morphine metabolism.

In the steady-state exposure, the fact that morphine is of lower specific activity than codeine, consistent with the known product-precursor relationship and the fact that morphine is present in much larger pool size, established that saturation of the alkaloid carbons with ^{14}C has not yet occurred. Since the pool of normorphine is only 4% of that of morphine, the specific activity of normorphine should be similar to but less than that of morphine. However, the essential identity (within experimental error) of the morphine and normorphine specific activities (implying equilibration of labeled carbon in each)

[10] F. R. STERMITZ and H. RAPOPORT, *Nature, Lond.* **189** (4761), 310 (1961).

from the $^{14}CO_2$ exposure suggests that at least one complete cycle has occurred in the degradative turnover of normorphine. The results from the normorphine feeding suggest that this turnover is only in the forward direction. This puts a maximum limit of 4 hr (the length of the CO_2 exposure) as the turnover rate for the total normorphine pool. Comparing this to the result found for both morphine feedings, it can be seen that all the non-alkaloidal metabolized activity can be accounted for by assuming that from 6–10 complete normorphine degradative cycles have occurred.* In addition, the level of activity recovered as normorphine is approximately equal to the relative mass abundance found in plants of that age. It appears, therefore, that the rate of normorphine formation is equal to the rate of its further breakdown. Another explanation, that normorphine also is formed by a second pathway directly from norcodeine, was discarded since norcodeine was not present in the plant.

An interesting result from the ontogeny studies is that normorphine is never the most abundant alkaloid. It is known that the other major alkaloids, thebaine, codeine, and morphine, all take their turn at being the most abundant alkaloid, the sequence reflecting the biosynthetic pathway.[12] That the normorphine concentration fails to show the same behavior with age seems to imply that the rate of turnover is fairly constant over the life cycle of the plant.

The rapid turnover observed here indicates that alkaloid biosynthesis plays an active role in the biosynthetic functions of the plant. The inclusion of normorphine as the final step in the alkaloid biosynthesis completes the sequence of demethylations from thebaine. These facts together lend support to the hypothesis that the alkaloids may act as specific methylating agents, the relative stabilities of the successive methyl groups affording the plant a potentially sensitive control of methylation processes. Other passive roles, such as protection from predators, or even active roles such as biochemical control factors, cannot be rationalized on the basis of our results, since they would not require the high turnover observed.

A final tentative conclusion can be drawn regarding the site of morphine biosynthesis and metabolism. The rapid equilibration of the morphine and normorphine activity pools seen in the $^{14}CO_2$ exposure of necessity implies that active synthesis and degradation are occurring in the entire plant. Since the plants were used prior to flowering, the same does not necessarily hold true for the capsulated plants. Therefore, it is possible, as suggested recently,[13] that only the stem latex is involved with the morphine and normorphine activity. Indeed, it was found that senescent, dying plants had almost no normorphine, despite the fact that morphine concentration was still quite high.

EXPERIMENTAL

Plants grown hydroponically from seed of *Papaver somniferum* L. var. *alba*, U.S.D.A. No. 40, grown at Somerton, Arizona in 1950, were used throughout.

* Fairbairn and El-Masry[11] have recently suggested that 'bound' alkaloids may be involved in morphine metabolism and seed development. If such forms were to exist in the plant, they would influence the interpretation of the results of the morphine feedings. We have studied plants of the ages used here (pre-flowering), and have found no evidence for either bound morphine or codeine. The subject of bound alkaloids in the plant and in the seed will be discussed in detail in a future publication.

[11] J. W. FAIRBAIRN and S. EL-MASRY, *Phytochem.* **7**, 181 (1968).
[12] R. O. MARTIN, M. E. WARREN and H. RAPOPORT, *Biochemistry* **6**, 2355 (1967).
[13] J. W. FAIRBAIRN and M. DJOTÉ, *Phytochem.* **9**, 739 (1970).

Radioactive morphine. Four 110-day-old plants were grown in a $^{14}CO_2$-labeled atmosphere for 4 days. The plants were frozen in liquid N_2 and then extracted in a Waring blendor with 10% aq. Na_2CO_3 and several portions of *n*-BuOH–C_6H_6 (1:1). The organic phase was extracted with 1 M H_3PO_4 (pH 2·5) and the aq. acid extract basified (pH 12) and extracted with $CHCl_3$ to remove the non-phenolics, and then the pH was adjusted to 8·6 and further extraction with $CHCl_3$ gave the phenolic alkaloids. The morphine was purified by preparative TLC, ($CHCl_3$–MeOH–NH_3, 3:1:1% on Silica) and converted to heroin with refluxing Ac_2O, and the heroin demethylated to normorphine using CNBr. Remethylation of the normorphine was accomplished with $ClCO_2C_2H_5$, followed by $LiAlH_4$ reduction to give morphine[nuclear-^{14}C] specific activity $3{\cdot}14 \times 10^7$ dpm/mmol.

Plant alkaloid isolation and analysis. The entire plant was frozen in liquid N_2, ground in a blender, and extracted with two portions of H_3PO_4 (1 M, pH 2·5), followed by washing with *n*-BuOH–C_6H_6 (1:1) to remove the pigments. The plant mash was centrifuged to separate the aqueous acid phase from the plant solids and the acid extract was brought to pH 8·6 with Na_2CO_3 and extracted with 6 portions of $CHCl_3$–isoPrOH (3:1). The latter was dried and evaporated, then acetylated with refluxing Ac_2O for 16 hr. This solution was evaporated to dryness and analyzed directly, or separated into its components for subsequent purification. The triacetylnormorphine and diacetylmorphine were separated and purified by TLC on silica gel, using C_2H_5OH–C_6H_6 (1:4) as developing solvent. Some additional purification of labeled samples was done with small-scale preparative GLC (column 1·8 m × 3 mm i.d. of 3% OV17 on Aeropak 100–120 mesh; 250°; with argon, 60 ml/min. For collection, the column temp. was reduced to 230°).

Opium extraction. A weighed sample of opium was dissolved in H_3PO_4 (1 M, pH 2·5) by heating and stirring. The pH of the solution was then brought to 8·6, and extracted as before with $CHCl_3$–isoPrOH (3:1). The dried residue was acylated and analyzed in the same manner as above.

Morphine feedings. In the first feeding, the labeled morphine was dissolved in a small amount of 1% H_3PO_4, and nutrient added to 300 ml. Three plants were transferred from hydroponic tanks to the feeding container, and left for 22 hr; the level of nutrient was maintained near 300 ml. After 22 hr, the plants roots were rinsed in 1% H_3PO_4 and H_2O, then frozen and extracted as usual after adding 10 mg of inactive carrier normorphine. The residual nutrient solution and the root washes were counted to determine the activity not taken up by the plants and morphine was recovered by adjusting to pH 8·6 and extracting in the usual manner. In the second feeding, the labeled morphine was dissolved in 1 ml of 0·1 M H_3PO_4, diluted to 13 ml with nutrient, and 4 ml were fed to each of three 70-day-old plants via the roots. Additional nutrient was added to maintain the level at 5 ml for the first 6 hr, at which point the plants were removed, their roots rinsed, and replaced in fresh nutrient for an additional 18 hr. The plants were then frozen and extracted, after adding 14·7 mg of inactive carrier normorphine.

$^{14}CO_2$ *'Steady-state' exposure.* Four 90-day-old plants were exposed to 50 mCi of $^{14}CO_2$ for a 4-hr period in an apparatus as described.[9] The CO_2 concentration was maintained at 0·035% throughout, and the specific activity was kept constant. At the end of the exposure, the plants were treated in the normal manner to isolate the alkaloids for specific activity determinations. The morphine was demethylated as previously for comparison of the nuclear carbons.

Labeled normorphine feeding. This was done similarly to the second morphine feeding, except that after 4 hr, the nutrient level was increased to 15 ml and maintained at that level for the rest of the feeding. There was no intermediate root rinse included, so the plants were exposed to the labeled normorphine throughout. After adding 100 mg morphine and 50 mg normorphine as carrier, the alkaloids were extracted and analyzed as normally.

Acknowledgements—This work was supported in part by the U.S. Atomic Energy Commission and Grant MH 12797 from the National Institute of Mental Health, U.S. Public Health Service.