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MORPHINE METABOLISM IN PAPAVER SOMNIFERUM

Robert John Miller
(Ph. D. Theis)

June 1972

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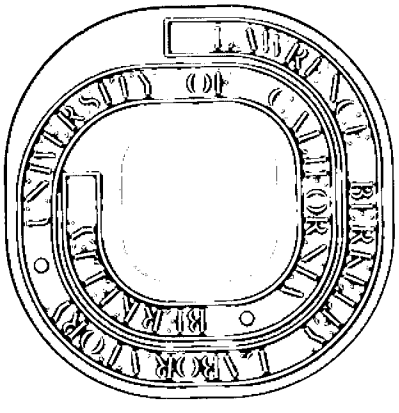

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DISCLAIMER

MORPHINE METABOLISM IN PAPAVER SOMNIFERUM

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MORPHINE METABOLISM IN PAPAVER SOMNIFERUM

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June 1972

ABSTRACT

Until recently, morphine (I) was considered to be a metabolic end-product of the opium poppy, P. somniferum. We were interested in substantiating other published results which suggested that morphine is indeed degraded by the plant, as well as learning something about the nature of the degradation products and the pathway leading to them. By feeding labeled morphine to poppy plants via the roots, we have demonstrated that morphine is metabolized. These experiments suggested that normorphine (II) is an active metabolite of morphine. A procedure was developed to isolate and analyze for normorphine found in poppy plants. When plants of various ages were extracted, significant amounts of normorphine were found throughout the life cycle of the poppy; normorphine has also been found in two different samples of raw opium. The hypothesis that morphine is being degraded via normorphine was tested by two methods, labeled morphine feedings, and labeled CO_2 steady-state exposures. The results of these experiments indicated 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, most likely as specific methylating agents.

The question of the existence of bound forms of alkaloids in the seeds of P. somniferum has also been raised. We were interested in determining whether our seeds contain any forms of alkaloids which can be released by acid hydrolysis, or if any free alkaloids are in the seeds. At the same time, our interest in morphine metabolism led us to a search of the poppy plant itself for bound alkaloids, since these could play a role in metabolism. Both seeds and plant material were extracted for their free alkaloids, then subjected to vigorous acid hydrolysis. The seeds did show traces of both free and bound alkaloids, one of the latter possibly being codeine. The plants were found to contain alkaloids which are released by acid treatment. However, no bound morphine or codeine were detected. The problem of distinguishing between bound alkaloids and aberrant products produced by the hydrolytic conditions is discussed.

The recent demonstration that neopinone is present in the poppy plant suggests that there may exist a parallel biosynthetic pathway to the morphine series, to produce neopine, neomorphine, and norneomorphine. It was desired to obtain authentic samples of each of these three alkaloids, as comparison standards to determine their presence or absence in the plant. When neopine·HBr (which was available) is treated with 15% HBr in acetic acid, 6-acetylneomorphine is the product, whereas treatment with 48% aqueous HBr

leads directly to neomorphine. Neomorphine is also obtained by basic hydrolysis of the 6-acetate. Conversion of either neomorphine or the mono-acetate to the diacetate, followed by treatment with diethylazodicarboxylate, and subsequent hydrolysis, affords a good yield of the new compound, norneomorphine. Initial searches of plant extracts for the neo-alkaloids have been inconclusive.

PART 1

MORPHINE METABOLISM AND NORMORPHINE

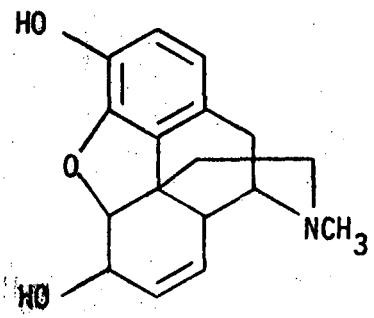
I. INTRODUCTION

Ever since the isolation of morphine from opium by Sertürner in 1805,[1] chemists have been interested in learning more about the class of natural products called alkaloids. Most of this interest has been directed toward the isolation and identification of new alkaloids, investigations concerning their biosynthesis, and the *in vitro* synthesis of some of the more important alkaloids. However, one major area of the study of alkaloids has received little investigative attention: the role that alkaloids play in the plants which produce them. Indeed, many different theories have been proposed regarding alkaloid function, including:[2,3] (a) inert end-products of metabolism; (b) protection from insects or other predators; (c) involvement in nitrogen metabolism; (d) links to general plant metabolism as methyl carriers; (e) and involvement as factors in the control of biochemical processes. Several fairly recent studies have presented specific evidence for alkaloid turnover and active metabolism of morphine in the opium poppy,[4-6] nicotine in *Nicotiana Rustica* (tobacco),[7] and the alkaloids of the hemlock.[8] This type of evidence strongly suggests that the metabolism of alkaloids is somehow involved with other plant metabolic processes.

The case of the opium poppy, *Papaver Somniferum*, is a typical one. The biosynthetic pathway leading from relatively simple metabolites to morphine (I) has been extensively studied, and is fairly

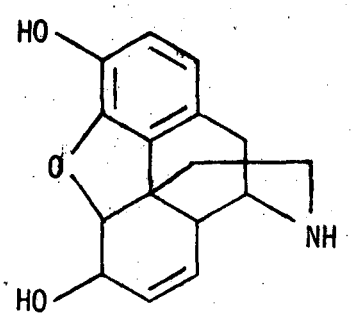
well established.[9,10] More recently, Fairbairn and Wassel have found marked variations in alkaloid content over periods as short as one or two hours.[5] In addition, feeding experiments using radioactive morphine have shown that the morphine molecule is degraded to other plant metabolites, as yet unidentified.[6] It was felt that further work along this line might give some insight into the metabolism and function of morphine.

The approach we have chosen has really been two-fold. First of all, by feeding ^{14}C-morphine to the poppy plants, we have obtained further evidence for the active metabolism of morphine, as well as obtaining some information concerning the nature of the metabolic products. Secondly, preliminary results from some of the feedings, along with a logical extension of the biosynthetic sequence leading to morphine, led to the suspicion that morphine could be degraded via an initial N-demethylation to normorphine (II). With this in mind, various studies have been done to establish the existence of normorphine as a native product of the poppy. Once normorphine was found, ^{14}C-morphine feedings and $^{14}CO_2$ biosyntheses were performed to establish the biosynthetic relationship between morphine and normorphine. The results which have been obtained seem to support an active role for normorphine in the metabolic breakdown of morphine. How these results were obtained, and the implications they have regarding morphine metabolism and function, are the subject of the first section of this thesis.



I

Morphine



II

Normorphine

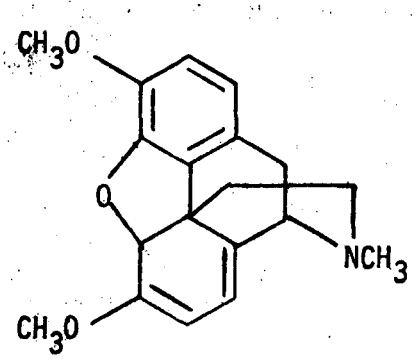
II. MORPHINE METABOLISM AND NORMORPHINE

A. Historical

The earliest evidence that suggested that morphine (I) is not a metabolic end-product came in 1961, when Pfeifer and Heydenreich published studies they had made on the absolute quantities of the morphine alkaloids as a function of the time of day.[4] Their results, though questionable with regard to sampling, seem to indicate a marked daily variation in the alkaloidal content. Prior to that study, Rapoport and co-workers had suggested that both thebaine (III) and codeine (IV) are active in some way in the plant's metabolism.[11] Their results from several $^{14}CO_2$ exposures indicate that the rate of synthesis of both these morphine precursors requires that they are necessarily metabolically active, since the concentration of neither of them increases to any extent. More recently, Fairbairn and Wassel have used radioactive tracer techniques to measure the changes in morphine alkaloid content.[5] They fed tyrosine[2-^{14}C] to the plants by allowing it to pass through a scraped portion of the pedicel immediately below the capsule. They then scraped small samples of latex from the capsules and tested the alkaloidal content and activity. Despite the very low incorporation of the labeled tyrosine, their results do indicate a turnover in the alkaloid content from day to day.

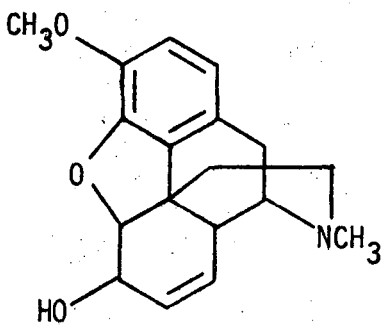
Fairbairn and El-Masry have also fed ^{14}C-morphine to plants through the pedicel, just below the capsule.[6] They then examined the plants for radioactivity after certain time periods. They noted (1) a fairly rapid translocation of the radioactivity from the fed area to other parts of the plants, and (2) that some of the radioactivity was apparently in non-alkaloidal metabolites, indicating a breakdown of the morphine molecule.

Evidence for alkaloid turnover has also been presented for the nicotine-related alkaloids. Tso and Jeffrey have fed nicotine doubly labeled with ^{14}C and ^{15}N to Nicotiana Rustica via the roots.[7] A significant amount of recovered activity was found in all forms of plant metabolites, indicating an active role for nicotine in the plant metabolism. Other workers have obtained similar results with the Nicotiana alkaloids.



III

Thebaine



IV

Codeine

Although normorphine has never been found in the poppy plant or in raw opium, nor as a metabolite of morphine in the plant, there is some precedence in nature for suspecting it. Numerous animal studies

have been performed, the results of which indicate significant N-demethylating activity after administration of morphine to the animal in question. In one case, normorphine itself was recovered (~4%) in the 48 hr urine from rats injected with ^{3}H-morphine.[12] Other workers have observed significant $^{14}CO_2$ expired from mice,[13] rats,[14] and man[15] after administration of morphine-N-$^{14}CH_3$ (expired activity ranging from 1-10%).

B. Methods Used

In the study of alkaloid biosynthesis, certain kinds of experiments have traditionally been used to establish specific biosynthetic sequences. For example, if one wishes to show that alkaloid A proceeds to alkaloid C via an intermediate alkaloid B, three kinds of experiments are helpful (and necessary, as will be pointed out). The first, and most obvious, is to demonstrate by isolation that alkaloid B does, in fact, exist in the plant as a natural product. Secondly, precursor feedings can be used to establish the fact that the plant is capable of producing B and C from A, as well as C from B. Precursor feedings are most commonly done using isotopic tracers, such as ^{14}C and ^{15}N, which provide a suitable analytical handle for such biosynthetic studies. The major drawback of precursor feedings is that the plant is necessarily subjected to an unnatural situation when the precursor in question is fed. Therefore, a third type of experiment can be used which, for all practical purposes, duplicates exactly the natural conditions, namely, $^{14}CO_2$ exposures. If a plant is exposed to $^{14}CO_2$ for a short time, the alkaloids in question will

become labeled in the same order in which they are synthesized by the plant. Their relative specific activities then reflect the natural biosynthetic pathway. Labeled carbon dioxide exposures are also the easiest way to obtain labeled natural products for feedings or other studies.

All three of these approaches were used in the work to be described herein. In particular, the study of the metabolism of morphine was first attacked by feeding radioactive morphine to the plants, and then effecting a crude separation of the plant products into several fractions by suitable liquid-liquid extractions. The sole method of feeding used was root feeding. There are many different feeding techniques which have been used for such work; however, it was felt that root feedings caused the least physical damage to the plant. Most other methods involve some physical means for introducing the precursor, such as injection, wick feeding, or applying the compound in solution to a leaf surface. In our case, the plants are routinely grown hydroponically. By supplying the labeled precursor dissolved in nutrient solution to the plant, we feel that natural conditions are maintained as close as possible. One drawback to root feedings is the chance of premature degradation caused by microorganisms on the roots or instability to the nutrient solution, which could lead to incorporation of undesired compounds. It is necessary, therefore, to check the integrity of the activity which is not incorporated by the plant, to make certain that no aberrant degradation is occurring.

Two different types of labeled carbon dioxide exposures are used in this work, as suggested previously. If the purpose is only to obtain labeled alkaloids for subsequent use, there need be no concern

about maintaining specific conditions. However, if the purpose is to study a biosynthetic pathway by determining labeling sequences, it is necessary that the exposure be "steady-state". Parker has described the requirements of a steady-state exposure in detail, as well as the equipment used for this purpose.[16] It will be sufficient for our purposes to mention that the major requirement is that the specific activity of the $^{14}CO_2$ must not decrease during the exposure. The latter type of exposure was used here to establish the biosynthetic relationship of normorphine and morphine. The non-steady-state exposure was used to prepare labeled morphine, which was subsequently employed in the feeding experiments, and as one means of establishing the existence of normorphine as a native plant product. The latter method proves to be an extremely useful tool for looking for suspected plant substituents which are present in trace amounts. Thus, by adding cold carrier normorphine, for example, and purifying the isolated normorphine to constant specific activity, the presence of activity is substantial proof of normorphine's existence.

C. Morphine Feedings, Sets 1 and 2

1. Preparation of ^{14}C-Morphine

A $^{14}CO_2$ biosynthesis was performed for the purpose of obtaining ^{14}C-morphine to be used for the feedings. Four poppy plants, average age of 3-3/4 months, were transplanted from vermiculite to nutrient solution, and exposed to a $^{14}CO_2$ atmosphere (50 mC) for 1 day, followed by normal air for three more days. The morphine isolated from the plants was purified by preparative thin layer

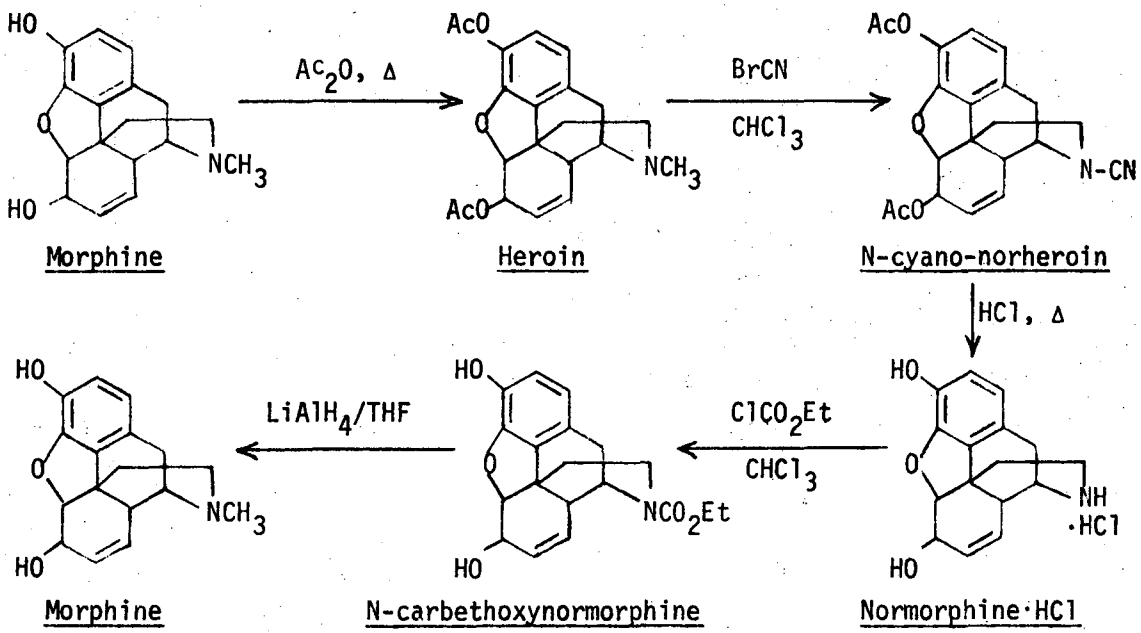
chromatography, to give 10 mg of morphine with a specific activity of 1.15×10^6 dpm/mg.

Since there was the possibility that active methylating agents could cause transmethylations to occur in the plant independent of the specific alkaloid biosynthesis,[11] it was decided that morphine labeled only in the ring, or nuclear, carbons should be used for the feeding experiments. We were mainly interested in the fate of the basic morphine skeleton, and not the peripheral methyl groups. The morphine which one obtains from a $^{14}CO_2$ exposure is obviously labeled in all carbons, including the N-CH_3. It was necessary, therefore, to remove the labelled methyl group and replace it with an unlabeled one. The sequence of reactions used for this purpose has been worked out in detail for small quantities of morphine;[17] it is outlined in Scheme I. The morphine is converted via heroin (V) to normorphine with BrCN (Von Braun reaction),[18] and the normorphine N-methylated via N-carbethoxynormorphine and subsequent reduction with lithium aluminum hydride to give back morphine.[19] After an appropriate dilution, the biosynthetic morphine was subjected to this demethylation-remethylation, to give 11 mg of nuclear-^{14}C-morphine, with a specific activity of 270,000 dpm/mg. This represented a 25% loss of label due to the N-methyl carbon, which compared favorably with results obtained in previous $^{14}CO_2$ exposures.[16]

2. Plant Extraction and Alkaloid Isolation

The main purposes of the initial feeding experiments were to: (1) establish that morphine is being degraded; (2) obtain information regarding the nature of the metabolites; and (3) learn what we could

Demethylation - Remethylation of Morphine



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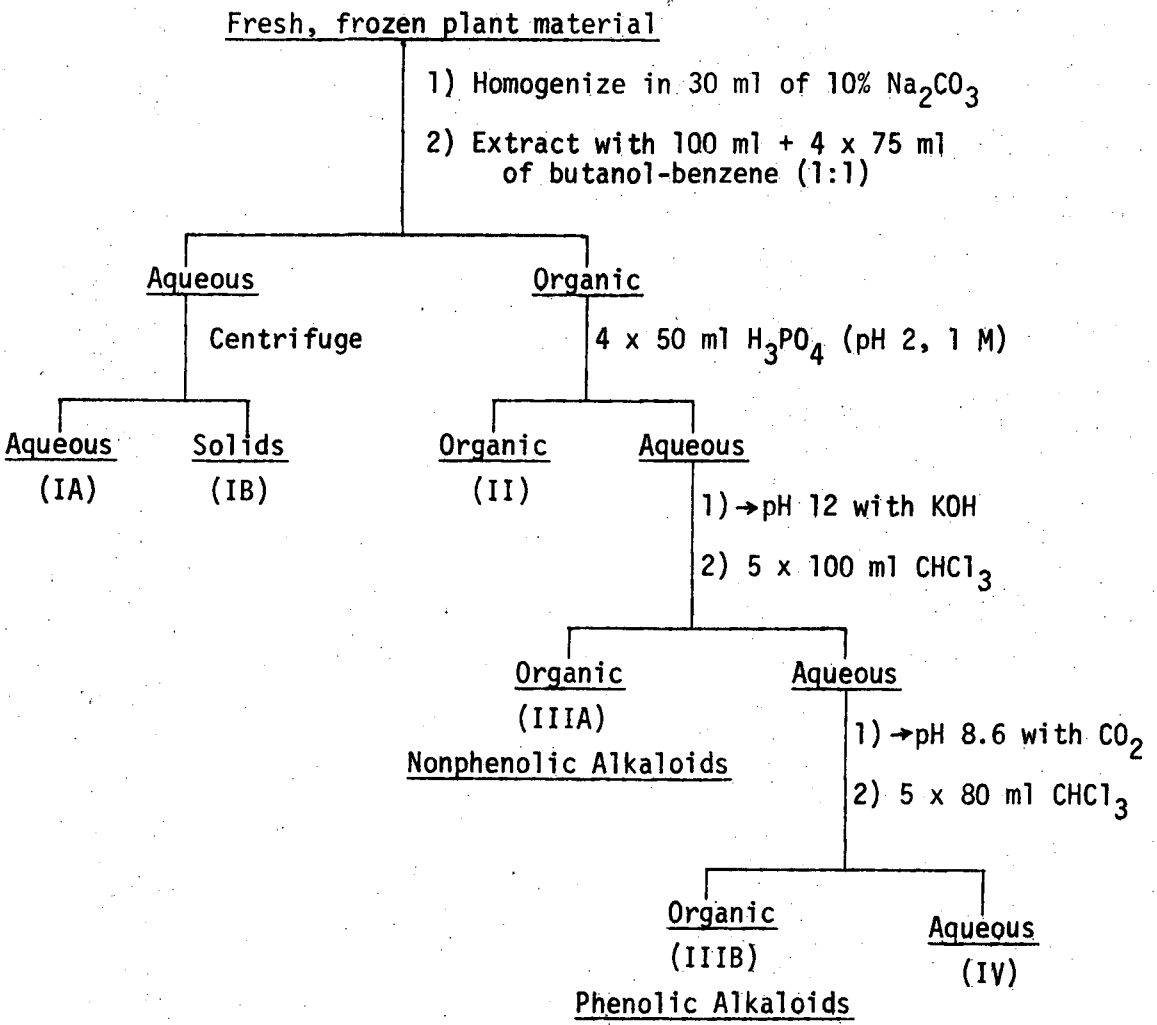
Scheme I

regarding the rate of morphine metabolism. Since we began with no prior knowledge of exactly what to look for, it was felt that any initial searches for metabolites should be limited to a relatively small number of fractions, each representing a rather broad, but somewhat characteristic, range of compound classes. It was of major interest, of course, to isolate the alkaloid fraction to determine whether or not any of them are involved in the breakdown of morphine. The standard alkaloid isolation procedure being used by previous workers here was adopted as a starting point for these studies, with several minor modifications.[20] This extraction scheme involves several consecutive liquid-liquid separations, which provide either five or six fairly characteristic fractions. The procedure is summarized in Scheme II. In addition to the two alkaloid fractions obtained, IIIA and IIIB, one obtains four other fractions, whose chemical constituents can roughly be assigned on the basis of separations performed. Thus, Fraction Ia should contain mainly water-soluble acidic and neutral materials, Fraction IB water- and organic-insolubles, Fraction II pigments, lipids, and high molecular weight acids, and Fraction IV any incompletely extracted alkaloids or residual acids and neutrals. In practice, the procedure was often simplified by extracting all the alkaloids into one fraction, by skipping the pH 12 extract.

The efficiency of this procedure for recovery of morphine was tested by extracting a known aliquot of ^{14}C-morphine with some cold plant material. About 80-85% of the labeled morphine ends up in the alkaloid fraction, while the rest is distributed fairly equally between Fractions IA and IV, and represents incompletely extracted

Standard Plant Extraction Scheme



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Scheme II

morphine. The same situation holds true for all the other major alkaloids encountered in the poppy.[20] The same does not hold for normorphine, however. This fact was discovered subsequent to these initial feeding experiments. If normorphine is subjected to a control extraction using this procedure, it will be distributed approximately equally to Fractions I and IV. More will be said concerning the extractability of normorphine in a later section. However, this fact can be kept in mind when the feeding results are presented and discussed.

3. Morphine Feedings, Set I

An initial series of ^{14}C-morphine feedings was conducted using several different masses of morphine in the feeding solution. It was desired to learn if the mass of morphine taken up by the plants had any effect on the distribution of degraded activity. It is conceivable to suspect that fairly large doses of morphine (relative to the existing plant pool) could lead to abnormally high local concentrations, which in turn could activate normally inactive degradative enzyme systems. On the other hand, one is limited on the low side by increased inaccuracies due to lower activity.

With these thoughts in mind, three sets of two plants each were fed 0.22, 2.2, and 21 mg, respectively. The feeding technique employed has been referred to as "starvation feeding", in that the precursor (labeled morphine, in this case) is dissolved in a minimal amount of nutrient solution, and maintained that way for the first 20-30 minutes of the feeding. This is to enhance the possibility of efficient adsorption and incorporation by the roots of the poppies.

The plants used for these feedings were 60-63 days old. At the end of 24 hours, the roots of the plants were rinsed with 1% H_3PO_4, followed by distilled water, to remove any morphine which had merely been adsorbed, but not incorporated. These washings, in addition to any residual nutrient solution remaining in the feeding flasks, were counted to determine their activities. By then subtracting this unincorporated activity from the fed activity, one indirectly determines the amount of activity actually incorporated.

Following the root wash, the plants were killed by immersing in liquid N_2, and then subjected to the extraction procedure outlined in Scheme II. In this case, the alkaloids were extracted together. The results of these feedings are summarized in Table I. Problems were encountered in trying to count the solid phase of the plant mash, so only the aqueous portion has been included here. It can be said with certain confidence, however, that a significant portion of the activity not accounted for in each case resides in the solids. It should also be noted that Runs 1 and 3 were done separately from Run 2, and, though the plants were of the same age, they were apparently not in the same condition. This fact is reflected by the relative inefficiencies of incorporation of Runs 1 and 3.

Despite the analytical weaknesses mentioned above, certain tentative conclusions can be drawn from these results. Of major import, of course, is the fact that the morphine is indeed being degraded, and to a significant extent in a single day. Secondly, even accepting the position that a portion of the activity in Fractions I and IV is due to normorphine, the results still suggest that degradation has

Table I

^{14}C-NUCLEAR LABELLED MORPHINE FEEDINGS: SET 1

DISTRIBUTION OF ACTIVITY AS A FUNCTION OF MASS FED

(METABOLIC TIME = 24 HOURS)

	RUN # / FRACTION	1	2	3
	AMOUNT FED (mg)	0.22	2.2	21
% OF FED	1. ROOT WASH	8%	39%	13%
	2. NUTRIENT	54%		67%
	3. INCORPORATED	38%	61%	20%
	A. MASS (mg)	0.084	1.3	4.2
% OF INCORPORATED	IA. PLANT MASH - AQUEOUS ONLY	48%	21%	20%
	II. BuOH/Bz	5%	4%	1%
	III ALKALOIDS	14%	33%	19%
	IV. AQUEOUS RESIDUE	13%	5%	16%
	TOTAL I-IV	80%	63%	56%

proceeded quite far, to give mainly water-soluble and insoluble metabolites. And finally, a comparison of the three feedings seems to indicate that there is in fact some difference between large and small doses. It appears as if metabolic breakdown has proceeded further in one of the two extreme cases, although one cannot say from this data which one, since this would depend on the nature of the unaccountable active degradation products. Due to this difference, it was concluded that future feedings be done using the small doses, since this more clearly represents the natural condition. The amount of morphine incorporated in Run 1 corresponds to approximately 1% of the total morphine pool in 60-day plants.

Some further studies were done on the aqueous portion of the plant mash isolated from Run 2. First of all, it was extracted with $CHCl_3$ over a broad range of acid and base strengths (pH 2-10.5). Only traces of activity were thus extracted (<1% for each pH tested). Secondly, a separation was made on the basis of the ionic character in acidic and basic solutions, by running the material first through a cation exchange column, and then running the basic materials through an anion exchange column. By this sequence of operations, it was found that about 70% of the water-soluble morphine degradation products were acidic or neutral in character, 21% were amphoteric, and only 9% basic. These results further support the conclusion that morphine is extensively degraded in the course of 24 hours.

4. Morphine Feedings, Set 2

A second set of labeled morphine feedings was performed with the intention of studying the effect of feeding time on the distribution

of activity. Six sets of 60-day old plants (2 per set) were fed identical amounts of ^{14}C-morphine, and then allowed to metabolize it for different lengths of time ranging from 3 to 24 hours. Each set of plants, at the end of its metabolic period, was harvested and analyzed in the normal manner to determine the distribution of the fed activity. Table II gives the results for the determination of incorporated activity. The fed morphine represents a total of 0.42 mg, or 0.21 mg per plant. As expected, the amount of activity actually taken up by the plants showed an increase with time. The inconsistently high value observed for 9 hours could be due to a higher rate of nutrient uptake by that set of plants, as indicated by the fact that practically no nutrient solution remained in the flasks at the time of harvest (thus, the low figure for nutrient activity). The figures for 20 and 24 hours are relatively consistent with previous feeding experiments of 1-day duration.

Table II also presents the distribution of activity in the various fractions of the plant extracts. Several tentative conclusions can be drawn from these results. The amount of degradation of morphine after three hours of feeding is already fairly extensive, on the order of 50%. In fact, it definitely appears as if there is an initial burst of degradative activity, followed by a slower, more constant degradation, so that after 24 hours, approximately 75% of the morphine has been metabolized. This result could be a reflection of an initial rapid degradation caused by the presence in the roots of an abnormally high concentration of morphine. Then, once an equilibrium concentration is restored, the normal metabolic rate is also restored. Without knowing the identity of any of the metabolites, it is rather

Table II

^{14}C-NUCLEAR LABELLED MORPHINE FEEDINGS: SET 2

DISTRIBUTION OF ACTIVITY AS A FUNCTION OF TIME FED

0.21 mg ^{14}C-MORPHINE PER PLANT (55,400 dpm)

	TIME (hr) / FRACTION	3	9	12	20	24
% OF FED	1. ROOT WASH	44%	21%	26%	17%	32%
	2. NUTRIENT	23%	---	23%	15%	6%
	3. INCORPORATED	33%	79%	51%	68%	62%
% OF INCORPORATED	I. PLANT MASH	17%	17%	10%	18%	15%
	II. BuOH/Bz.	3%	1%	18%	1%	---
	III. ALKALOIDS	37%	16%	24%	22%	15%
	IV. AQUEOUS RESIDUE	17%	26%	24%	31%	31%
	TOTAL I-IV	74%	60%	76%	72%	61%

difficult to predict whether the initial degradation is an unnatural, induced one, or if it is still being degraded by the normal process. The only real trend which one sees in these results is a general shift in activity from the Alkaloid Fraction III to the Aqueous Residue (IV). The amount of activity in water-soluble and insoluble materials (which are here combined as "Plant Mash") appears to remain relatively constant, suggesting that the initial burst of degradation may indeed be an artifact caused by the feeding technique. This thought was kept in mind in subsequent morphine feedings, when the object was to search specifically for normorphine as a metabolite. In particular, in an attempt to avoid high initial concentrations, one feeding was done with a dilute solution of morphine in nutrient. This method had been used with success in work with tobacco plants.[21] Further discussion on this problem will be included when those feeding results are presented.

The suspicion that normorphine was involved in the metabolism of morphine was given further impetus by the results of this second set of feedings. The decision was therefore made to concentrate on this particular problem. The remainder of this part of the thesis will discuss the work done concerning normorphine. After those results are presented and discussed, their implications in terms of general morphine metabolism and the results presented up to this point will be discussed further.

D. $^{14}CO_2$ Biosynthesis - Search for Normorphine

1. Methods Used

In light of the preliminary findings in regard to the presence of normorphine in the poppy plant, a decision was made to run a $^{14}CO_2$ biosynthesis with the express purpose of isolating radioactive normorphine, by adding cold carrier normorphine to the plant workup. Cold carrier morphine would also be added, in hopes of deducing the relative quantities of these two alkaloids in the plant. Prior to doing this experiment, an efficient means of extracting normorphine from aqueous solutions was discovered. By shaking the solution containing normorphine at pH 8.6 with six portions of $CHCl_3$/isopropyl alcohol (3:1), one can effect an 80% recovery. Since morphine is quantitatively recovered using this solvent, the decision was to replace $CHCl_3$ with $CHCl_3$/IPA in the plant extraction procedure (for the phenolic alkaloids, only).

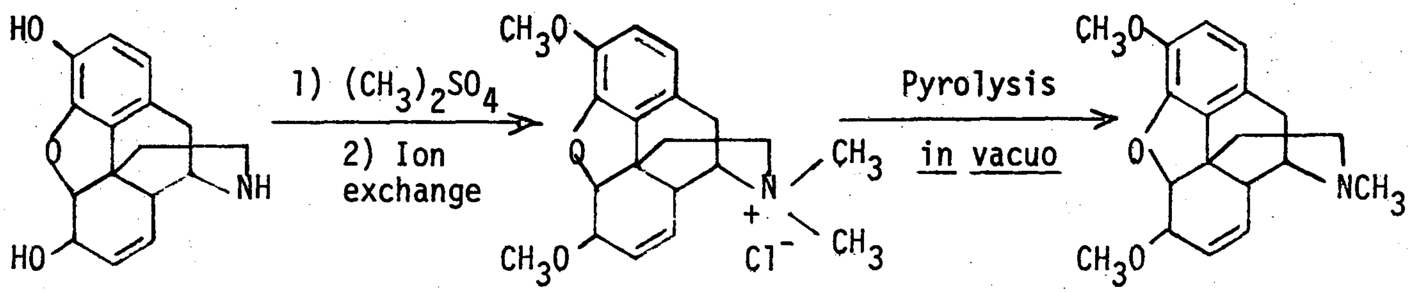
The method of purification and analysis chosen for this run was to purify the normorphine first by preparative tlc, to remove all traces of morphine, and then convert it to codeine methyl ether (VI) with dimethylsulfate (Scheme III).[23] The latter would then be purified to constant specific activity by preparative thin-layer chromatography and sublimation. The morphine would be demethylated to normorphine, so that one would be comparing only the ring carbons.

2. Results

Since the object of this biosynthesis was to get as much activity as possible into the normorphine (if it's there) to make the detection

Scheme III

Conversion of Normorphine to Codeine Methyl Ether



Normorphine | Codeine methyl ether methochloride | Codeine methyl ether

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easier, two approaches were possible, based on previous biosyntheses. One was to feed a moderate amount of $^{14}CO_2$, initially, and let the plants metabolize for a longer time (4 days) with $^{12}CO_2$; or a large amount of $^{14}CO_2$ could be used, so there would be enough to have a hot atmosphere throughout the entire (shorter) biosynthesis. The latter approach was chosen for several reasons; one, it is easier to keep the plants healthier for a shorter time (2 days, in this case); and, secondly, this approach would be expected to give fairly comparable specific activities in the morphine and normorphine, if the conversion is relatively facile, that is. Therefore, 2 days was chosen, and 120 mC of $^{14}CO_2$ was fed to the 10 plants, aged 75-90 days, over a period of nearly 30 hours. A small amount of $^{12}CO_2$ was needed at that time to restore the CO_2 concentration to 0.04%, as it was allowed to drop to 0.02% in order to incorporate as much $^{14}CO_2$ as possible. The plants were worked up nearly as usual at the end of 48 hours, with the exception being that the phenolic alkaloids were extracted at pH 8.6 with $CHCl_3$/IPA (3:1), rather than $CHCl_3$. 100 mg each of morphine and normorphine were added as carrier to the ground plant material before the extraction. The crude phenolic alkaloid product was immediately separated into its components by preparative tlc. The tlc, as expected, showed mainly normorphine (Rf 0.10) and morphine (Rf 0.30) with a few other very faint spots. The morphine and normorphine were well separated, and each was scraped from the plates. To assure that the elution from the silica gel was efficient, it was stirred with MeOH, twice, for 15 minutes, before filtering through a Millipore filter. The mass of the eluted alkaloids was

determined by vpc. This indicated recoveries of 13% and 36% overall for normorphine and morphine, respectively.

Since the step we were depending upon for establishing the presence of normorphine was the conversion to codeine methyl ether, we had to be absolutely certain that there were no impurities in the normorphine that could also be converted to CME. This included morphine and codeine, both of which give CME by the procedure used here. Codeine was safely eliminated, since it was extracted at pH 12.0 with the non-phenolics, and also since it has an RF even higher than that of morphine. Morphine was more of a problem. The only adequate analytical method available at the time for detecting traces of morphine in normorphine was a liquid-liquid chromatographic system. A good separation was obtained, using a one-foot column of silica gel, and eluting with $CHCl_3$/MeOH/Et_3N (2:1:0.1%). Morphine had a retention time of about 1 minute, while normorphine's was nearly five. However, the normorphine peak was very broad, making it difficult to quantify. Due to other problems, the lower limit of morphine detection was not too good, being on the order of 2 µg. Anyway, an aliquot of the ^{14}C-normorphine from above was run through this column, corresponding to about 100 µg normorphine. No morphine peak was observed; however, there was another very strongly uv absorbing band at about 30 seconds. A crude collection of the two peaks was made, and each was counted; this indicated that 65% of the activity was in the compound with short retention time, while only 35% was in the normorphine peak. However, this was of no great concern, as long as this material did not give CME upon methylation (which would seem quite unlikely).

The important fact established was that there was less than 2% morphine in this crude ^{14}C-normorphine.

With this in mind, a preparative thin-layer was run on 10 mg of cold normorphine intentionally contaminated with 0.4 mg (4%) ^{14}C-morphine (480,000 dpm). Three bands were cut from the plate after development and eluted as before with MeOH: normorphine, the band between normorphine and morphine, and morphine itself. The latter was visualized by running a morphine standard along the edge of the plate. Table III contains the counting results of this tlc. Vpc

Table III

Band	Activity (dpm)	%
Normorphine	12,300	2.4
(Norm-Mor)	122,000	24
Morphine	381,000	74
Total:	515,000	100

indicated a recovery of only 2.5 mg of normorphine. This was chromatographed once more, with the results shown in Table IV. Only about 1 mg of the normorphine was recovered. This result seems rather

Table IV

Band	Activity (dpm)	%
Normorphine	4000	100
(NM-M)	0	0
Morphine	0	0

curious at first glance, and one would seem to be led to the conclusion that it is impossible to eliminate all the morphine from normorphine. However, a more likely explanation is that there was a ^{14}C-normorphine contamination in the ^{14}C-morphine being used, since the latter was obtained from the former in the demethylation-remethylation of biosynthetic ^{14}C-morphine. So the conclusion from this study was that one preparative tlc was enough to completely eliminate a 4% morphine impurity from normorphine. Therefore, one more tlc was done on the crude ^{14}C-normorphine from the biosynthesis.

The conversion of the normorphine to CME was carried out in a manner similar to that used by Blaschke on 50-100 mg of normorphine.[24] A large excess of dimethylsulfate was used to assure complete conversion to CME-methosulfate, the ratio of Me_2SO_4 to normorphine being on the order of 100:1. A total of 580,000 dpm (65% of starting activity) was eluted from the ion exchange column as the methochloride salt of CME. This was then pyrolyzed to give CME. Two overlapping spots were observed on the tlc of this product, CME at Rf 0.41, and a second compound at Rf 0.39, initially presumed to be codeine. Since codeine and CME co-chromatograph on vpc, and give essentially the same mass responses, one could deduce from vpc that there was about 2.8 mg of CME (plus codeine). However, there was a second peak in the vpc, with a retention time of 4:00 minutes, compared to 5:05 for CME. Therefore, it was decided to try and purify the CME by preparative tlc. An analytical thin-layer chromatograph showed CME (Rf 0.41) and codeine (0.28) well separated, and also gave the first indication that the impurity was not codeine. The CME band was eluted with MeOH, as were a band immediately preceding CME (Rf 0.35), and a third band

at Rf.75. These were each counted, and analyzed by vpc, with the results shown in Table V. It is quite obvious that the Pre-CME compound from the prep. tlc is the 4:00 peak on vpc, and, therefore, is

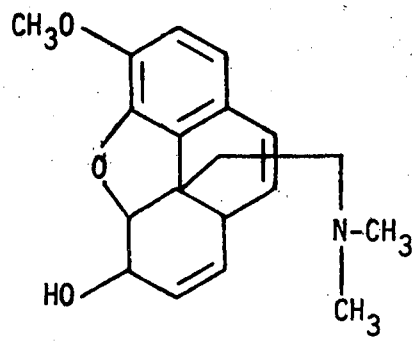
Table V

Band	Activity	VPC: Ret. time (pk. ht)	Mass (mg)	Spec.Act. (dpm/mg)
CME	55,400	5:05 (8.5), 4:00 (1.5)	2.5	21,000
Pre-CME	4,500	5:05 (0.2), 4:00 (1.0)	---	(15,000)
Rf 0.75	43,900	No peaks	---	---

not codeine. The figure for specific activity of the Pre-CME compound was calculated assuming a similar mass response as CME. The fact that the two specific activities are fairly close would lead to the conclusion that Pre-CME is also derived from normorphine.

Further information on this aspect of the purification was obtained when the ^{14}C-normorphine derived from the biosynthetic ^{14}C-morphine was converted to CME. This CME showed nearly the same behavior on tlc and vpc as that obtained from the biosynthetic normorphine. Thus, it showed two major spots on tlc, with R_f's of 0.41 (CME) and 0.35, as well as two minor spots, R_f's of 0.02 and 0.45. The CME recovered from a single preparative tlc gave two peaks on vpc, at 4 minutes and 5 minutes (CME), in the ratio of 1 to 12 respectively. The band with R_f 0.35 was also eluted, and it showed a UV spectrum with λ_{max}^{MeOH} 279, 302, and 313 nm; the major peak on vpc was the one at 4 minutes. One product we might expect from this reaction is a methine (as in the Hoffman degradation). Indeed, α-codeimethine (VII)[25] has a

UV spectrum with λ_{max}^{EtOH} 275 nm, ε = 10,700; 315 nm, ε = 2,700. The methylation reaction would yield the methyl ether of α-codeimethine, which should have a similar UV. It was decided, therefore, that UV mass determination was not the best choice in this case.



VII

α-Codeimethine

After extensive purification of both the normorphine and morphine from the biosynthesis, the purified codeine methyl ether from each was sublimed, and a portion was accurately weighed, and counted by directly dissolving it in scintillation solution. The final results thus obtained are summarized in Table VI. Despite the concerns with impurities, these data definitely imply that normorphine does exist in the poppy plant. If one makes the assumption that the specific activities of morphine and normorphine from the biosynthesis are the same (which proves to be fairly accurate subsequently), then the fact that equal quantities of each was added as carrier should mean that their final relative specific activities reflect their relative native abundances. This would say, therefore, that the normorphine/morphine mass ratio is on the order of 1-2%.

Table VI

$^{14}CO_2$ - BIOSYNTHESIS

	Compound	Specific Activity (dpm/mmole)
I.	$^{14}CO_2$ fed (120 mC total)	120×10^9
II.	Morphine	
	A. Total	105×10^6
	B. Nuclear-label (as CME)	96×10^6
III.	Normorphine (as CME)	1.12×10^6
IV.	Ratio: Morphine/Normorphine	86/1

E. Normorphine Isolation, Purification and Analysis

The preliminary search for normorphine from the $^{14}CO_2$ biosynthesis pointed out numerous flaws in the overall procedure, the major ones being the low recovery of normorphine using the standard alkaloid extraction procedure, purification of the isolated normorphine, and lack of a sensitive analytical means for detecting normorphine. All of these factors become extremely limiting when one is searching for small amounts of a natural product. How these problems were overcome is the subject of this section.

1. Normorphine Isolation

As mentioned previously, the problem of extracting normorphine from aqueous solution had been solved by switching to $CHCl_3$/IPA (3:1) as the extracting solvent. However, it was obvious from the low

recovery of normorphine in the $^{14}CO_2$ biosynthesis that at least one other step in the plant extraction was inefficient for normorphine. To check for the inefficient step(s), a known amount of labeled normorphine was added to some ground plant material. The plant was then ground almost as usual, after adding 21 ml of 10% Na_2CO_3 and 100 ml of BuOH/benzene (1:1). One difference was that the grinding was done for a longer time (15 minutes for first and second grinds, and 5 minutes for third). Another difference was that only three butanol/benzene extracts were made, and each was kept and counted separately, to give some idea of the effectiveness of each grinding. The countings were made by dissolving known aliquots in aqueous scintillation solution (125 ml Biosolv. BBS-3, 50 ml of Fluor Concentrate II, and 825 ml of toluene). This aliquot was one ml for all the butanol-benzene extracts and the aqueous portion of the plant mash, and 100 µl for the initial ^{14}C-normorphine in 10% Na_2CO_3. The results of this experiment appear in Table VII.

Table VII

Sample	Count (dpm)	Percent of Total
1. ^{14}C-Normorphine added to blender	6,200	100
2. BB Extract I (100 ml)	2,400	39
3. BB Extract II (100 ml)	630	2
4. BB Extract III (75 ml)	110	2
5. Aqueous Residue (after removal of solids)	3,000	48
6. Total Recovered	6,140	99

Two conclusions can be drawn from these results concerning this first step of the extraction procedure. First, normorphine is definitely being left behind in the aqueous mash to a large extent, much greater than one would like. And secondly, one appears to approach a limit as far as removing more of the normorphine. In hand with this, it is apparent that the traditional grinding with five portions of butanol-benzene is unnecessary, that two, and at most three, such extracts are enough. If one assumed that the normorphine was remaining intact through the process, then such a limit as one observes here would seem highly unlikely. Thus, a likely explanation of this problem could be that the normorphine is being destroyed or converted in some manner as to render it more water-soluble. One other possibility might be that the pH conditions of the aqueous mash are not suitable for efficient normorphine extraction, or that these conditions change as a result of the extraction process. A second experiment was performed with this question in mind, taking the logical approach that if the pH is maintained by using a buffer, this variable can be overcome.

The buffer chosen was Clark and Lubs' borate,[26] prepared from two stock solutions, one being 0.1 M boric acid in 0.1 M KCl, the other 0.1 M NaOH. This buffer was chosen since it is reported to be highly effective over the pH range of 8 to 9, which is precisely the range desired here. A single poppy plant, about 90 days old, was extracted as before with butanol-benzene, but the 10% Na_2CO_3 was replaced with 50 ml of borate solution buffered at pH 8.6, to which had been added 10.6 mg of cold normorphine. Two BB extractions were done, each for 10 minutes. A check on the pH of the filtered aqueous residue showed

it to be 8.55. In order to analyze for normorphine, it was necessary to extract the butanol-benzene phases with H_3PO_4 (1 M, pH 2.5), adjust the pH of the latter to 8.6, and re-extract with four portions of $CHCl_3$/IPA (3:1). From previous checks, the latter process, from BB to $CHCl_3$/IPA, yields about 50% recovery of normorphine. Therefore, the amounts of normorphine found in the $CHCl_3$/IPA extracts have to be doubled for comparison with the previous radioactive experiment. The results are summarized in Table VIII. Analysis was done by vpc,

Table VIII

	mg Normorphine	Percent of Original
1. Initial normorphine	10.6	100
2. Extract I		
- $CHCl_3$/IPA	1.45	(14)
- BB (by extrapolation)	2.9	27
3. Extract II		
- $CHCl_3$/IPA	0.55	(5)
- BB (by extrapolation)	1.1	10
4. Aqueous (by subtraction; 1 - (2 + 3))	---	63

by comparing with a standard curve of normorphine quantity versus detector response. These results can be seen to be very similar to the radioactive, unbuffered case, especially if we include a factor for the 33% shorter grinding periods in the buffered experiment. Obviously pH control is not the determining factor for increasing extraction efficiency.

It becomes apparent from these additional controls that the first step of the extraction process is the culprit. The original thought was that a change of organic solvent might be called for. Another approach, however, is to change the pH of the aqueous portion of the extract, and make it acidic. The alkaloids will be extracted from the plant into the aqueous acid, in which they are extremely soluble. At the same time, butanol-benzene can be retained in the grinding procedure, as a means of removing any neutral and acidic plant products, including the plant pigments. The aqueous acid, after separation from the plant residue by centrifugation, can then be adjusted to the proper pH, for extraction into organic solution, from which the alkaloid extract can be directly analyzed, either free, or after acetylation. This procedure essentially eliminates the formerly troublesome step, and should (and does: see below) give a much higher recovery of normorphine. Of course, this procedure is not generally applicable as an alkaloid extraction, since thebaine, and possibly other alkaloids, undergo degradation in the presence of strong acid.[27] However, since all we are interested in now is the identification of normorphine as a native plant constituent, this procedure is adequate.

An initial study was performed to test the feasibility of the proposed extraction procedure. A single poppy plant, about 60 days old, to which had been added 19.4 mg of cold normorphine, was extracted with 1 N HCl, in the presence of butanol-benzene. The procedure used was otherwise similar to that used formerly, in terms of grinding and removing BB supernatant. Two portions of BB were

used, but only a single portion of acid. Centrifugation effected a separation of the aqueous acid from the plant mash. Extraction at pH 8.6 with $CHCl_3$/IPA (3:1) then gave the alkaloids, which were dried, and analyzed by vpc. Comparison of the extracted normorphine with normorphine standards indicated the recovery of 9.8 mg (50%) of normorphine by the extraction scheme. Another 0.5 mg was recovered from the BB extract, implying that some was again left behind in the plant mash. However, if one assumes the extraction from aqueous to $CHCl_3$/IPA (3:1) to be 75% effective, then, by interpolation, one can conclude that the initial extract from plant to acid is also about 75% effective. It seems likely that extraction of the residual plant mash with a second portion of squeous acid may bring the latter figure to 90%, and, hence 60-70% recovery overall.

A final, complete extraction of a plant, with added carrier normorphine, was performed, to test the recovery of normorphine. A single 20-gram plant was used, and 10 mg of cold normorphine added to the ground plant before work-up. The work-up included two separate portions of 1 N HCl for the three-phase grinding process. Calculation of the recovery of normorphine was done by comparison of vpc peak heights of known amounts of normorphine. This indicated a recovery of 7.3 mg, or 73%, which is the best recovery of normorphine ever obtained in such a control experiment. This compares with a 50% recovery obtained using just a single portion of HCl in the grinding. Since the final extraction step from aqueous solution to $CHCl_3$/IPA (3:1) has been shown to be only 75% effective, it appears as if the initial extraction from plant mash into aqueous acid is 100% effective with the two portions of acid used.

2. Purification and Analysis

The approach used for the biosynthesis to purify and analyze the normorphine is acceptable if one has a relatively large quantity of it. However, if only several hundred micrograms is isolated, losses must be minimized, and a sensitive analytical method employed. In fact, if the analysis can be made in the presence of other alkaloids, then purification is not as major a problem. Hope for such an approach came with the discovery of a gas chromatographic system which separated morphine and normorphine. The column was 6 feet of 3% OV-17 (silicone, 50% methyl, 50% phenyl) on Aeropak (100-120 mesh), packed in a glass column. The use of the all glass system has been found to enhance the sensitivity and reduce the tailing of the morphine alkaloids.[15] At a temperature of 250°C, morphine has a retention time of 5:10, and normorphine 6:00. The desire was to analyze for small amounts of normorphine in morphine. In practice, however, the limits of normorphine resolution combined with the morphine tailing made quantitation difficult.

Anders and Mannering have reported using the peracetates of the morphine alkaloids for gas chromatographic analysis.[28] They obtained their information by direct on-column acetylation, by co-injecting acetic anhydride. This was tried with a morphine-normorphine mix, and gave a series of peaks, not unexpectedly. This was discarded in favor of a complete acetylation, using the conditions by which morphine is totally converted to diacetylmorphine (heroin).

To test this approach, 100 mg of normorphine was treated with one ml of refluxing acetic anhydride. The reaction was followed by tlc.

After only one-half hour of reflux, two distinct products were observed, at R_f's of 70 and 75 (compared to 5 for normorphine), and the products were already present to the extent of 70-80%. After two hours, the two product spots had coalesced, mainly due to a larger quantity of the higher R_f spot. There was still a small spot for normorphine (~5%). The refluxing was continued for 18 hours, following the published recipe; in retrospect, this long time period is not necessary -- six hours is probably enough. After 18 hours, only a single spot existed on tlc, at R_f 75. Vpc analysis was done on the product after work-up (see Table IX). The peak at long retention time was the only one in the product. NMR of the product clearly indicated that it was the tris-acetate.

Table IX

Compound	Vpc Retention Time
Normorphine	5:40
Acetylated normorphine	32:20
Heroin	8:30

A mixture of morphine and normorphine was then acetylated in the same manner. Tlc of the reaction mixture just prior to the onset of reflux showed the normorphine completely acetylated, and the morphine was gone after a total of six hours of reflux. The normorphine ester had an R_f of 70, and heroin an R_f of 63 (the latter was compared with a standard sample of heroin). Vpc analysis of the product is summarized in Table X. The peak at 5:45 in the product is apparently

Table X

Compound	Vpc Retention Time
Normorphine ester	32:00
Heroin	8:20
Morphine + normorphine acetylation product	32:00, 8:20, 5:45

due to unreacted morphine, or possibly mono-acetylated morphine. The same peak was observed, in smaller relative amount, in the supposedly pure sample of heroin, and was also seen on tlc, as a spot just trailing heroin. The R_f is higher than expected for morphine, so it is most likely a mono-acetylated morphine.

The gc separation thus achieved by acetylation is remarkable. But several other benefits were gained at the same time. The resolution of the normorphine was significantly enhanced, the lower limit of detection decreasing from about 200 nanograms to 50 nanograms. The peracetates were also found to be easily separable by thin-layer chromatography, which provided a good means of purification. And, finally, the normorphine-Ac_3 was stable to tlc conditions, and to gc conditions below 235°C, both of which help eliminate the problem of losses encountered with free normorphine base.

To find the best tlc system for separating the two peracetates in question, several systems were tried. Table XI lists the results of this testing. The choice was made to use absolute EtOH/ϕH (1:4) for the initial separation of the acetylated mixture of crude plant alkaloids, when the desire was to purify the peracetates for

Table XI

Tlc of Morphine and Normorphine Peracetates

Plate Characteristics	Solvent System	R_f's Morphine-Ac_2	R_f's Normorphine-Ac_3
1. Silica gel, Eastman, 100 μ thick, w/fluor. ind.	abs. EtOH/benzene (1:4)	45	68
2. "	$CHCl_3$/MeOH/NH_3 (3:1:tr)	65	72
3. "	abs. EtOH/ϕH (1:9)	32	43
4. "	abs. EtOH/ϕH (1:2)	42	61
5. "	95% EtOH/ϕH (1:4)	42	58
6. Homemade silica gel, 250 μ	abs. EtOH/ϕH (1:4)	27	53

subsequent specific activity analysis (as for ^{14}C-morphine feedings and $^{14}CO_2$ exposures.)

3. Control Experiments

The results concerning the presence of normorphine, to be presented shortly, depend heavily on proof that normorphine is not just an artifact of the work-up procedure or the analysis. To provide this proof, it is necessary to show experimentally that no normorphine is produced from morphine by any step in the plant work-up, or in the subsequent analytical manipulations. It is also of interest to know that normorphine is not being produced from some other unknown or unsuspected

native plant product; however, I can conceive of no experiment at this point that could answer that question. Therefore, it is felt that if one shows that no normorphine is produced from morphine by the work-up procedure, control criteria will be adequately met.

Before any checks were made on the extraction procedure, the morphine which would be used was tested for purity; vpc showed that it did, indeed, contain at least two impurities, one which looked suspiciously like normorphine. When this bottled morphine was acetylated, there was found a significant amount of normorphine-Ac_3 (about 1.3%). Purification of this morphine by sublimation eliminated practically all of this normorphine impurity (see Table XII), so the re-sublimed morphine was used in the control experiments. It should also be noted that the heroin which has been used as standard material also contains a significant amount of normorphine-Ac_3. This, therefore, was not used further as a standard for vpc analysis. To prepare standards for the latter purpose, a known amount of normorphine was mixed with various quantities of morphine to give samples with normorphine/morphine mass ratios of approximately 2.5 - 7.5%. The ratios of the vpc peak areas were then determined for each sample, and a plot of peak area ratios versus mass ratios prepared (Figure 1).

Two different checks were run on the total extraction procedure, using 1 N HCl as the aqueous phase. Control no. 6 was done with re-sublimed morphine in the blender, but without plant material. Control no. 7 was done with ^{14}C-morphine, with plant material. Both these checks produced significant amounts of normorphine (2 - 2.5%). Further

Table XII

Control Experiments

No.	Procedure Tested	Results NM/M Ratio (vpc)	Other
1.	Pure morphine from bottle.	---	Trace of normorphine
2.	Heroin from stock.	1-2%	---
3.	Acetylation of bottled morphine.	1.3%	---
4.	Sublimed morphine, acetylated.	0.4%	---
6.	Morphine through HCl/BB extraction procedure, without plant.	2.5%	82% recovery
7.	^{14}C-morphine + plant + carrier normorphine.	2.2%	---
9.	Total procedure without plant or blender, + morphine.	0.3%	---
10.	Morphine + HCl + BB + air, 70°C for 15 minutes .	0.8%	---
11.	Morphine + H_3PO_4 (1 M, pH 2.5) + BB + air, 70°C, 30 minutes.	0.4%	---
12.	Repeat 10, + hydroquinone, 70°C for 25 minutes.	1.8%	---
14.	Normorphine extracted via modified procedure, without plant, with blender.	---	50% recovery
15.	Morphine + blender, modified procedure, temp. less than 40°C.	0.4%	100% recovery

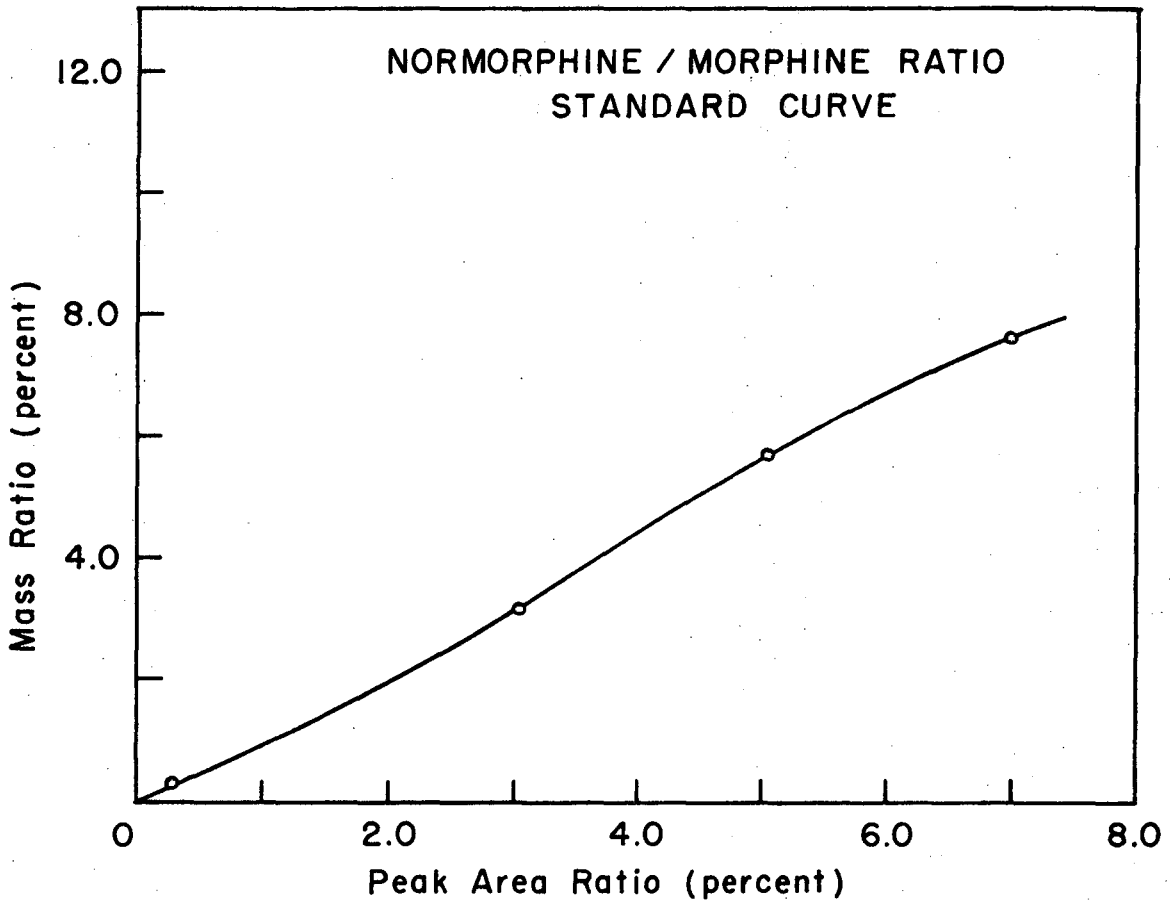
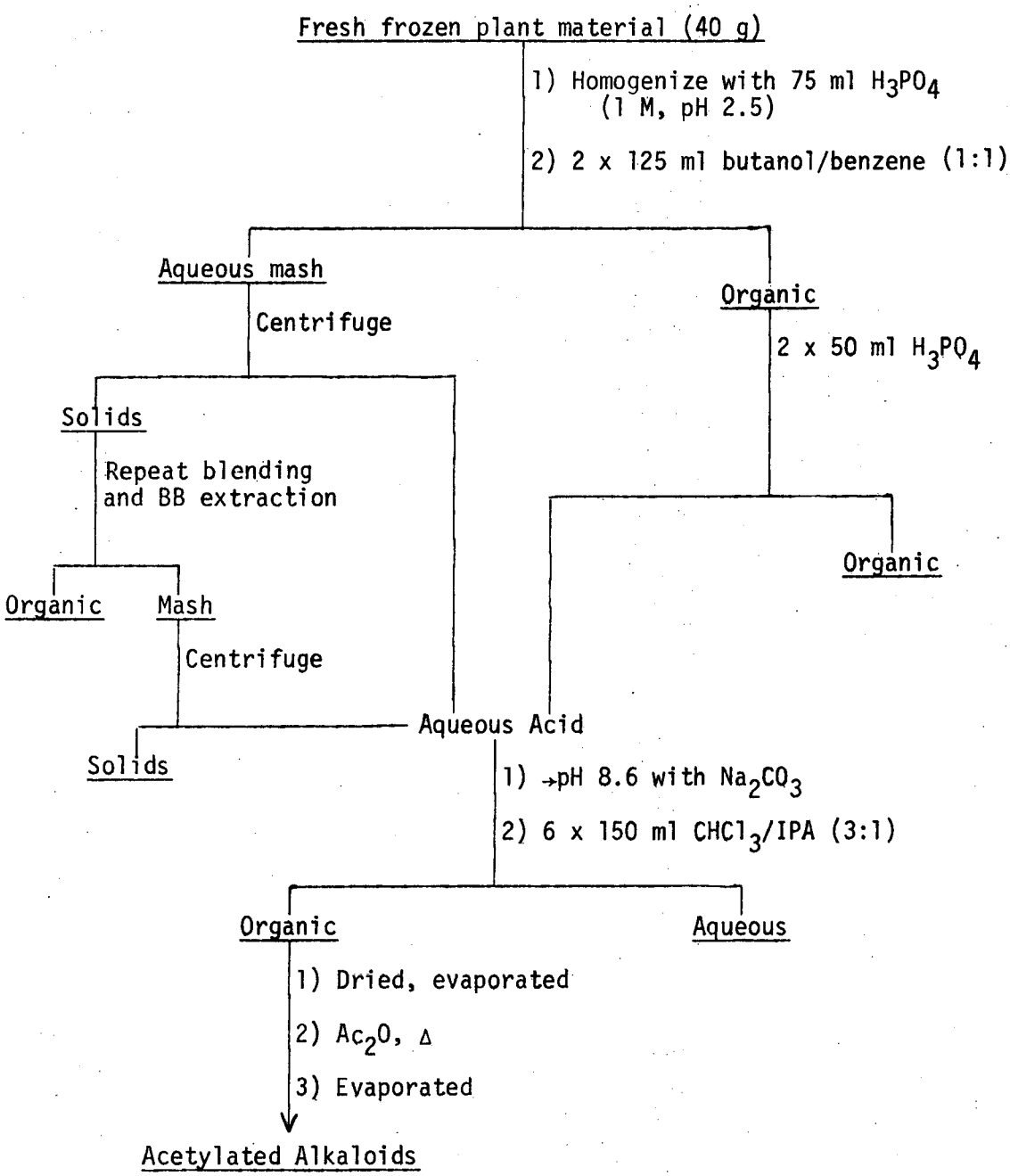

NORMORPHINE / MORPHINE RATIO
STANDARD CURVE
12.0
8.0
4.0
Mass Ratio (percent)
0
2.0
4.0
6.0
8.0
Peak Area Ratio (percent)
XBL725-4658

Figure 1

control experiments (nos. 9-12) seemed to implicate the blending process as the culprit, i.e. the combination of heat, air, and 1 N HCl. Thus, when no heat is applied (no. 9), there is no significant production of normorphine; likewise, when the aqueous phase was changed to a buffered acid, no normorphine was produced (no. 11). Finally, when morphine was subjected to a complete extraction, using H_3PO_4 (1 M, pH 2.5) instead of 1 N HCl, and keeping the temperature of the blend below 40°C by putting the blender on ice when necessary, there was again no significant production of normorphine. This modification was subsequently used for the $^{14}CO_2$ biosynthesis, as well as for checks on the ontogeny studies. As seen in controls no. 14 and no. 15, the relative recovery efficiencies for this modified procedure now favor morphine by a factor of 2. This represents a significant loss of efficiency for normorphine, when compared to that obtained using HCl.

The studies of normorphine isolation and analysis, and the controls, led to a definitive procedure for subsequent normorphine studies. The modifications of the standard plant extraction procedure include only a change to an acidic phosphate buffer for the initial plant extraction, and direct acetylation of the crude alkaloid residue for subsequent purification and analysis. The entire process is outlined in Scheme IV. It should be emphasized again that this procedure is not necessarily useful for general plant studies. The experience gained here from the control experiments is adequate testimony to the care which must be taken in such biochemical studies.

Modified Plant Extraction Scheme for Normorphine Studies



XBL 725-4634

Scheme IV

F. Presence of Normorphine in Poppies and Opium

Since normorphine had never been reported as a native plant alkaloid, it was of interest not only to establish that it did exist, but also to determine its relative abundance as a function of plant age. The hope of such a study was that it might give some clue to the biosynthetic relationship of morphine and normorphine, especially in conjunction with the results from morphine feedings and $^{14}CO_2$ exposures.

A series of plants of various ages was, therefore, extracted via the modified alkaloid extraction procedure, and the ratio of normorphine to morphine at each age determined by vpc analysis of the acetylated extracts. A certain number of these extractions were performed using 1 N HCl as the initial aqueous phase, before it was discovered that this led to aberrant normorphine production. The normorphine/morphine mass ratios found for these initial extracts were corrected for this aberrant normorphine. Subsequent checks using buffered H_3PO_4 as the aqueous phase showed the corrections to be valid within experimental error.

As mentioned previously, to avoid any errors caused by irreproducible vpc analyses, the normorphine/morphine mass ratio was determined by a single sample injection, by determining the relative area ratios and comparing with standard mixtures. The mass of morphine was determined at the same time. The results for the morphine concentration as a function of plant age appear in Figure 2, and the normorphine/morphine mass ratios in Figure 3. The youngest plants looked at were 20 days old. At this age, the plants are only a few centimeters tall, and still in the immature stage with respect to

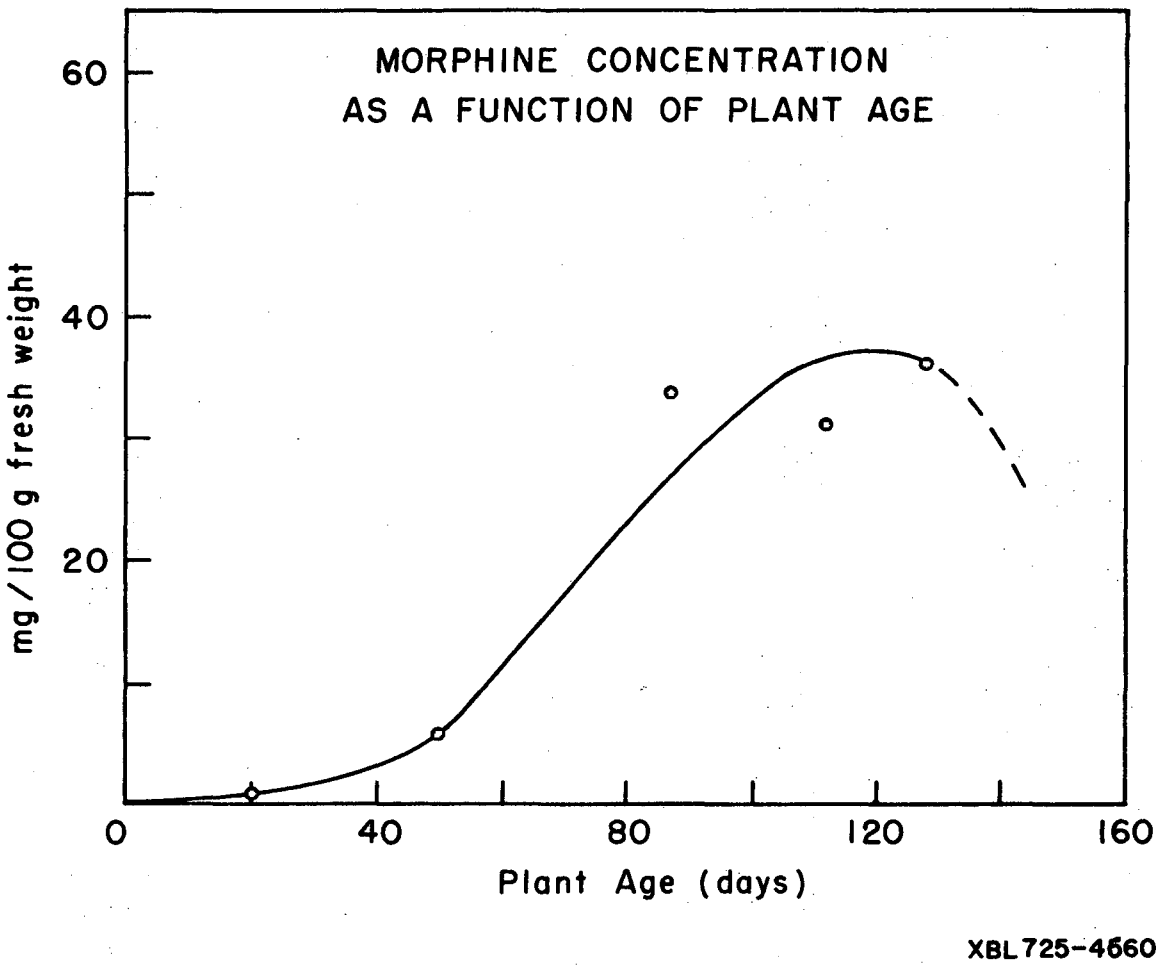
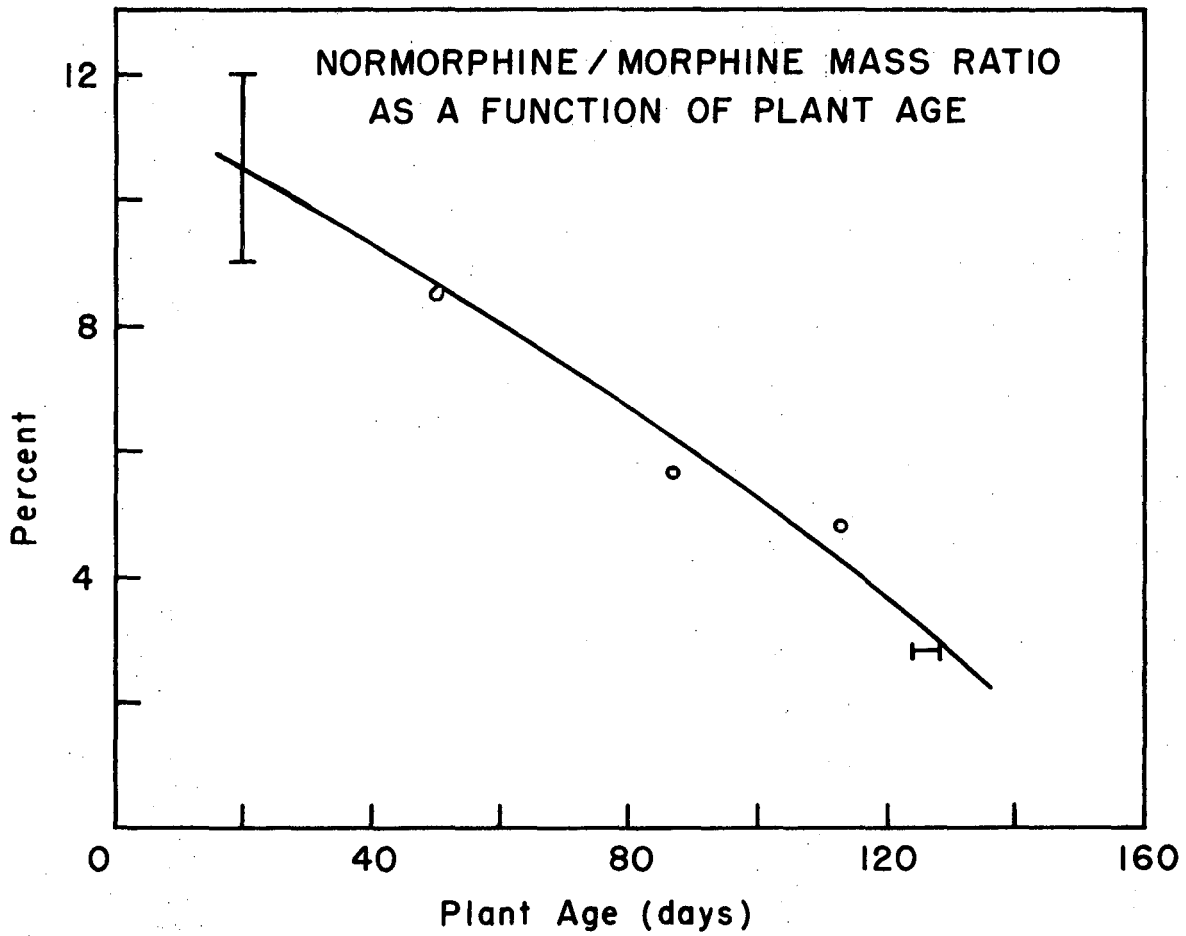

MORPHINE CONCENTRATION
AS A FUNCTION OF PLANT AGE
mg / 100 g fresh weight
60
40
20
0
40
80
120
160
Plant Age (days)
XBL725-4660

Figure 2



XBL 725-4659

Figure 3

morphine biosynthesis. Codeine and thebaine are much more abundant than morphine. Though there was very little of each, an approximation could be made that normorphine was present to the extent of 10% of the mass of morphine. All the other figures are more significant, especially with respect to one another. One problem with studies of this type is the great variability of the plants. Two plants of the same chronological age can vary greatly in extent of development, size, etc. The plants studied here were all grown within three months of each other, to try to avoid differences caused by seasonal conditions. The major point, therefore, to be drawn from these results is the significant downward trend of the normorphine/morphine mass ratio with age. Although the ratio decreases, however, the absolute concentration is increasing slowly. What these results might mean in terms of morphine and normorphine metabolism will be discussed after presenting the biosynthetic studies performed.

In addition to the plants, two samples of raw opium were available, so both of these were also searched for normorphine. A sample of Turkish Opium showed a normorphine/morphine mass ratio of 3.1%, with morphine comprising 12.5% of the weight of the raw opium. Indian Opium, on the other hand, had less morphine (7.0%), but a significantly higher normorphine/morphine mass ratio, 8.0%. The fact that these are different is undoubtedly a reflection of the manner in which the opium is harvested and treated. That the two samples were different was obvious, since the Turkish Opium was of a dark, very hard glassy appearance, while the Indian was granular in texture, and a light tan in color. It may also be that different species of P. somniferum

have different levels of the various alkaloids. What is important from the study of opium is that normorphine is apparently being thrown away during the commercial isolation of morphine. These results also explain why there was a trace of normorphine in our commercial morphine.

G. Morphine Feedings, Set 3

With the knowledge in hand that normorphine does exist in the plant, two labeled morphine feedings were performed in an attempt to show that morphine is, indeed, the direct precursor to normorphine. The first one employed the dilute feeding technique, with aeration.[21] It was hoped that this method would give good incorporation, and be somewhat closer to the natural greenhouse conditions under which the plants were grown. Unfortunately, the morphine was subsequently found to be somewhat unstable to the aerated conditions. Though this instability appeared to be of no major concern regarding the incorporation of activity into normorphine, nevertheless a second feeding was done using the "starvation" technique as a further check.

1. Morphine Feeding Number 5

Three 72-day-old plants, each weighing about 25 grams, were fed a solution containing about 10 mg of ^{14}C-morphine (3.14×10^7 dpm/mmole) in 300 ml of poppy nutrient. The incorporation of activity was followed for the first 4.5 hours by counting an aliquot, and estimating the volume of remaining nutrient. Enough nutrient was added periodically thereafter to maintain the level near 300 ml. The plants were killed after 22 hours, and the activity remaining in the nutrient and

in a root wash were determined to calculate the total activity incorporated by the plants. This incorporation data is summarized in Table XIII. As has been commonly true in the past, a good portion (~50%) of the residual unincorporated activity is removed by an acidic wash from the plant roots.

Table XIII

Incorporation of ^{14}C-Morphine

Time (hrs)	Total Count in Nutrient	As Percent of Fed
0	1,170,000 dpm	100
1	640,000 "	55
2	720,000 "	61
3	600,000 "	51
4.5	466,000 "	40
22	406,000 "	35
Total ^{14}C incorporated (by subtraction):	764,000 dpm	65%

Work-up of the plant material was done by the aqueous acid-butanol-benzene procedure, after adding 10 mg of cold carrier normorphine to the plant material. The distribution of the incorporated activity into the four major fractions obtained by the extraction procedure is summarized in Table XIV.

To check whether the integrity of the morphine in the feeding solution had been maintained, the residual nutrient and root wash were

Table XIV

Distribution of Incorporated Activity

Fraction	Total Activity	As Percent of Incorporated
I. Plant Mash	358,000 dpm	47
II. Butanol/benzene	32,000 "	4
III. Residual aqueous	157,000 "	20
IV. Crude alkaloids	229,000 "	30
Total Recovered:	776,000 dpm	101%

combined and extracted at pH 8.6 with $CHCl_3$/IPA (3:1). This removed a total of 108,000 dpm, or 69%, of the residual activity. The material was purified once by tlc. The compound so obtained gave a single peak on vpc, with a retention time of 5:53 (morphine has a retention time of 4:40). Assuming a mass response identical to that of morphine, the specific activity of this compound was quite close to that of the morphine originally fed. Subsequent treatment of it with refluxing Ac_2O gave a peracetate which co-chromatographed with heroin. To check whether this conversion of morphine was caused by the method of feeding, 11.7 mg of morphine was dissolved in 300 ml of nutrient solution, to which was added portions of roots from several plants and an air bubbler, as in the feeding experiment. After four hours, the solution was extracted at pH 8.6 to recover the alkaloids. The dried alkaloid residue gave two peaks on vpc, at 4:40 (morphine) and 5:40 (same as above). The latter peak was twice as large as the morphine peak.

The morphine degradation product was separated from the morphine by preparative tlc; the R_f's were 40 and 20, respectively. The chromatographic data seems to indicate a mono-acylated type of compound. The mono-acetate which one sees first in the acetylation of morphine to heroin has similar chromatographic properties. In addition, nmr of the degradation product, though not definitive with regard to absolute structure, shows the N-CH_3 still intact. The fact that the bulk of the unincorporated activity resides in this product seems to imply that it is taken up by the plants very slowly (if at all) relative to the morphine. This, along with the preliminary data on structure, makes it highly unlikely that the degradation product will lead to aberrant normorphine production in the plant.

The normorphine-Ac_3 and morphine-Ac_2 were separated by preparative tlc, using EtOH/benzene (1:4) as developing solvent. Subsequent vpc analysis of the eluted normorphine-Ac_3 showed only one other peak, with a retention time of about 3 minutes. Therefore, a second preparative tlc was done, developing this time with $CHCl_3$/MeOH/NH_3 (3:1:trace). Only a trace (<1%) of the 3-minute peak remained. This material was used as is to determine the specific activity of normorphine. The morphine-Ac_2 obtained from the first preparative tlc was contaminated with codeine-Ac. Since the conversion of morphine to codeine does not occur,[20] the specific activity of the morphine-Ac_2 could be determined from this product, by assuming all the activity is in the morphine-Ac_2. These results are summarized in Table XV.

Table XV

Feeding Number 5 Results

Sample	Mass (mg)	Specific Activity (dpm/mmole)	Total Activity (dpm)
I. Morphine incorporated	7.0	3.14×10^7	760,000
II. Recovered alkaloids			
A. Morphine	5.4	1.07×10^7	202,000
B. Normorphine (10 mg carrier added)	4.75	0.097×10^7	17,100
C. Total	---	---	219,100
III. Crude alkaloids (before purification)	---	---	229,000

2. Morphine Feeding Number 6

Three plants, about 70 days old, were fed a total of 6.8 mg nuclear-^{14}C-morphine (751,000 dpm). The amount of nutrient was kept to a minimum for the first six hours, at which point the roots were rinsed in dilute acid. The plants were then allowed to metabolize the incorporated morphine for an additional 18 hours, or a total of 24 hours. The plants were worked up as usual. The incorporation and distribution results are summarized in Table XVI. The distributive results are not much different than from the aerated feeding, except for a drop in the activity in the aqueous residue. The incorporation compares favorably with that which has been observed for a six-hour feeding in the past.

Table XVI

Incorporation and Distribution of Activity

Fraction	Activity (dpm)	As % of Fed
Fed morphine	751,000	100
Nutrient and root washes	470,000	63
Incorporated	281,000	37
		As % of Incorporated
I. Plant mash	102,000	36
II. Butanol/benzene	11,500	4
III. Aqueous residue	24,400	9
IV. Crude alkaloid	111,000	39
Total recovered:	249,000	88

Before the crude alkaloids were acetylated, the mass recoveries of morphine and normorphine were determined by vpc comparison with standards. Of a total of 14.7 mg of carrier normorphine added to the work-up, approximately 6.4 mg were recovered (43%); the morphine mass was 5.6 mg. This meant a normorphine/morphine mass ratio of 1:1. After acetylation, the mass ratio was 1.08, which, within experimental error, indicates that no morphine is transformed to normorphine by the acetylation conditions, nor is there any other significant destruction of either of the two.

Both the morphine and the normorphine (as their peracetates) were purified by preparative tlc to constant specific activity. The total results are summarized in Table XVII. The activity recovered as morphine and normorphine represents only 77% of the total activity recovered in the crude alkaloid fraction. However, the bulk of the remaining activity came at the origin of the first preparative tlc, and is apparently non-alkaloidal impurities.

Table XVII

Feeding Number 6 Results

Sample	Mass (mg)	Specific Activity (dpm/mmole)	Total Activity (dpm)
I. Incorporated morphine	2.6	3.14×10^7	281,000
II. Recovered alkaloids			
A. Morphine	5.6	3.9×10^6	83,000
B. Normorphine	7.8	0.075×10^6	2,000
III. Total plant alkaloids			
A. Morphine*	7.5	3.9×10^6	103,000
B. Normorphine**	~0.4	$\sim 3 \times 10^6$	4,000
IV. Metabolized morphine	---	----	174,000

*Based on 75% recovery of morphine

**Based on 50% recovery, and a 6% native normorphine/morphine mass ratio

A similar check, as done for the first feeding, was made on the integrity of the residual unincorporated activity. Analysis of the extract from the nutrient and root washes by vpc and tlc indicated only morphine, and its specific activity was unchanged.

Table XVIII presents a condensed summary of both feedings. The total activity existing in the plants as morphine was calculated by assuming a 75% isolation recovery to the crude alkaloid stage. The normorphine activity could be determined quite accurately by extrapolation back to the amount of carrier added (assuming the native pool to be small, relative to the carrier). It should be noted that the alkaloid activity in both feedings resides only in morphine and normorphine, within experimental error. This suggests that the metabolic breakdown of morphine proceeds either solely *via* normorphine, or both *via* normorphine and directly from morphine. The differences between the two feedings in terms of normorphine production is no doubt a reflection of the difference in feeding techniques. The starvation technique is the more severe, and may cause significantly reduced or altered biosynthetic activity. These results, nonetheless, do support the hypothesis that normorphine is formed from morphine, and is an active participant in the metabolic degradation of morphine. Further reference to these results will be made after presenting those obtained from a $^{14}CO_2$ "steady-state" exposure.

Table XVIII

^{14}C-NUCLEAR LABELLED MORPHINE FEEDINGS: SET 3

Feeding Number	5	6
Method of root feeding	Dilute solution, with aeration	"Starvation"
Feeding Time (hours)	}24	6
Metabolic Time (hours)		18
Fed: Activity (dpm)	1,170,000	751,000
Mass (mg)	10	6.8
Incorporated: Activity (dpm)	764,000	281,000
Percent	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

H. $^{14}CO_2$-Biosynthesis No. 3

This biosynthesis was run for the sole purpose of establishing the biosynthetic relationship of morphine and normorphine. Four plants were used, each of them 90 days old (two of them had already developed immature capsules) and weighing about 25 grams. Steady-state conditions were maintained as closely as possible throughout the 6-hour run. A small amount of $^{12}CO_2$ was added at the beginning of the run, along with the $^{14}CO_2$, to insure that the specific activity would not decrease. The CO_2 concentration was set at 0.035% at the start, and dropped only slightly during the last half-hour of the exposure. It was planned to use a total of 120 mC of $^{14}CO_2$; however, about 50 mC was lost from the pressurized $^{14}CO_2$ cylinder at the start of the experiment, leaving only about 70 mC for the exposure. Of this, about 20 mC was left in the chamber at the end of six hours, meaning 50 mC ($\sim$100 ml) was taken up by the plants. It should also be noted that the actual time of active metabolism was more like three or four hours. The plants were quite wilted at the start of the exposure. It was noticed that the humidity was around 10%, which is much drier than these plants are accustomed to. Beginning after one hour, therefore, a tray of water was placed in the chamber, and the humidity rose to about 40% at the end of the second hour, and finally to 50%, where it was maintained. The plants responded favorably to this change, and looked fairly normal again after two hours.

The plants were worked up *via* the modified acid/butanol/benzene procedure, with one slight change. Rather than including the

butanol/benzene during the grinding process, the plants were ground with only the aqueous acid phase, centrifuged to remove the solids, and the aqueous extracted by hand with several portions of butanol/benzene. The end result is undoubtedly nearly the same, but the number of manipulations is significantly decreased (an important factor when one has to extract in the hot box). The normal procedure was followed once the aqueous acid phase had been washed with butanol/benzene.

The specific activities of the isolated morphine and codeine were determined by collecting aliquots from several gc injections of the crude acetylated alkaloids, and then determining the mass of the particular alkaloid by re-injection, and the activity of the collected material. In addition, the total mass of each alkaloid in question was determined by gc of the crude mixture. The normorphine presented a slightly different problem in determining its specific activity; in particular, the small mass present, and the presence of several hot impurities chromatographing very close to normorphine-Ac_3 in the vpc. Therefore, the normorphine-Ac_3 was purified first by three separate preparative tlc's. During this purification process, it was discovered that one particular colored (pale green) plant product gave a gc peak which was only four minutes different from that of normorphine-Ac_3 (32 min for the former and 36 min for the latter, at a column temperature of 240°C, which was the temperature used for collecting). This compound was also found to be extremely hot, relative to the alkaloids. However, the three separate preparative tlc's did remove most of this impurity, as well as others. The final

activity determination was then done by collecting a known aliquot of the normorphine-Ac_3 from the vpc. To assure that only that activity due to normorphine-Ac_3 itself was being counted, the collection was done over a 20-minute period (the normorphine-Ac_3 peak in the middle) at one-minute intervals. Thus, a plot of activity collected for each interval versus time showed clearly the actual activity as a peak corresponding to the mass peak for normorphine-Ac_3.

The final piece of information desired was obtained by demethylating a portion of the morphine, to determine the activity residing in those carbon atoms common to both morphine and normorphine. The standard Von Braun reaction was used for this purpose. The normorphine so obtained was purified by preparative tlc, to remove any traces of norcodeine produced from codeine, of which there was some in the morphine. The system used for this purpose was silica gel layers, developed with EtOH/dioxane/benzene/NH_4OH (1:8:10:1).[29] In this system, normorphine has an R_f of 0.11, and norcodeine 0.24, while morphine's is about 0.4.

The results of these analyses appear in Table XIX. The relative specific activities of the codeine and morphine demonstrate satisfactorily that the exposure was indeed maintained under steady-state conditions, since the codeine is significantly hotter (even considering the extra 3-O-CH_3 group, which is typically on the order of 25% of the total activity for this length of exposure). The results for the nuclear carbons of the morphine and the normorphine do, within experimental error, further support the hypothesis that the normorphine comes about as a result of a demethylation of morphine. The near

Table XIX

$^{14}CO_2$ Steady-State Exposure

Compound	Specific Activity (dpm/mmole)	Total Alkaloid (mg/100 g fresh weight)
Codeine	440×10^6	4
Morphine	38×10^6	26
Morphine (minus N-CH_3)	26×10^6	--
Normorphine	27×10^6	~1

identity of the specific activities of each also supports the proposed hypothesis that the further metabolism of normorphine occurs at least as fast as its formation from morphine. In addition, this identity leads one to conclude that the formation of normorphine potentially plays a very active role in the metabolism of morphine; i.e., in a period of only 4-6 hours, the normorphine and morphine pools are in equilibrium with respect to activity, which requires at least one complete cycle in the degradative turnover of normorphine. Comparing this result with those obtained from the feeding experiments leads one strongly to the tentative conclusion that the natural degradation of morphine via normorphine is, at the least, a major contributor and, indeed, could be the sole major mechanism which the plant can use for the further metabolism of morphine.

I. Discussion

Although it is fairly difficult to make definitive conclusions from the kinds of results presented here, nevertheless, certain implications are apparent. As already suggested, the results of the morphine feeding experiments could be explained almost entirely by a metabolic breakdown *via* normorphine, if the latter's turnover rate is on the order of 2-4 hours (as suggested by the $^{14}CO_2$ exposure). The initial burst of degradation seen in the early feedings can be postulated to occur as a result of high local concentrations of morphine, implying that control mechanisms are at work to maintain an equilibrium concentration of the various alkaloids. This would seem to imply that the level of morphine is a major controlling factor in the synthesis of the morphine alkaloids.

One fact that is extremely interesting 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 specific biosynthetic pathway.[20] That the normorphine concentration fails to show this same behavior with age seems to imply that the rate of turnover of morphine is fairly constant over the life cycle of the plant. In particular, it means that the rate of formation of normorphine is nearly equal to the rate of breakdown. The relative increase in normorphine concentration with age might, then, merely reflect an increase in degradative sites.

The rapid turnover seen here without a doubt implies 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 (III). This fact lends support to the hypothesis that the alkaloids are involved as specific methylating agents, the relative stabilities of the successive methyl groups affording the plant a specific control of methylation processes. All other passive roles, such as protection from predators, or even simple biochemical control factors, cannot be justified on the basis of our results, since they would not require the high turnover observed.

A recent study by Fairbairn and Djote on isolated stem and capsule latex, led to the conclusion that morphine metabolism is mainly associated with stem latex.[30] Although our results neither support nor refute this hypothesis, the indications here are that, prior to flowering, there is total equilibration of both the morphine and normorphine pools throughout the plant. This would require that active synthesis and degradation are occurring in the entire plant. It may be that flowering, or maturation of the seeds, is the signal for the plant to shut off alkaloid biosynthesis, and the alkaloids in the capsule latex may no longer be required for methylating activity. It would indeed be of interest to do a $^{14}CO_2$ exposure with mature plants, and see if, in fact, there is a difference between the stem and leaves, and the capsule, with regard to morphine and normorphine equilibration.

III. CONCLUSION

The work presented in this part of the thesis has established beyond a doubt that morphine is an active metabolite of P. somniferum. The presence of normorphine, and the results of morphine feedings and $^{14}CO_2$ exposures, lead one to the conclusion that morphine is degraded to other non-alkaloidal metabolites solely via normorphine. The rate of this turnover is such that the normorphine pool is completely recycled about six to ten times daily, while the morphine pool may undergo complete turnover within a two-day period. This active metabolism, as well as the completion of the demethylation sequence from thebaine to normorphine, lends substantive support to the hypothesis that the morphine alkaloids function as specific methylating agents, and as such are important to the plant's economy.

More information concerning the effect of plant age on rate of metabolism might be sought from further $^{14}CO_2$ exposures. It also remains to be shown that normorphine is not methylated by the plant back to morphine. This irreversibility has been shown to be true for the steps leading up to morphine.[31] Future work concerning morphine and normorphine metabolism would be much more feasible if cell-free extracts can be isolated which perform these vital functions.

IV. EXPERIMENTAL

A. $^{14}CO_2$ Biosynthesis

Four poppy plants (average age 3-3/4 months) were transplanted from vermiculite to nutrient solution 3 days prior to the start of the hot run. On the morning of the hot run, the plants were placed in silvered flasks (250 ml) containing nutrient solution and transferred to the growth chamber of the hot box in the Round House. The growth chamber had been purged of its natural CO_2 overnight, by passing the chamber's air through a freshly-charged Ascarite chamber. The CO_2 level prior to charging the chamber was approximately 0.01% (as compared to 0.04% normally). $^{14}CO_2$ was passed into the growth chamber until the level of CO_2 was restored to 0.04%. The $^{14}CO_2$ flow rate was adjusted to about 1.5 ml/min. After about four hours, it was necessary to add $^{12}CO_2$ to maintain the total CO_2 level at 0.04%. The level of radioactivity had dropped to about 10% of its maximum value by the end of the day. For the next 3-1/2 days, the lights in the growth chamber and the $^{12}CO_2$ flow were both shut off for a period of 12 hours during the night, and turned on for the day cycle. The plants were killed on the morning of the fourth day by freezing in liquid nitrogen.

Extraction of the plants was done using the standard plant extraction procedure. Three separate batches were required, due to the amount of material. All the alkaloids were extracted together initially, before removing anything from the hot box. This organic

extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was redissolved in 20 ml of chloroform, and then washed with 3 x 20 ml of KOH (pH 13). The combined aqueous extract was brought to pH 8.6 with dry ice. It was extracted overnight with methylene chloride to give the phenolic alklaloids. The labeled morphine was isolated from this fraction by silica gel preparative thin-layer chromatography, using $CHCl_3$/MeOH/NH_3 (3:1:1%). The thus purified morphine was dissolved in a measured amount of chloroform for purposes of counting (total counts were 11,500,000 dpm). The UV absorption of a methanol solution of the residue at 288 nm, when compared to a standard, indicated the presence of about 10 mg of morphine. As a test of purity, a small amount of the residue dissolved in chloroform was spotted on an analytical tlc plate, along with authentic samples of morphine and narcotine. Ascending development was performed using chloroform/methanol/1% NH_3 (3:1) as above.

B. Standard Plant Alkaloid Extraction Procedure

Up to 50 gm (wet weight) of plant material can be accomodated conveniently by this extraction procedure. The plants are first cut into liquid N_2 and frozen. The frozen material is then ground briefly in a Waring blender. 100 ml of butanol/benzene (1:1) and 30 ml of 10% Na_2CO_3 are then added, and the mixture ground for about 5 minutes. The green organic supernatant is transferred to a 500 ml separatory funnel. The plant mash is then extracted in the same manner with five additional 75 ml portions of butanol/benzene, the last portion containing 10 ml of 10% Na_2CO_3 as well. The combined organic phase is

extracted with 4 x 50 ml of H_3PO_4 (pH 2.5, 1 M). This aqueous extract is then brought to pH 12 with the addition of 8 M KOH, and extracted with 5 x 100 ml of $CHCl_3$. The latter extract is dried over Na_2SO_4, and evaporated to dryness in vacuo to give the non-phenolic alkaloids. The aqueous residue is brought to pH 8.5 with dry ice, and extracted with 5 x 100 ml of chloroform, to give the phenolic alkaloids.

Alternatively, if separation of the phenolic and non-phenolic alkaloids is not necessary or desired, the aqueous acid phase can be brought directly to pH 8.5 with saturated Na_2CO_3 and extracted with 5 x 100 ml of $CHCl_3$. This gives all the alkaloids in a single extract.

C. Morphine Demethylation-Remethylation Sequence

1. Diacetylmorphine (Heroin)

About 50 mg of morphine is refluxed in 500 µl of acetic anhydride for 16 hours, in a N_2 atmosphere (bath temperature of 160°C). The excess volatiles are evaporated in vacuo to give an oily residue. This residue is dissolved in 25 ml of benzene, washed with 2 x 10 ml of 0.5 M K_2CO_3 and 2 x 10 ml of H_2O, dried over Na_2SO_4, and evaporated to dryness to give a semicrystalline residue. Analytical tlc shows this residue to be heroin. The heroin can be purified by recrystallization from ethyl acetate. For the purposes of the small-scale demethylations, however, the heroin was used as is in the Von Braun reaction.

2. Normorphine (via N-cyanonorheroin)

The heroin (from above) is redissolved in 10 ml of $CHCl_3$ (redistilled), and transferred to a dropping funnel. This is added dropwise, over a period of 15 min, to a stirred, ice-cold solution of 400 mg of

BrCN in 5 ml of $CHCl_3$, under a N_2 atmosphere. After rinsing the funnel with an additional 2 ml of $CHCl_3$, the reaction flask is fitted with a reflux condensor, stirred at 0°C for 30 min, at room temperature for 30 min, and finally refluxed at 75°C for 2.5 hr. The solution is cooled and evaporated in vacuo. The residue is twice redissolved in 5 ml of $CHCl_3$, and evaporated to remove lingering volatiles. This residue is redissolved in 20 ml $CHCl_3$, and washed with 3 x 10 ml of 0.1 M H_3PO_4. Each acid wash is back-washed with 10 ml of $CHCl_3$. The combined organic phase is dried over Na_2SO_4 and evaporated. The residue, N-cyanonorheroin, is dissolved in 1.5 ml of concentrated HCl by heating at 80°C for 5 min. Twelve ml of H_2O is added, and the resulting mixture heated at 80°C for 1 hr, refluxed (118°C) for four hours, cooled, and evaporated in vacuo to a slightly smaller volume. The pH of this solution is adjusted to 9.1 with 10% Na_2CO_3. It is then extracted with 6 x 50 ml of $CHCl_3$/EtOH (4:1). The organic extract is dried over Na_2SO_4, and evaporated to dryness. To obtain chromatographically pure normorphine, this latter residue is spotted on a preparative tlc plate (silica gel, 1000 microns thick), and developed in $CHCl_3$/MeOH/NH_3 (3:1:trace). The normorphine band (R_f = 0.05) is scraped off, and eluted from the silica with $CHCl_3$/MeOH (1:1). A second preparative plate can be run, the latter assuring greater than 99% purity.

3. Normorphine to Morphine (via N-carbethoxynormorphine)

To the normorphine is added 5 ml of H_2O and 0.5 g of NaOH, and the resulting mixture stirred at 0°C until solution is complete. To this is added 10 ml of $CHCl_3$, followed by 1.0 ml of $ClCO_2Et$, and the mixture

stirred vigorously at 0°C for 20 min. The aqueous layer is separated, and washed with 10 ml of $CHCl_3$. The combined organic phases are washed with 2 x 10 ml of 0.1 M NaOH and 2 x 10 ml of 0.1 M H_3PO_4. Each aqueous wash is backwashed with 5 ml $CHCl_3$. The total organic phase is dried over Na_2SO_4, and evaporated in vacuo.

The carbethoxynormorphine residue is dissolved in 5 ml of dry THF, and stirred at 0°C. Nine ml of $LiAlH_4$ in THF (0.25 mmoles/ml) is added from a dropping funnel over a period of 5 min. The mixture is stirred at 0°C for 1 hr, and refluxed for 2 hr (in N_2 atmosphere). The solution is then cooled in ice, and 3 ml of 12 N HCl in 10 ml H_2O added (slowly at first), followed by 3 g of sodium potassium tartrate in 10 ml of H_2O. The solution is filtered, and the precipitate washed with H_2O. The pH of the solution is then adjusted to 8.5 with concentrated NH_4OH, and extracted continuously with CH_2Cl_2 for 24 hours. The extract is dried over Na_2SO_4 and evaporated in vacuo. The morphine thus obtained can be purified by preparative tlc on silica gel, developing with $CHCl_3$/MeOH/NH_3 (3:1:trace). This effectively removes any trace of unreacted normorphine.

D. Morphine Feedings, Set 1

Six poppy plants were transplanted from Vermiculite to hydroponic tanks three days before each feeding. On the day of the feeding, the plants were removed from the hydroponic solution, their roots patted with paper towels to remove the bulk of water, and then placed in dry, blackened 50 ml flasks equipped with a glass tube for insertion of feeding solution. To one set of two plants was fed a total of 0.22 mg

of radioactive morphine, 2.1 mg to the second, and 21 mg to the third, beginning at 12:30 PM. This was done by dissolving the morphine in a minimal amount of 1% H_3PO_4 (approximately 150 λ), diluting to 2 ml with nutrient solution, and then giving 1 ml of solution to each plant. Additional cold nutrient solution was given to the plants according to the schedule in Table XX. At the end of the 24-hour metabolic period, the plants were removed from the flasks, the roots rinsed first in 1% H_3PO_4, followed by distilled water, and then cut up into liquid N_2 and frozen. The two washings were combined to comprise the neutral residue. These two solutions were diluted to a known volume, and an aliquot of each counted in dioxane scintillation solution. The counter used for this, and all subsequent radioactivity analyses was a Packard Model 3375 Tri-Carb Liquid Scintillation Spectrometer.

Table XX

Time	Amount to Each	Description
0	1 ml	Morphine solutions
5 min	1 ml	Cold nutrient solution
20 min	1 ml	"
35 min	2 ml	"
50 min	5 ml	"
2 hours	5 ml	"
6 hours	10 ml	"
20.5 hours	5 ml	"
24 hours	Harvested	---

The cut-up plants were transferred to a Waring blender, and extracted *via* the standard alkaloid extraction procedure, to give the various plant fractions. Again, all aqueous fractions were counted by adding an appropriate aliquot to dioxane scintillation solution. The latter is prepared from 50 ml of Fluor Concentrate II (Research Supplies Laboratory), 250 ml absolute ethanol, 400 ml p-dioxane, 50 g naphthalene, and toluene to bring the final volume to 1 liter. Those solutions which were soluble in toluene (such as organic extracts) were counted in cocktail made from 50 ml of Fluor II, diluted to 1 liter with toluene.

The aqueous solution obtained by centrifugation of the plant mash from Feeding Number 2 was brought to pH 3 with concentrated H_3PO_4, and applied to a cation exchange column (AG-50W-X8, 200-400 mesh, hydrogen-ion form). The column was washed with distilled water to neutral pH, and then with 1.5 N NH_4OH until about 300 ml of basic eluent was collected. All eluents were saved (in about 150 ml fractions) for subsequent counting. Fraction 1 from this column (containing the bulk of non-basic active substances) was reapplied to the regenerated cation exchange column and eluted in the same manner. Counting of the eluents showed that nearly all the sample passed through prior to eluting with base, indicating little if any column overload.

The first 150 ml of basic eluent from above was evaporated *in vacuo* until the pH was reduced to 8. It was then applied to an anion exchange column (150 ml of AG-1-X8, 200-400 mesh, hydroxide-ion form), the column eluted with distilled water to neutral pH, and finally with 2 N HCl to remove any adherants. Again all eluent was collected in fractions for subsequent counting.

E. Morphine Feedings, Set 2

Eighteen poppy plants, aged 60 days, were fed 0.21 mg of labeled morphine each. The feedings were done in the same manner as for Set 1. Two plants were harvested at the end of each metabolic period (3, 6, 9, 12, 20, and 24 hours, respectively). An entire day-night cycle was thus included in the experiment. The harvested plants were treated in the same manner as previously, to obtain the various plant fractions for counting.

F. Codeine Methyl Ether from Normorphine

Fifty mg of normorphine is dissolved in 250 µl of H_2O, 50 µl of 5 N NaOH and 25 µl of dimethylsulfate. The reaction is carried out in a small vial (~5 ml volume), fitted with N_2 bubbler. The vial is placed in an ice bath for 4 hr, with constant stirring. At the end of each hour, 40 µl of 5 N NaOH and 25 µl of Me_2SO_4 are added (for a total of 125 µl Me_2SO_4). Finally, the solution is stirred at room temperature for about 2 hr, and 1 N NaOH added when necessary to maintain the pH near 8. The solution is then filtered through an anion-exchange resin (15 gm of AG-1-X8, 200-400 mesh, rinsed previously with 1 N NaOH H_2O, 1 N HCl, NaCl solution, and H_2O until the eluate was Cl-free), eluting with H_2O to a Cl-free eluate (about 100 ml is needed). The eluate is evaporated in vacuo to dryness, then transferred to a micro-sublimer. It is heated for 20 min at 220°C, 0.2 mm, and then 40 min at 280°C, 0.2 mm. The sublimate is rinsed from the cold finger with EtOH, evaporated to a smaller volume, then spotted on preparative tlc plates for purification of the codeine methyl ether. The developing solvent used here is $CHCl_3$/MeOH/NH_3 (3:1:trace).

G. $^{14}CO_2$ Biosynthesis - Search for Normorphine

A two-day $^{14}CO_2$-biosynthesis was done, using 10 poppy plants, ages 75-90 days. A total of 120 mC of $^{14}CO_2$ was added over the two-day period. Approximately 40 mC was in the growth chamber at the time the plants were added. In order to maintain the CO_2 concentration near 0.04%, another 35 mC had to be added 3 hours into the biosynthesis. The remainder (45 mC) was added at about 22 hours, after the lights were turned on the second day. The CO_2 concentration was then allowed to drop to about 0.02%, at which time (30 hrs) cold CO_2 was added to restore it to 0.04%. The plants were kept on a 12-hr light/dark cycle; at least 95% of the activity had been taken up at the end of 48 hours.

Due to the quantity of plant material, it was divided into two batches, which were extracted separately to the organic alakloid extracts. The following cold carriers were added (total amounts): normorphine (100 mg), morphine (100 mg), neopinone (300 mg), codeine (50 mg) and thebaine (50 mg), the latter three for H. Parker's use. They were added to the ground plant mash in slightly acidic solution (1% H_3PO_4), and rinsed in with BuOH/benzene (1:1). The normal alkaloid extraction scheme was followed to get an acidic solution of the alkaloids. This solution was brought to pH 12 with 8 M KOH, and extracted with 5 x 100 ml of $CHCl_3$ to obtain the non-phenolics. The pH was then adjusted to pH 8.6 with concentrated H_3PO_4, and the resulting solution extracted with 6 x 90 ml of $CHCl_3$/isopropyl alcohol (3:1). This organic extract was dried over Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was taken up in a minimal amount of MeOH/10% $CHCl_3$, and spotted on four preparative silica gel plates (dried previously for

1/2 hour at 100°C). After development in $CHCl_3$/MeOH/NH_3 (3:1:trace), the morphine and normorphine bands were located by spraying the edges with iodoplatinate. These were scraped off, and eluted by stirring with 15 ml of MeOH for 15 minutes, filtering on a millipore filter, and repeating once more. The resulting solutions were evaporated to dryness. A second preparative tlc of the normorphine from above was performed in the same manner to give morphine-free normorphine.

Conversion of the ^{14}C-normorphine to ^{14}C-codeine methyl ether was carried out using the procedure described in Section F above. Subsequent purification of the CME was done by preparative tlc, as above, and sublimation at 120°C, 0.2 mm. Mass analysis of the purified material was done by vpc and UV at 286 nm (ε = 1350).

The ^{14}C-morphine isolated from the plants was converted first to normorphine, and then to codeine methyl ether. The latter was also purified by tlc. A final purification of both CME samples (i.e., from ^{14}C-morphine and ^{14}C-normorphine) was made by subliming each for 8 hrs, at 120°C, 60 microns. Some of each was scraped from the cold finger, and portions then precisely weighed and counted.

H. Normorphine Extraction and Purification Studies

1. ^{14}C-Normorphine Extraction

A sample of ^{14}C-normorphine (containing about 20,000 dpm) was dissolved in 10 ml of Na_2CO_3. A 100 λ aliquot was removed and counted, and 9 ml of the remaining solution immediately transferred to the Waring blender. 100 ml of BuOH/benzene (1:1) was added, along with 21 ml of 10% Na_2CO_3. Finally, a frozen 53 g plant, 60 days old, was

added to the blender mix, and the whole was ground for 5 minutes. 60 ml of BuOH/benzene had to be added to break up the emulsion, and the mixture was ground for 10 minutes more. 100 ml of the dark-green supernatant was removed by pipetting into a volumetric flask. 80 ml more BuOH/benzene was added, and the mixture ground for 15 minutes. After removing another 100 ml of organic supernatant, 60 ml more BuOH/benzene was added to the mash, the mix ground for 5 minutes, and the remaining supernatant removed and diluted to 100 ml. Samples were then removed from each extract for counting purposes. The aqueous mash was centrifuged at 2500 rpm for 15 minutes, and the aqueous (along with a small amount of solids which remained on top of the water) decanted and filtered. The solids were rinsed with water, recentrifuged, the aqueous decanted and added to the former aqueous solution, and the remaining solids dried in a dessicator.

2. Normorphine Extraction - Cold

Two stock solutions were prepared as follows: Solution A, 0.1 M boric acid in 0.1 M KCl; and Solution B, 0.1 M NaOH. To 25 ml of stock Solution A were added 10.6 mg of cold normorphine, and the mixture stirred until the normorphine was completely dissolved. To this solution were added 6 ml of stock Solution B, and 19 ml of H_2O, to give 50 ml of aqueous normorphine solution buffered at pH 8.6.

A single poppy plant, about 90 days old, was cut-up, frozen in liquid N_2, transferred to a Waring blender, and ground briefly. The normorphine solution from above was then added, and the mix ground again for about 30 seconds. Finally, 100 ml of BuOH/benzene were added, and the mix ground for 10 minutes, stopping it at 5 minutes to

scrape material from the sides of the blender. The mash was allowed to settle, and then the organic supernatant was decanted, yielding about 75 ml of solution. An additional 75-ml portion of BuOH/benzene was added to the mash, and the grinding resumed for 10 more minutes. Again, the organic supernatant was decanted. The plant mash was then filtered, with suction, and a check on the deeply colored filtrate showed the pH to be 8.55, indicating the buffer was effective.

Each of the organic extracts was extracted with 4 x 20 ml of H_3PO_4 (1 M, pH 2.5). The aqueous extracts were brought to pH 8.6, using KOH pellets and concentrated H_3PO_4, and extracted with 4 x 50 ml $CHCl_3$/IPA (3:1). The organic extracts were dried over Na_2SO_4, filtered, and evaporated to dryness in vacuo. Subsequent analysis was done by gc (on OV-17), after dissolving each residue in 5 ml of $CHCl_3$/MeOH (1:1).

3. Normorphine Acetylation

To 105 mg of normorphine in a 25-ml pear-shaped flask was added one ml of acetic anhydride. The flask was fitted with condenser and nitrogen bubbler, and refluxed. Samples were removed after various time intervals for tlc analysis. The refluxing was continued for 16 hours (bath temperature ~160°C). The residual acetic anhydride and acetic acid were removed by evaporating in vacuo; the residue was taken up in 25 ml of benzene (it dissolved slowly), then washed with 2 x 20 ml of 0.5 M K_2CO_3. The combined aqueous washes were back-washed with 25 ml of benzene. The combined organics were dried over Na_2SO_4 and evaporated to dryness in vacuo. The residue was redissolved in 25 ml of $CHCl_3$ for vpc analysis.

4. Morphine-Normorphine Co-acetylation

To a three-necked pear-shaped flask (25 ml), fitted with nitrogen bubbler and condenser, was added 100 mg each of morphine and normorphine, and 2 ml of acetic anhydride. It was placed in an oil bath, and the solution brought to reflux (bath temperature of 155°C). Aliquots were removed and checked by vpc immediately after Ac_2O addition, and 45 minutes after addition (just prior to boiling). Reflux was continued for about six hours. The product was worked up the same as above for the normorphine case, to give a residue of the acetylated products.

5. Normorphine-Plant Extraction with Acid

To a single frozen, ground poppy plant, ~20 gm wet weight, 50 days old, in a Waring blender, was added 19.4 mg of normorphine. 75 ml of butanol/benzene (BB) was added, and the mixture blended briefly. Finally, 50 ml of 1 N HCl was added, and the mix blended for five minutes. The BB supernatant was removed by pipetting to a separatory funnel. 75 ml more BB was added, the mixture ground again for 5 minutes, and the supernatant transferred to the separatory funnel. The remaining plant mash was centrifuged, after rinsing thoroughly from the blender with 1 N HCl, for 20 minutes at 2400 rpm. The aqueous layer was removed by syringe. Additional HCl was added to the residual solids, the mix stirred briefly, and then recentrifuged. The aqueous phases were combined, brought to pH 8.6 with solid Na_2CO_3, and extracted with 6 x 70 ml $CHCl_3$/IPA (3:1). The total organic extract was dried over Na_2SO_4, and evaporated to dryness. Vpc analysis was performed directly on this residue, after dissolving in 5 ml of MeOH. The sample was subsequently redried, then acetylated by refluxing for 16 hours at 155°C

in acetic anhydride. This was used to study the feasibility of analyzing plant extracts as the acetates.

A second check of the acid-butanol/benzene extraction procedure was done by adding 10 mg of normorphine to a ground poppy plant (fresh weight about 20 gm), and the mixture extracted using the new acid-butanol/benzene procedure. Two separate 75-ml portions of 1 N HCl were used to remove the alkaloids. The first BB phase was back-extracted with 1 M (pH 2.5) H_3PO_4. All the aqueous acid phases (after centrifugation) were combined, brought to pH 8.6, and extracted with 6 portions of $CHCl_3$/IPA (3:1). The organic phase was dried over Na_2SO_4, filtered, and evaporated to dryness in vacuo. The residue was acetylated in refluxing Ac_2O (3 ml) for 16 hours, and the product evaporated to dryness.

I. Control Experiments

1. Control No. 7

To a sample of radioactive morphine (150,000 dpm, ~3 mg) was added 9.7 mg of cold normorphine. The mixture was dissolved in a small amount of 1 N HCl, and added to some ground plant material (~35 gm), for extraction via the acid-butanol/benzene procedure. The final alkaloid residue, after acetylation, was separated on preparative tlc, to give the morphine-Ac_2 and normorphine-Ac_3. After counting and mass analysis, the normorphine-Ac_3 was rechromatographed a second time, the developing solvent being $CHCl_3$/MeOH/NH_3 (3:1:trace), as opposed to EtOH/benzene (1:4) for the first plate.

As a check blank, a sample of the radioactive morphine used in this control was acetylated, and the percentage of normorphine present determined. This determination was included as a correction factor in the plant extraction control.

2. Control No. 9

This control experiment was performed in three parts, by adding morphine at each of three distinct points in the extraction procedure. For the first one, 5.4 mg of morphine was dissolved in 37 ml of 1 N HCl. This was extracted with 2 x 75 ml of BuOH/benzene. The latter extract was back-washed with 2 x 25 ml of 1 N HCl. A second portion (~40 ml) of 1 N HCl was extracted with 75 ml of BB. Each BB-acid extraction was shaken for about 5 minutes. The total aqueous acid portion was brought to pH 8.6 with solid Na_2CO_3, and extracted with 6 x 75 ml $CHCl_3$/IPA (3:1). The latter was dried over Na_2SO_4, and evaporated *in vacuo* to dryness.

For the second control, 125 ml of 1 N HCl was extracted with 150 ml of BB. To the aqueous phase was then added 4.8 mg of morphine, the pH of the solution brought to pH 8.6, and extraction performed with $CHCl_3$/IPA as above. And finally, for the third control, everything was identical as in the second one, except the morphine (5.2 mg) was added to the $CHCl_3$/IPA extract prior to drying and evaporating.

All three residues were acetylated as usual, and analyzed for NM/M ratio by vpc.

3. Control No. 10

Five mg of morphine was dissolved in 35 ml of 1 N HCl. This solution was combined with 70 ml of BB in a round-bottom flask, fitted with air bubbler and reflux condenser. It was then heated for 15 min at a bath temperature of 75°C, with air bubbling through at a rate limited only by the capacity of the condenser. After cooling, the aqueous phase was separated, and extracted as usual to give the alkaloid residue.

4. Control No. 11

This experiment was run just like No. 10, after combining 73 mg of morphine, 75 ml of 1 N HCl, 130 ml of BB, and 250 mg of hydroquinone, the heating time being extended to 25 minutes. Work-up was done as usual for normorphine/morphine analysis.

5. Control No. 12

This was again a repeat of No. 10, substituting 1 M H_3PO_4, buffered at pH 2.5, for 1 N HCl. Conditions were identical, except the heating time was extended to 30 minutes.

J. Gas Chromatography of the Alkaloids

All vpc analyses for the normorphine studies were done using 6-foot, 3 mm i.d. glass columns filled with Aeropak 30 (100/120 mesh) coated with 3% OV-17. The latter is a silicone rubber with 50% methyl and 50% phenyl side chains. The preparation of these columns has been described in detail by Parker[16] (according to the procedure of Kruppa et al.).[41] An F&M Scientific Model 402 High Efficiency Gas Chromatograph was employed. This equipment is designed to take glass columns, and avoids any contact of the injected sample with metal until it reaches the flame detector. The latter has been proven necessary to avoid tailing and loss of sensitivity. The conditions used for most of the analytical work were: column temp., 250°C; flash heater and detector temp., 290°C; carrier gas, argon; flow rate, 60 ml/min. Sensitivities of about 50 nanograms for both triacetyl normorphine and diacetylmorphine were achieved under these conditions.

The column conditions had to be altered when it was desired to trap compounds from the vpc. Apparently the integrity of triacetylnormorphine is not maintained at 250°C. Good recoveries were obtained at a column temperature of 230°C (identical flow rate as above).

K. Modified Plant Extraction Procedure

The plant material (40 gm) is frozen in liquid N_2, then ground briefly in a Waring blender. After adding 75 ml of H_3PO_4 (1 M, pH 2.5) and 150 ml of butanol/benzene, the mixture is blended for five minutes, then cooled by putting the blender on ice. The organic supernatant is removed, and the grinding repeated with a second portion of BuOH/benzene. After removing the green supernatant, the aqueous plant mash is centrifuged to separate the acid portion from the solid. The latter are returned to the blender, and extracted as above with a second portion of acid. The combined organic supernatants are extracted with 2 x 50 ml H_3PO_4, and then discarded (except in feeding experiments). The total combined aqueous extracts are brought to pH 8.6 with solid Na_2CO_3, and extracted with 6 x 150 ml $CHCl_3$/iso-PrOH (3:1). The latter extract is dried over Na_2SO_4 and evaporated to dryness. The residue is dissolved in 3 ml of acetic anhydride, and refluxed at 160° for 16 hours. The excess anhydride is removed *in vacuo*, to give the crude alkaloid peracetates.

L. Normorphine-Morphine Ontogeny Studies

Plants of various ages were extracted *via* the modified extraction procedure (the initial ones using 1 N HCl as the aqueous phase). The

resulting alkaloid residues were analyzed by dissolving in 3 ml of $CHCl_3$, and injecting appropriate aliquots onto an OV-17 vpc column.

The opium samples were extracted by dissolving 800 mg of the raw opium in 200 ml of 1 M H_3PO_4 (pH 2.5) with vigorous magnetic stirring. The pH of the solution was then brought to 8.6 with sodium carbonate, and extracted as before with six portions of $CHCl_3$/iso-PrOH (3:1). The residue was acylated and analyzed in the same manner as the plant extracts.

M. Morphine Feedings, Set 3

1. Morphine Feeding No. 5

The nuclear ^{14}C-morphine (110,000 dpm/mg) was dissolved in 1 ml of $CHCl_3$/MeOH (1:1). 500 µl of this was transferred to the feeding vessel. The chamber used for this feeding was a 500-ml crystallization dish, fitted with a bubbler and a cover with holes for 3 plants. The solvent was evaporated in a nitrogen stream; the residue was redissolved in approximately 700 µl of 1% H_3PO_4. Poppy nutrient was then added, with stirring, to a total volume of 300 ml. Three plants (72 days old, each weighing about 25 gm) were transferred from hydroponic tanks to the feeding container. The nutrient level was maintained at 300 ml for the first two hours, then allowed to drop (to about 250 ml) for the next four hours, at which time it was brought to 350 ml to last overnight. The plants were removed from the chamber after 22 hours total, their roots rinsed in dilute H_3PO_4 (1%), and the plants frozen in liquid N_2.

The plant material was extracted in two batches, after adding about 5 mg cold normorphine to each batch. Again, the BB phase was back-extracted once with aqueous acid. All the aqueous acid portions were

combined for alkaloid extraction at pH 8.6. Acetylation by the normal procedure gave the peracetates.

After attempting to collect the normorphine triacetate from the vpc for specific activity analysis, and failing, it was decided that a tlc system should be sought to separate the NM-Ac_3 from heroin, as well as from the abundant low-R_f, highly colored impurities. Ethanol/benzene (1:4), with silica gel, was found to give such a separation. The crude acetylated alkaloid mixture was therefore purified by a preparative tlc, using EtOH/ØH as the developing solvent. The normorphine triacetate so obtained was purified once more by preparative tlc, developing with $CHCl_3$/MeOH/NH_3 (3:1). Vpc showed that the first tlc had effectively removed all the morphine-Ac_2, so the second tlc was just a precautionary measure. Analysis of the specific activity was then accomplished by determining the NM-Ac_3 mass by vpc, and counting a known aliquot of the NM-Ac_3 material.

2. Morphine Stability Study under Feeding Conditions of MF-5

To test for the integrity of the morphine in the residual nutrient solution and root wash from MF-5, the two were combined and brought to pH 8.6 with solid Na_2CO_3. The resulting solution was extracted with 3 x 250 ml $CHCl_3$/IPA (3:1), and the organic extract dried over Na_2SO_4 and evaporated to dryness. Vpc analysis showed this extract was not morphine (see Results), although subsequent acetylation gave a major product whose retention time is identical with heroin.

With this result in hand, it was decided that a check on the stability of morphine under these feeding conditions should be made. To do this, 11.7 mg of cold morphine was dissolved in a minimal amount of

0.1 M H_3PO_4, and diluted to 300 ml with poppy nutrient solution. To this solution were added portions of roots from the poppy plants (aged 70-90 days), and the whole fitted with an air bubbler. After 4 hours of bubbling, the roots were removed and rinsed in about 25 ml of 0.1 M H_3PO_4; as much liquid as possible was squeezed from the roots. The acidic root wash and remaining nutrient solution were combined, and the pH brought to 8.6 with solid Na_2CO_3. The solution was extracted with 3 x 150 ml of $CHCl_3$/IPA (3:1). At this point, the pH was re-checked and found to be about 9.5, so it was brought back to 8.6 with H_3PO_4 (conc.). After readjustment, the solution was extracted with three more 150-ml portions of $CHCl_3$/IPA (3:1). The total organic extract was dried over Na_2SO_4, filtered, then evaporated to dryness.

The residue, containing morphine and morphine-Dl, was separated into its two components by preparative tlc on silica gel, developing with $CHCl_3$/MeOH/NH_3 (3:1:trace).

3. Morphine Feeding No. 6

The remaining nuclear ^{14}C-morphine was purified once more by preparative tlc. The resulting morphine gave a single spot on tlc, and a single peak on vpc. The entire sample was taken up in one ml of 0.1 M H_3PO_4. To this solution was added 12 ml of poppy nutrient. A 100 λ aliquot was removed for scintillation counting. Four ml of the remaining feeding solution was transferred to each of three feeding flasks (blackened 50-ml Erlenmeyer flasks). Three poppies, about 70 days old, were removed from the hydroponic tank, their roots blotted with paper towels, and placed in the feeding flasks. After 5 minutes, one ml additional nutrient was added to each flask. Additional nutrient was added

periodically to maintain the level near 5 ml in each flask. After six hours, the plants were removed from the flasks and their roots rinsed in 0.1 M H_3PO_4. The flasks, after rinsing with acid, were filled with nutrient solution, and the plants replaced. They were kept this way for an additional 18 hours, for a total of 24 hours. They were then removed, rinsed briefly in nutrient solution, and frozen in liquid nitrogen. Extraction of the plants was done as normal, via the acid-butanol/benzene procedure, after adding 14.8 mg of cold normorphine as carrier. The crude alkaloid extract was acetylated with 3 ml of refluxing acetic anhydride. The product from this was separated by preparative tlc on silica gel, developing with ethanol/benzene (1:4). Bands were scraped off, eluted with $CHCl_3$/MeOH, and evaporated to dryness.

Counting of most of the samples was straightforward. However, the plant mash was counted by suspending it in scintillation solution with Cab-O-Sil (a gel agent), and the aqueous residue (from which the alkaloids are removed at pH 8.6) was counted in a cocktail of "Oxifluor-H_2O" to insure solubility.

The residual nutrient and root washes were combined, and brought to pH 8.6, by first adding about 100 ml of a pH 8.6 borate buffer, then adding 8 M KOH to the proper point. This solution was extracted with 3 x 200 ml of $CHCl_3$/IPA, followed by 3 x 200 ml of $CHCl_3$. The combined organic phase was dried over Na_2SO_4 and evaporated to dryness. The residue, after being counted, was purified by preparative tlc (silica gel, developing solvent $CHCl_3$/MeOH/NH_3, 3:1:trace). The morphine band was eluted as usual.

N. Morphine Biosynthesis No. 3

Four poppy plants, 90 days old, were exposed to $^{14}CO_2$ under "steady-state" conditions for a total period of six hours in the light. There was a slight drop in the CO_2 concentration over the last one-half hour, but the specific activity of the CO_2 reamined fairly constant throughout. At the end of the exposure time, the plants were cut up and frozen in liquid nitrogen. They were then ground briefly in a Waring blender (in two batches), 150 ml of H_3PO_4 (1 M, pH 2.5) added, and the mixture ground for an additional 5 minutes. The aqueous phase was separated from the solids by centrifugation, and the latter blended again, with a second portion of H_3PO_4. After separating, the total aqueous phase was extracted with 3 x 200 ml of butanol/benzene (1:1); the organic extracts were back-extracted once with 50 ml of H_3PO_4. The combined aqueous phase was brought to pH 8.6 with solid Na_2CO_3 (to about pH 6) and 8 M KOH. It was then extracted with 6 x 150 ml $CHCl_3$/IPA (3:1). It was necessary at times to add more organic to break up the emulsions which occurred. The total organic extract was dried over Na_2SO_4, filtered, and evaporated to dryness in vacuo.

The alkaloid residue was immediately esterified with Ac_2O (16 hours at reflux) and evaporated. A portion of the acetylated alkaloid mixture was separated by preparative tlc on silica gel (developing solvent, EtOH/benzene, 1:4) to give one fraction containing the normorphine-Ac_3, and another the morphine-Ac_2; the latter also including codeine-Ac. The normorphine-Ac_3 was further purified by tlc, using $CHCl_3$/MeOH/NH_3 (3:1:trace) on one plate, and EtOH/benzene on a final plate. The final determination of specific activity was done by collecting a known quantity of the normorphine-Ac_3 from a vpc injection, eluting, and counting.

The specific activities of the morphine-Ac_2 and codeine-Ac were determined by vpc trapping of known aliquots of each, and counting. The total fraction was then purified by a second preparative tlc, developing with $CHCl_3$/MeOH/NH_3, 3:1:trace; the morphine-Ac_2 was eluted, and evaporated to dryness. This residue was dissolved in 5 ml of freshly distilled $CHCl_3$. The resulting solution was then added, over a period of 20 minutes, to a stirred ice-cold solution of 200 mg of BrCN in 2.5 ml of $CHCl_3$. The funnel was rinsed with one ml of $CHCl_3$. The reaction mixture, under nitrogen, was stirred at 0°C for 30 minutes, room temperature for 30 minutes, and finally refluxed (75°C bath) for 2.5 hours. After cooling, the solution was evaporated to dryness in vacuo, twice redissolved in $CHCl_3$ and reevaporated, and finally dissolved in 10 ml of $CHCl_3$, washed with 3 x 10 ml of 0.1 M H_3PO_4, dried over Na_2SO_4, and evaporated to dryness.

The residue was dissolved in 0.75 ml of concentrated HCl by swirling and heating at 80°C for 15 minutes. To this solution, 6 ml of water was added, and the resulting solution heated at 80° for one hour, then refluxed (118°) for 3.5 hours. After cooling, the pH of the solution was brought to 8.6 with 10% Na_2CO_3, and it was extracted with 6 x 20 ml of $CHCl_3$/IPA (3:1). The organic extract was dried and evaporated. The normorphine was then purified by preparative tlc (developing solvent, EtOH/dioxane/benzene/NH_4OH, 1:8:10:1), and its specific activity determined (mass by tlc).

PART 2

BOUND ALKALOIDS

I. INTRODUCTION

A report concerning the presence of "bound" alkaloids in the seeds of several varieties of P. somniferum[32] led to our interest in determining whether or not bound forms of alkaloids exist in the poppy plants themselves, or in our variety of seeds. If, indeed, bound morphine is present in the plants as a metabolite of morphine, this would most certainly be reflected in labeled morphine feedings. Any activity ending up in water-soluble metabolites could then be claimed to be "bound" morphine.

With these questions in mind, a definitive procedure was established for extracting seeds and plants which could distinguish between free and "bound" forms of alkaloids. In this case, "bound" is defined as being initially water-soluble, but releasing a free alkaloid upon acid hydrolysis. Both seeds and 2-month old plants were subjected to this procedure. Evidence was sought to establish the presence or absence of bound forms by comparing pre-hydrolysis and post-hydrolysis alkaloid extracts. In the case of the plants, it was of greatest concern to determine if morphine is found in some bound form, although a qualitative estimate of the extent to which non-phenolic alkaloids were present was also made. Some doubts were raised concerning the ability to distinguish actual bound alkaloids from incompletely extracted free alkaloids and aberrant products produced by the hydrolytic conditions. Some discussion of this problem will be included with the presentation of the results of this study.

II. RESULTS AND DISCUSSION

A. Historical

In 1968, Fairbairn and El-Masry reported the discovery of "bound" alkaloids in the seeds of several varieties of P. somniferum.[32] They claim that upon acid hydrolysis or pepsin digestion of ground poppy seeds, alkaloid-like substances are liberated, among them being codeine and a phenolic alkaloid similar to morphine. These results are somewhat contradictory to those obtained in this laboratory by Martin concerning the gradual appearance of alkaloids in seedlings, which leads one to the conclusion that no alkaloids are present in the seeds.[20,33] There is one report in the literature of a Russian woman who ate several tablespoons of ground poppy seeds, and found her psychological state to change from irritability to euphoria.[34] Fairbairn and El-Masry claim this could be due to the release of bound alkaloids by the action of the stomach acid-pepsin.[32]

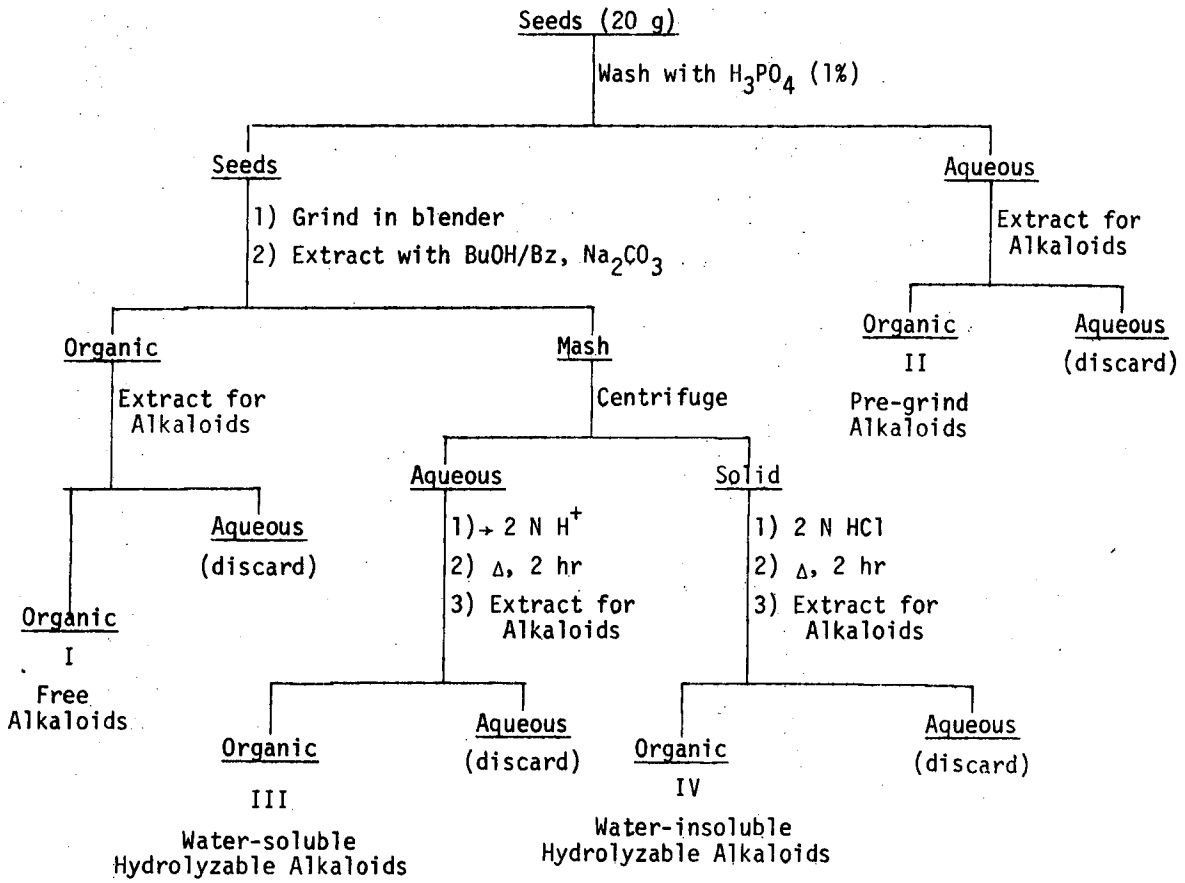
No evidence exists in the literature for the presence of bound alkaloids in the poppy plant itself. The existence of such forms has been indicated for the alkaloids in hemlock.[35,36] Bound forms of the morphine alkaloids are found as metabolites in animal and human studies.[37,38] This has been shown to be a monoglucuronide, in the case of morphine.[39,40] Of course, no direct correlation can be drawn from these studies to the situation occurring in plants, but they do indicate the presence in nature of mechanisms which render such compounds as alkaloids more water-soluble.

B. Methods Used

The major concern in a search for "bound" alkaloids, whether in seeds or in the plant, is to adequately distinguish between free and bound forms. Since the determination of the bound forms is accomplished by an acid hydrolysis to yield the free alkaloids, it is necessary to remove all naturally free alkaloids first. Fairbairn and El-Masry accomplished this by exhausting the ground seed with EtOH.[32] Subsequent hydrolysis and EtOH extraction gave their alkaloid extract. Since our interest was in both seeds and plants, this extraction method was inadequate. In addition, it failed to distinguish between free alkaloids which remained on the external surfaces of the seeds, and those inside the seeds. The standard plant extraction scheme (as described in Part 1 of this thesis) was, therefore, modified to answer the question of free versus bound alkaloids.

In the case of the seeds, we were interested in three types of alkaloids: 1) the free alkaloids within the seeds, 2) the residual alkaloids outside the seeds, and 3) the water-soluble hydrolyzable alkaloids. The procedure outlined in Scheme V was adopted for this purpose. An initial acid wash removes any residuals, a normal grinding and extraction removes the free, and an hydrolysis, followed by an alkaloid extraction, gives the bound. In addition, an hydrolysis of the solid phase was included, as a check for any water-insoluble bound forms. The extraction for alkaloids from aqueous phase was performed with $CHCl_3$ at pH 8.6 (or first at 12, then 8.6, if the non-phenolics and phenolics were separated).

Seed Extraction Scheme



XBL 725-4636

Scheme V

A similar procedure was adopted for plant material, with the exception that no pre-grind wash was necessary. It was already apparent at the time these extractions were made that $CHCl_3$ was not totally effective in removing 100% of the alkaloids. In certain cases, therefore, a second extraction with $CHCl_3$/EtOH (4:1) was performed, at pH 9. As noted in Scheme VI, a second complete grinding and blending process was included, to provide a check on the efficiency of removal of all free alkaloids.

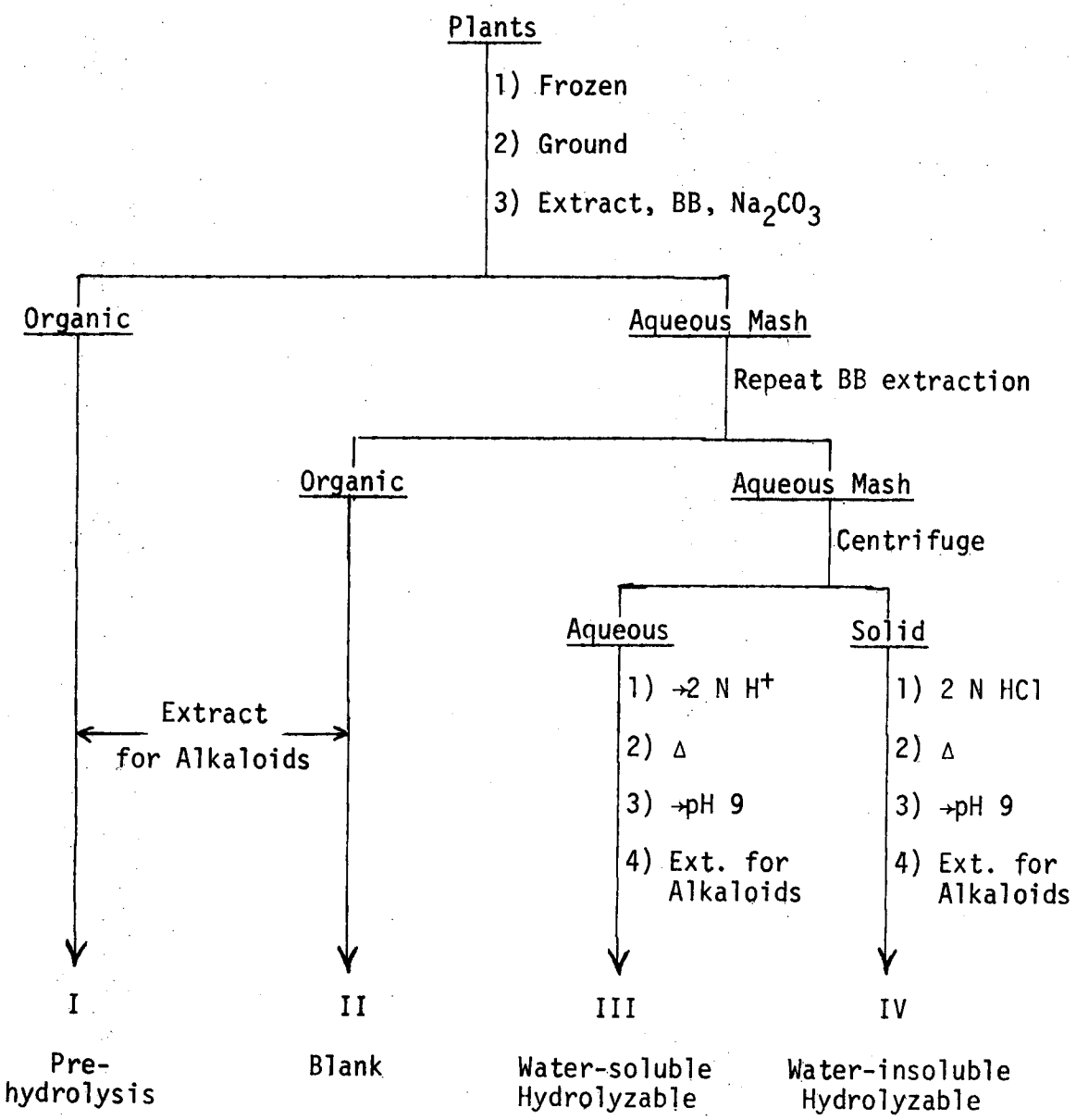
Analysis of the various isolated alkaloid extracts was done using tlc and gas chromatography. Some secondary identification was attempted by purifying the compounds in question by preparative tlc. However, the main effort of the analysis was directed toward determining whether morphine or codeine exist in bound forms, and to give a qualitative idea of the relative importance of them to general alkaloid metabolism.

C. Seed Alkaloids

A 20 gm batch of seeds was extracted and hydrolyzed according to Scheme V. Each crude alkaloid residue was analyzed both by gc and by tlc. The gc results are depicted graphically in Figure 4, and Table XXI lists the tlc data. Fraction IV showed no peaks on vpc or alkaloid spots, so was not included. On the gas chromatographic system used here, morphine gives a retention time of 6:30, and codeine 5:00.

Comparing the gc spectra of Fractions I and II, only one new peak appears in the pre-hydrolysis alkaloids (17:30). The other

Plant "Bound-Alkaloid" Extraction Scheme



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Scheme VI

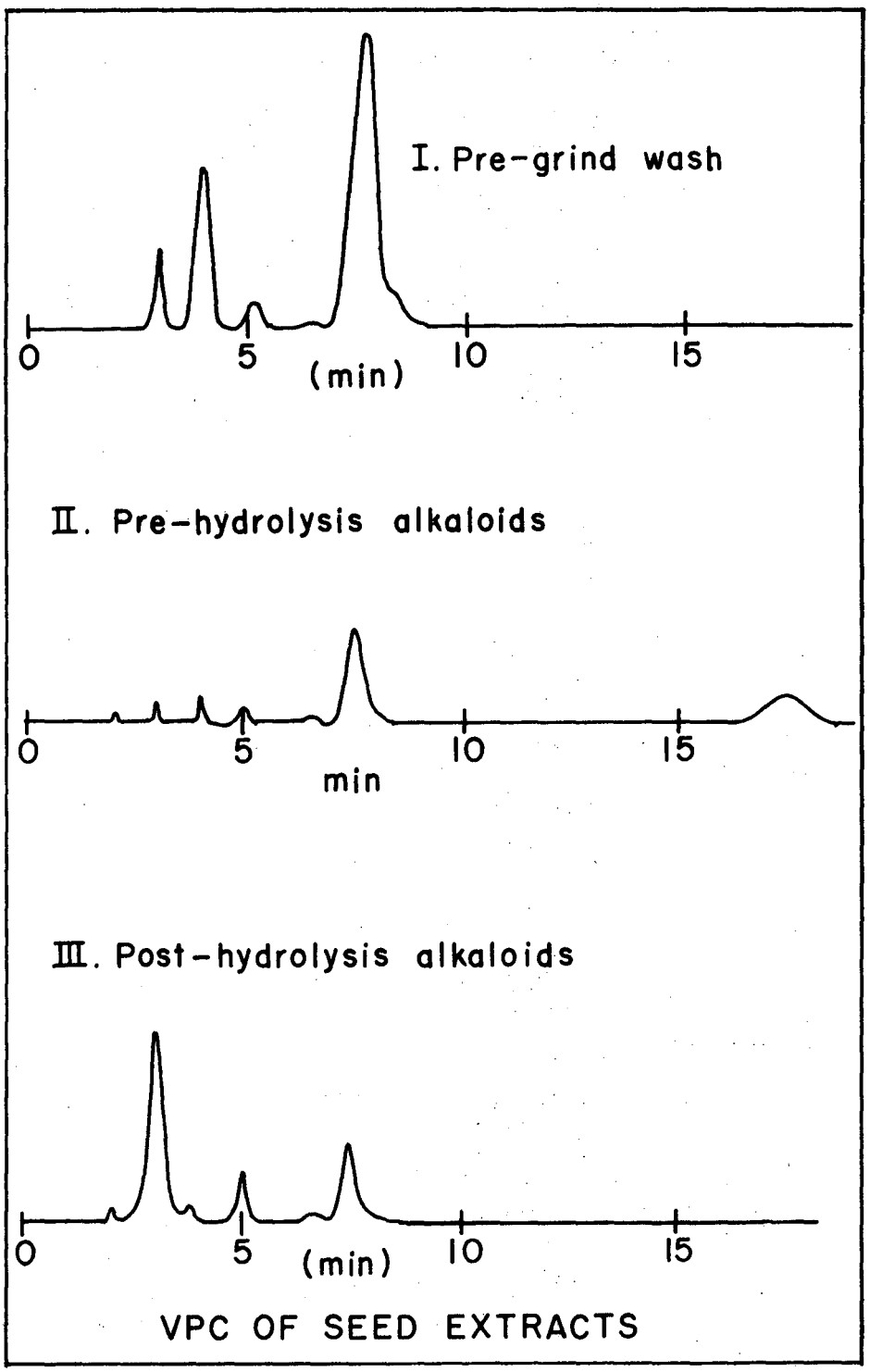

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Figure 4.

Table XXI

Fraction	Spot R_f(s)
I. Pre-Grind Wash	77
II. Free Alkaloids	15, 77
III. Bound Alkaloids	3, 77
Standards	
1. Morphine	19
2. Codeine	37

peaks may represent actual free alkaloids, but more likely they represent incompletely removed residual alkaloids. Morphine and codeine are both apparently present, in trace amounts, in Fraction II, though they do not appear in the tlc. Likewise, the codeine peak appears enhanced in the post-hydrolysis alkaloids, but tlc does not substantiate that fact. However, there is definitely a new spot sppearing on tlc as a result of hydrolysis (R_f = 3); in addition, the gc spectrum shows a definite increase in the amount of the compound with a 3 min retention time. It does appear, therefore, that there do exist certain unknown alkaloids in the seeds, at least one in a free form, and one or two in bound forms. However, the amount of alkaloid we are dealing with here is extremely low. For instance, the peak at 5:00 in the post-hydrolysis alkaloids, if it is codeine, would imply a concentration of about 250 mg codeine per gram of seeds. There are about four grams of seeds per plant, and about 1 mg codeine in

the mature plant. The amount of seed codeine would represent only one-tenth of one percent (1/1000) of the total plant at maturation. Thus, it appears as if some other more extensive use is made of the alkaloids.

The fact that there do seem to be trace alkaloids in the seeds is not necessarily a contradiction of the results obtained by Martin and co-workers, which indicate that no alkaloids are synthesized until about three days after germination. The limits they reported for detection were on the order of 1-5 μg/gm of seedlings. The seed concentrations reported here fall below these limits, especially if one assumes that there is at least a five-fold weight increase in 3-day seedlings relative to dry seeds.

Certainly more intensive study of the identity, biosynthetic source, and metabolism of these seed alkaloids is needed to determine their importance to the growth and development of the poppy plant. Fairbairn's hypothesis[32] that these seed-alkaloids are somehow involved in the process of germination can hardly be tested by simple experiments like the one just discussed.

D. Plant Alkaloids - Free versus Bound

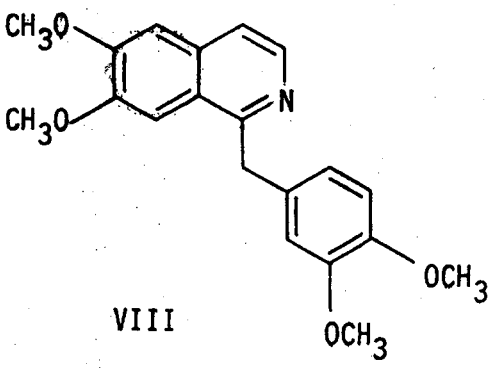
Two batches (50 gm per batch) of poppy plants, aged 60 to 70 days, were ground, extracted, and hydrolyzed according to Scheme V. In addition, the aqueous residues from which the non-phenolics and phenolics were extracted at pH 12 and 8.6, respectively, of paths I and III were continuously extracted with $CHCl_3$/EtOH (4:1) for 4 days, in hopes of recovering any normorphine in the plants. These

samples are referred to as IB aq. and IIIB aq. As will be seen subsequently, this additional extraction demonstrated the inefficiency of the initial $CHCl_3$ extraction. All alkaloid extracts were separated into their non-phenolic and phenolic fractions, for easier analysis.

An analytical tlc of each isolated fraction was run. The result is depicted in Figure 5.

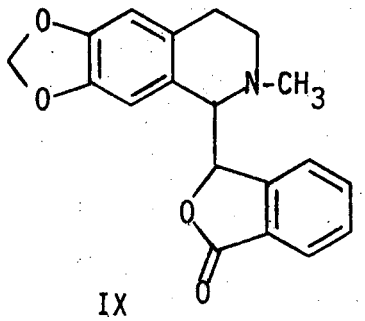
The following spots in IA and IB can be assigned with some certainty: codeine (streak in IA and R_f 0.35 and 0.34 in IIA and IB, respectively); thebaine (R_f 0.57); papaverine (VIII) and/or narcotine (IX) (R_f 0.80); and morphine (R_f 0.20). In addition, IB contains two spots (R_f's 0.26 and 0.39) which are not presently identified. The appearance of the yellow spot in nearly all the samples, including the hydrolyzed alkaloids, seems to eliminate it as a bound alkaloid, since it appears to be merely a matter of incomplete extraction.

Fractions IA, IB, IIIA, IIIB, IB aq., and IIIB aq. were all run on preparative tlc, and bands corresponding to the observed spots were eluted with MeOH and $CHCl_3$. After drying, the eluted residues were analyzed by vpc. The results of this analysis appear



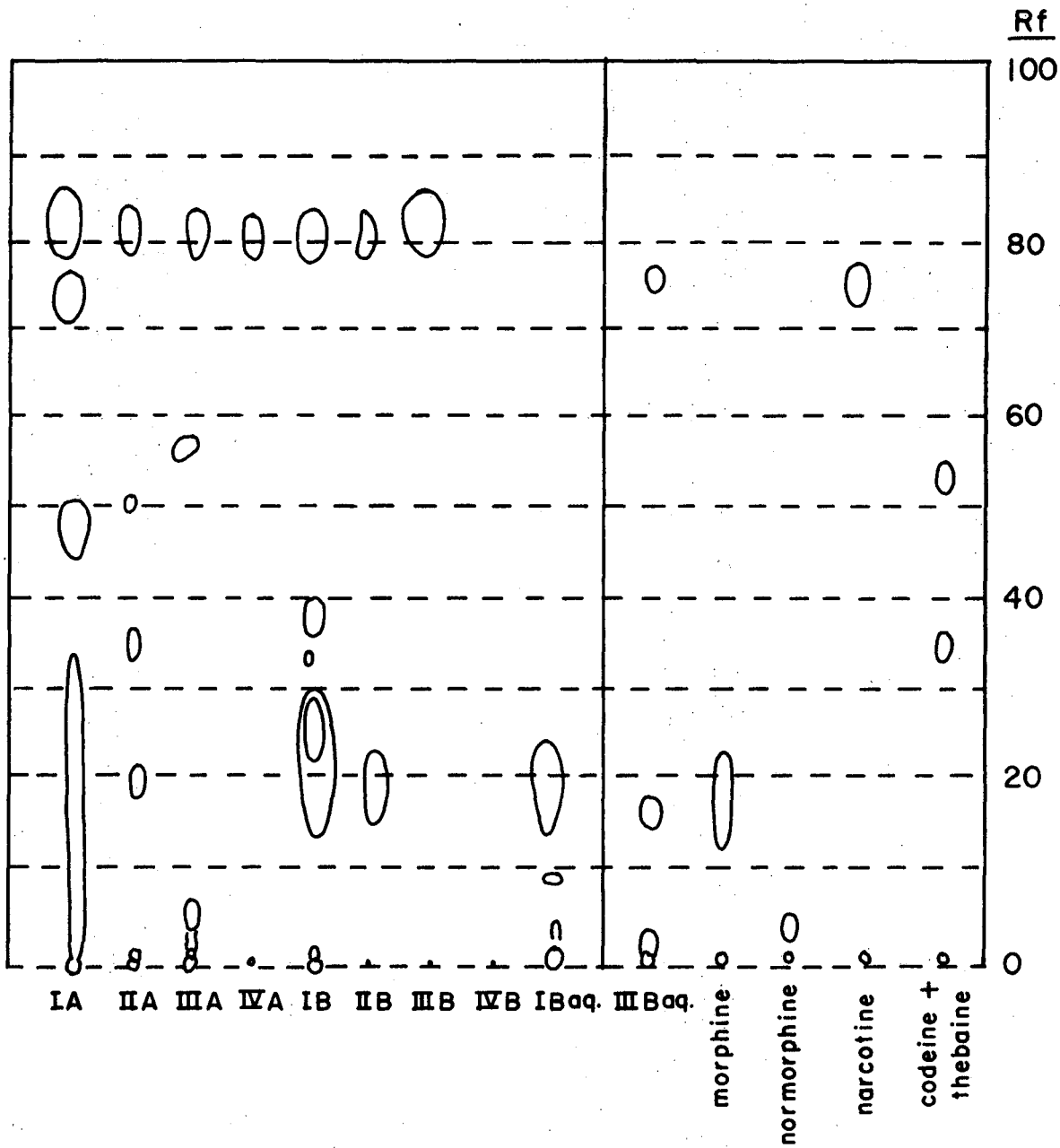
VIII

Papaverine



IX

Narcotine



TLC OF PLANT ALKALOIDS

XBL726-4670

Figure 5.

in Table XXII (non-phenolic alkaloids), Table XXIII (phenolic alkaloids), and Table XXIV (residual alkaloids extracted at pH 9.2). The numbers in the tables under the peak retention times are the areas of the corresponding vpc peaks, relative to a reference. For the non-phenolics, the reference is codeine = 100 (retention time = 4:30, band IA-c). For the phenolics, it is morphine = 1,000 (retention time = 6:00, band IB-b). In Table XXIV, it is the peak at 5:40, band IB aq-c. This is presumed to be residual morphine.

The vpc spectra of Fraction II (the check on the efficiency of the initial plant extract for free alkaloids) are not included here, but it should be noted that significant amounts of several phenolic alkaloids were obtained by this second grinding. In fact, all four of the peaks observed for the Fraction IIIB-c also appear in Fraction IIB (as well as IB-d). However, on the basis of the relative abundances of each of these compounds in the three fractions, one can conclude that the peak at 7:20 (Table XXIII) is the only one that is definitely enhanced by hydrolysis. All the others seem to be lingering, incompletely extracted free alkaloids.

It is of the greatest importance to our metabolism studies to find that no morphine is released by the acid hydrolysis. The data presented here make that conclusion absolutely definitive (compare the 6-minute peak for Fractions IB and IIIB).

Turning finally to the non-phenolic alkaloids (Table XXII), it is quite apparent that the acid treatment has released three major compounds, and one minor one, which were not present in the pre-hydrolysis alkaloid spectrum. Again, as in the phenolic case, there

Table XXII

VPC of Non-phenolic Alkaloids

Sample	TLC Rf range	Peak Retention Time (Min) 4:30	6:30	7:40	11:20	12:45	15:00	16:00	31:30
IA (pre-hydrolysis):									
a	0 → 10	2.4	6.8	---	---	---	---	86.0	88.0
b	10 → 19	---	2.6	---	---	---	---	---	---
c	19 → 37	100.0	7.2	---	3.9	---	---	---	---
d	37 → 58	---	2.3	---	3.9	---	---	---	---
e	58 → 100	---	---	---	---	15.7	---	---	---
IIIA (post-hydrolysis):									
a	0 → 7	---	2.0	---	---	---	---	---	---
b	7 → 65	---	0.7	75.0	---	---	---	---	---
c	65 → 100	3.1	23.5	---	---	---	20.8	---	---

Table XXIII

VPC of Phenolic Alkaloids

Sample	TLC Rf range	Peak Retention Time (Min) 3:00	4:30	5:30	6:00	7:20	16:40
IB (pre-hydrolysis):							
a	0 → 11	3.6	---	---	---	---	---
b	11 → 30	---	---	---	1,000	---	---
c	30 → 42	9.7	---	3.2	---	---	---
d	42 → 100	---	5.7	13.3	---	14.2	59.0
IIIB (post-hydrolysis):							
a	0 → 6	9.7	---	---	---	---	---
b	6 → 64	tr.	---	---	---	---	---
c	64 → 100	---	2.4	8.2	---	14.9	35.2

Table XXIV

VPC of Alkaloids Extracted at pH 9.2

Sample	TLC Rf range	Peak Retention Time (Min) 2:00	2:30	3:00	4;30	5:40	7:20	14:40	16:40
IB aq. (pre-hydrolysis):									
a	0 → 6	---	---	17	---	---	---	---	---
b	6 → 19	---	---	---	---	---	---	---	---
c	19 → 28	---	---	---	---	100	---	---	---
d	28 → 100	---	30	---	41	---	20	35	30
IIIB aq. (post-hydrolysis):									
a	0 → 10	---	---	---	---	---	---	---	---
b	10 → 32	---	---	---	---	---	---	---	---
c	32 → 100	35	---	---	---	---	---	---	---

was evidence of some lingering alkaloids (in Fraction II). However, these do not correspond directly with the compounds produced by hydrolysis. This brings us to the point regarding the distinction between bound alkaloids and acid-sensitive alkaloids. It is known that thebaine undergoes rearrangement in the presence of hot acid.[27] Whether some unknown plant alkaloids might also be sensitive to acid is impossible to determine, without more definitive controls (such as treating the normal free alkaloids with acid, and observing the spectral changes). In retrospect, it would be best to repeat the entire grinding process until absolutely no free alkaloids are left. This would definitely eliminate any concern for this problem.

It is fairly obvious that neither codeine, thebaine, nor morphine exist in "bound" form in the plants of the age studied here. If bound alkaloids do exist, as suggested by the results, they must be alkaloids which occur much earlier in the biosynthetic sequence, or differ entirely from the morphine-type alkaloid. The implications, therefore, are that bound forms of the alkaloids are not directly involved with morphine metabolism at this stage in the plant's development. It would definitely be interesting to do a similar study on mature plants, as a check on the hypothesis of Fairbairn and co-workers that bound alkaloids do play a role in the production of viable seeds.[32]

III. CONCLUSION

The results presented here do lend some support to the hypothesis[32] that acid-hydrolyzable bound forms of alkaloids exist in the seeds of P. somniferum. Whether or not such forms also exist in the plant is still in question, though our results do indicate their presence. In the case of the plants, however, the acid-released alkaloids do not correspond to the more abundant free alkaloids. In particular, no morphine, codeine, or thebaine is released, although one would not expect to find the latter, due to its acid sensitivity. It is apparent from this that the formation of bound alkaloids does not play a role in the metabolism of morphine prior to flowering. Examination of plants beyond that stage should be made, to determine whether alternate metabolic fates are available to morphine in the mature plant.

The levels of alkaloids found in the seeds, both free and bound, represent only a small fraction of the total alkaloid content in the capsule at the time of seed development. Whether these traces of alkaloids are necessary to the propagation of successive generations is impossible to determine from these results. However, the implications are that this is a minor role, at most, for the morphine alkaloids.

IV. EXPERIMENTAL

A. Seed Alkaloid Extraction

Twenty gm of poppy seeds were picked free of capsule debris, and then washed with 100 ml of 1% H_3PO_4, followed by 50 ml H_2O. The combined aqueous washes were brought to pH 8.6 and extracted with $CHCl_3$ (5 x 75 ml). The latter extract was dried over Na_2SO_4, and evaporated to dryness to give the "Pre-Grind Alkaloids" (II).

The washed seeds were transferred directly to liquid N_2, frozen briefly, then ground in a Waring blender with 30 ml of 10% Na_2CO_3 and 100 ml of butanol/benzene (1:1). The extraction then proceeded in the same manner as that for plant alkaloids, to give the alkaloids extracted into $CHCl_3$ at pH 8.6. The aqueous phase at this point was readjusted to pH 9.2 with 8 M KOH, and extracted with three portions of $CHCl_3$/EtOH (4:1). These two organic extracts were dried over Na_2SO_4, and evaporated to dryness, to give the "Free (pre-hydrolysis) Alkaloids" (I). The remaining seed mash was completely extracted a second time, as a blank check on the efficiency of the free alkaloid extraction.

The seed mash was then centrifuged to separate the solid and aqueous phases. The solids were washed once with water, recentrifuged, and again separated. The combined aqueous phase was taken to approximately 2 M in acid by adding concentrated HCl, then heated and stirred on the steam bath for 2 hr. The solid phase was heated

in the same manner after the addition of 60 ml of 2 N HCl. The aqueous portion, after cooling, was brought to pH 8.6 with 10% KOH and solid Na_2CO_3. It was then extracted with 5 x 100 ml of $CHCl_3$, and the latter extract dried and evaporated to give the "Water-soluble, Hydrolyzable Alkaloids" (III). The remaining aqueous phase was then brought to pH 9.2 and extracted with $CHCl_3$/EtOH (4:1); this extract was also analyzed as water-soluble hydrolyzable alkaloids. The solid material, after heating in acid, was cooled and brought to pH 8.6 with 10% KOH and solid Na_2CO_3. The solution was blended with 200 ml of butanol/benzene (1:1), the organic supernatant withdrawn, and the mash extracted twice more with 100 ml portions of butanol/benzene. The combined organic phase was treated as normal, to give the $CHCl_3$ extract at pH 8.6, and a $CHCl_3$/EtOH (4:1) extract at pH 9.2. The two dried and evaporated organic extracts were then the "Water-insoluble Hydrolyzable Alkaloids" (IV).

Both analytical and preparative tlc's of the four extracts were developed in $CHCl_3$/MeOH/NH_3 (3:1:trace). The vpc spectra were obtained by dissolving each sample in 25 µl MeOH, and injecting a 2 µl aliquot onto the column. The column used was 6 feet of 3% OV-17 on Gaschrom Q, operated at 218°C.

B. Plant "Bound-Alkaloid" Extraction

Fifty gm of fresh plant material were cut up and frozen in liquid nitrogen, and then blender-extracted in the normal manner. A second complete butanol/benzene extract was performed on the plant mash as a check on the efficiency of alkaloid removal. Both organic extracts

were extracted with 4 x 50 ml H_3PO_4 (1 M, pH 2). The pH of the aqueous phase was adjusted to 12 with KOH (8 M) and solid Na_2CO_3, and extracted with 5 x 80 ml $CHCl_3$ to give the free phenolic alkaloids. Both organic extracts, as well as subsequent ones, were dried over Na_2SO_4, and evaporated to dryness in vacuo.

The aqueous plant mash was centrifuged, and the aqueous phase removed. The solids were washed once with water, centrifuged, and separated. To the combined aqueous portions was added 25 ml of concentrated HCl, to bring the acid concentration near 2 N. The resulting solution was heated on the steam bath for two hours, with stirring. It was cooled and brought to pH 12 with KOH, and extracted with 5 x 80 ml $CHCl_3$. The pH was readjusted to 8.5 with dry ice, and extracted with $CHCl_3$. These two extracts gave, thus, the non-phenolic and phenolic water soluble hydrolyzable alkaloids, respectively. To the solid phase was added 50 ml of 2 N HCl. The mixture was heated on a steam bath for two hours, cooled, the pH adjusted to 9 with KOH, and the mixture transferred to the Waring blender. It was then extracted as normal with butanol/benzene, to a total organic extract of 400 ml. The butanol/benzene phase was extracted as above to give the water-insoluble, hydrolyzable non-phenolic and phenolic alkaloids.

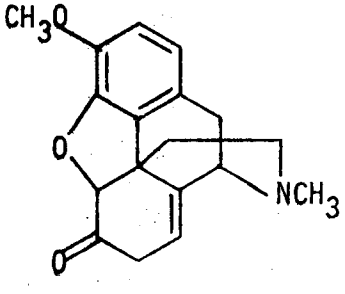
Vpc analysis of these extracts was done with a 6-foot silanized column of 4.5% UC-W-96 on Aeropak 30 (100-120 mesh), at a column temperature of 218°C.

PART 3

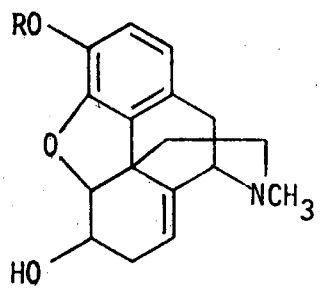
SYNTHESIS OF NORNEOMORPHINE

I. INTRODUCTION

Parker has shown that neopinone (X) is present in the poppy plant, and is an intermediate in the biosynthetic conversion of thebaine (III) to codeine (IV).[16] Its presence leads one to suspect that an alternate biosynthetic fate might be conversion to neopine (XI) and thence to neomorphine (XII) and norneomorphine (XIII), by a path paralleling the normal morphine biosynthesis. The possibility also exists that norneomorphine, due to its similarity to normorphine, may have been the plant-derived normorphine impurity which was encountered in some of the earlier $^{14}CO_2$ exposures. With both these thoughts in mind, it was decided to synthesize some authentic normorphine and neomorphine, for use as comparison standards for subsequent plant work. It should be noted that none of these alkaloids (other than neopinone) has been found as a native poppy constituent. Neopine, however, has been found in extracts of raw opium.[42]

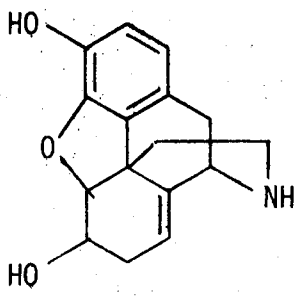


X



XI: R=CH_3

XII: R=H

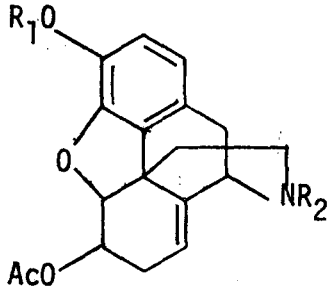


XIII

The synthetic approach taken was to convert neopine to neomorphine with HBr in acetic acid. Acylation of the neomorphine, followed by treatment with diethylazodicarboxylate, would afford, upon hydrolysis, norneomorphine. Some preliminary searches were made to determine whether or not any of these compounds exist in the poppy plant.

II. RESULTS AND DISCUSSION

Neomorphine (XII) has been prepared by Small by conversion of neopine-HBr to 6-acetylneomorphine (XIV) with 15% HBr in acetic acid, and hydrolysis of the latter in 2 N NaOH to neomorphine.[43] Norneomorphine (XIII), however, has never been reported in the literature. The common method of N-demethylation, the Von Braun reaction, will not work on neomorphine, due to the position of the double bond.[18] A recent patent, however, describes the conversion of thebaine to northebaine, as well as diacetylmorphine to normorphine, using diethylazodicarboxylate as the demethylating agent.[44] It was hoped that diacetylneomorphine (XV) could be treated in the same manner to give norneomorphine, upon hydrolysis of the intermediate adduct (XVI).



XIV: R_1=H, R_2=CH_3

XV: R_1=Ac, R_2=CH_3

XVI: R_1=Ac, R_2=CH_2-N(NH-CO_2Et)(-CO_2Et)

The first step in the conversion sequence was the treatment of neopine·HBr with 15% HBr in glacial acetic acid. Unfortunately, these conditions were apparently misinterpreted, and I made up the reagent by mixing 3.6 ml of concentrated HBr (48%) with 8.4 ml of glacial acetic acid. Small had reported a crude yield of about 75% of the 6-acetylneomorphine, whereas I was able to obtain only a 35% yield. In addition, he had reported gas evolution, when the temperature of the solution reached 115°C, the gas supposedly being CH_3Br. No such gas evolution was observed under the aqueous conditions used here, probably due to hydrolysis of the resulting CH_3Br. The product obtained was a cream-colored precipitate, which had a broad melting point of 200-245°C, with decomposition. Small reported a melting point of 243-251°C (evac. tube, decomp.) for once-crystallized 6-acetylneomorphine.

The crude 6-acetylneomorphine (300 mg) was hydrolyzed by boiling in 2 N NaOH for 10 minutes. The work-up of the product was done differently than Small had done. Small had saturated with CO_2, evaporated to dryness, and digested the residue with $CHCl_3$. I adjusted the pH of the reaction mixture to 8.6, and extracted the neomorphine with $CHCl_3$/IPA (3:1). The residue obtained upon evaporating the latter extract consisted of part crystals and part oil. The melting point of the crystals was 90-93°C, which compares with a reported melting point of 107°C for the pure $CHCl_3$-solvated neomorphine. The crystals were sublimed at 100°C, 100 microns, for 2 hours, then at 120°C for six hours. The sublimate (20 mg) had a melting point of 107°C; however, crystals (80 mg) had also developed on the residue

in the bottom of the sublimer, and these melted at 210-215°C with decomposition (lit. mp 240-241°, evac. tube, decomp.).

Attempts were made to improve the initial O-demethylation step on neopine·HBr. Two different reactions were run. The first involved demethylation directly to neomorphine, using 48% HBr (aqueous). This was mentioned by Small as an alternative to using 15% HBr/HOAc, which yields 6-acetylneomorphine. The reaction was carried out on one gram of neopine·HBr, using a 10-fold molar excess of HBr. The problem of water-solubility of the neomorphine, mentioned as a deterrent by Small, was overcome by extracting the neomorphine from the aqueous reaction mixture with $CHCl_3$/IPA (3:1) at pH 8.6. This was preceded by a $CHCl_3$ extraction at pH 11 to remove any unreacted neopine. Analysis of the latter extract by tlc did show neopine as the only spot. The extraction process gave a crude phenolic extract (90% yield), which upon drying at 75° under high vacuum for one hour, gave a pale-yellow powder, 0.44 gm (60%), which melted over a broad range from 110°-150°C. This implies that the product is partially solvated ($CHCl_3$-solvate, which has a melting point of 107°C, compared to 240°C for the free neomorphine). Tlc of the product showed only a single spot, R_f = 34, which compares to an R_f = 36 for morphine. Vpc of the product gave a single peak, with a retention time of 5:18 (compared with 5:07 for morphine).

A final attempt was also made to repeat Small's preparation of 6-acetylneomorphine, after obtaining the non-aqueous HBr (30-32%) in HOAc from Eastman Chemicals. 15% HBr in HOAC was prepared by mixing one gram of the commercial HBr with one gram (0.95 ml) of

glacial acetic acid. Using this for demethylating neopine·HBr (one gram), under Small's conditions and work-up procedure, gave a 66% yield of crude 6-acetylneomorphine. Analysis by tlc and vpc indicated the presence of about 10% neopine, and the melting point of 190-250°C (decomposition) confirmed this lack of purity.

To prepare diacetylneomorphine for the subsequent conversion to norneomorphine, 6-acetylneomorphine was prepared using the original conditions tried (i.e., 15% aqueous HBr in glacial acetic acid). A 55% yield of crude product was obtained, and tlc showed it to be much more pure than previously. This was converted directly to diacetylneomorphine by acetylating with acetic anhydride in pyridine, at room temperature for 32 hours.[43] The product, recrystallized once from Ligroin, was a white, crystalline solid, with a melting point of 124-128°C (lit. mp 127-127.5°C). It gave a single spot, at R_f = 64, on tlc; this compared with an R_f of 70 for the morphine analog, heroin.

It should be noted that diacetylneomorphine was also prepared from both 6-acetylneomorphine and neomorphine, using refluxing acetic anhydride. Although the crude product was pure diacetylneomorphine by tlc and vpc (R_f = 8:40), several attempts to purify it further by recrystallization and sublimation failed. However, enough pure material was on hand for the final conversion to norneomorphine, so this investigation was dropped.

Treatment of diacetylneomorphine with 1.8 equivalents of diethylazodicarboxylate in benzene, followed by a basic hydrolysis of the crude product, gave a 72% yield of norneomorphine (m.p. 120-150°). This product was pure by tlc (R_f = 22, compared to R_f = 19 for

normorphine). It was indistinguishable from normorphine by vpc. However, conversion to the triacetyl derivatives with refluxing acetic anhydride accomplished a definitive separation by vpc (R_f = 28:40 for norneomorphine-Ac_3, 36:40 for normorphine-Ac_3). It should also be mentioned that the diacetates of morphine and neomorphine are well separated by vpc (8:55 and 7:00, respectively).

Since the nmr spectrum for the neomorphine-type compound has never been published, I have summarized it here in Table XXV, taken as the diacetyl derivative (neoheroin). The spectra of heroin, 6-acetylneopine,[45] and 6-acetylcodeine[45] are included for comparison. As can be seen from this data, morphine and neomorphine are readily distinguishable by nmr. The same is true for normorphine and norneomorphine, whose nmr spectra are summarized in Table XXVI.

No definitive search has been made for the neo-series in the plant. One plant extract, of 90-day-old plants, did show a peak in vpc which co-chromatographed with authentic norneomorphine (as the peracetates). A crude approximation of abundance indicated about 0.6% relative to the morphine concentration. A subsequent extract of 87-day-old plants failed to confirm this finding, so the results are still inconclusive.

No neomorphine has been seen, although the presence of large quantities of both morphine and codeine interfere considerably with its detection. A peak has been observed in several plant extracts which does correspond to neopine. Further evidence for the presence of these alkaloids is being sought using a gas chromatography-mass spectrometer system.

Table XXV

Chemical Shifts of Definitive Protons in Morphine/Neomorphine (δ)

	Neoheroin	Heroin	6-AcNeopine	6-AcCodeine
1,2-H	6.70	6.64	6.59	6.67
5β-H	4.70	5.1	4.66	5.02
6-H	5.4	5.1	5.4	5.1
7-H	---	5.6	---	5.62
8-H	5.46	5.45	5.44	5.36
9α-H	3.58	3.34	3.59	3.36
10β-H	3.2	3.06	3.28	3.08
3-OCH_3	---	---	3.85	3.85
N-CH_3	2.44	2.45	2.44	2.43
3-O_2CCH_3	2.27	2.28	---	---
6-O_2CCH_3	1.53	2.14	1.49	2.15

Table XXVI

Proton(s)	Chemical Shifts (δ) Triacetyl-normorphine	Triacetyl-norneomorphine
1,2-H	6.68	6.75
5-H	5.1	4.8
6β-H	5.1	5.6
7-H	5.6	---
8-H	5.45	5.6
9α-H	3.4	3.5
10β-H	3.1	3.1
3-OAc	2.25	2.30
6-OAc	2.15	1.55
N-Ac	3.32	3.5

III. CONCLUSION

The synthetic procedures tested here do provide a suitable means for preparing both neomorphine and norneomorphine. If neomorphine is the desired product, the best O-demethylation procedure seems to be treatment of neopine·HBr with aqueous 48% HBr. The preparation of diacetylneomorphine for subsequent N-demethylation to norneomorphine, however, is best done by making the 6-acetylneomorphine, in non-aqueous HBr/HOAc, and converting this to the diacetate. As noted in the discussion, the conditions used here left some unreacted neopine. It seems likely that extending the time of the reaction should bring it to completion. This could easily be followed by vpc to determine optimum conditions.

The use of diethylazodicarboxylate as a demethylating agent proves to be very successful for this type of compound. In fact, the entire procedure from diacetate to norneomorphine is much easier than a corresponding Von Braun demethylation of morphine. Under the conditions used here, it appears as if the demethylation is nearly quantitative.

Additional work is definitely called for to determine whether or not the neo-series of alkaloids exist in the poppy plant. The availability of authentic standards and a suitable extraction and analytical procedures make such studies feasible.

IV. EXPERIMENTAL

A. Preparation of Neomorphine

A suspension of 2 grams of neopine hydrobromide in 12 ml of HBr in glacial acetic acid (3.6 ml of 48% HBr and 8.4 ml of glacial acetic acid) was stirred and heated to 145°C over a period of one hour. This was followed by 15 minutes of refluxing at 145°C. After cooling the product mixture to room temperature, 12 ml of H_2O was added, followed by concentrated NH_4OH to near pH 7. Additional NH_4OH was added to the solution at 0°C, until a copious precipitate was obtained (about pH 8). This precipitate was filtered, to give 420 mg of a cream-colored solid, crude 6-acetylneomorphine.

300 mg of this crude material was dissolved in 2 ml of 2 N NaOH. This solution was boiled for 10 minutes. After cooling only slightly, the solution was added to 2 ml of 1 M H_3PO_4 (pH 2.5), and the resulting solution was brought to pH 8.6 with additional 1 M H_3PO_4. Water was also added to bring the total volume to about 15 ml. The solution was then extracted with 5 x 20 ml of $CHCl_3$/IPA (3:1). The organic extract was dried over Na_2SO_4, and evaporated to dryness *in vacuo*. The residue was part crystalline and part oil. The crystals were removed, and dried for two hours at 100°C, 100 microns, and then at 120°C, 100 microns, for 6 hours. This yielded 80 mg of neomorphine, with a melting point of 210-215°C. In addition, 20 mg of $CHCl_3$-solvated neomorphine was obtained as a sublimate; this melted at 107°C.

An alternative method for preparing neomorphine directly from neopine was also tried, with some success. To 1 gm of neopine·HBr was added 3.0 ml of 48% aqueous HBr. The suspension was heated slowly to 105°C, at which time gas evolution was observed. The temperature was then increased to 145°C over a period of an hour. After cooling the solution, it was brought to pH 11, with concentrated NH_4OH and 8 M KOH. The solution was then extracted with 2 x 25 ml of $CHCl_3$ to remove any unreacted neopine. The pH of the aqueous solution was then brought to 8.6 with H_3PO_4 (conc.), and extracted with 4 x 25 ml of $CHCl_3$/IPA (3:1). The organic extract was dried over Na_2SO_4, and evaporated to dryness. The residue was dried for one-half hour at 75°C, 100 microns, yielding 0.44 gm of a dry brownish powder, which melted over a broad range of 110-150°C. Chromatographic analysis (tlc and vpc) showed only neomorphine.

B. Preparation of Diacetylneomorphine

The procedure used by Small[48] was modified slightly in the work-up. To 10 gm of neopine·HBr was added 60 ml of aqueous 15% HBr (in acetic acid (18.8 ml of 48% HBr diluted to 60 ml with glacial acetic acid). The suspension was heated slowly to 115°C, where boiling commenced, and then to 145°C over the next hour. The solution was cooled, and brought to neutrality with NH_4OH (conc.). The solution was kept at 0°C for 2 hours, and the precipitate formed was collected, to give 1.13 gm of a solid melting at 250-259°C (dec.); the reported melting point is 245-251°C (dec.). The pH of the aqueous residue was adjusted to 8.6, and the solution extracted continuously with CH_2Cl_2

for 20 hours. The extract was evaporated to dryness, and the residue recrystallized from 95% EtOH, to give 1.02 gm of pale yellow crystals, m.p. 250-266°C (dec.).

The combined products from above were acetylated with 8 ml of pyridine and 4 ml of acetic anhydride. This mixture was allowed to sit at room temperature for 24 hours, at which time tlc of the reaction mixture showed a single spot, corresponding to the diacetate. The solution was evaporated in vacuo to reduce the volume as much as possible. It was then dried over H_2SO_4 (conc.), under high vacuum, overnight. The syrupy residue was emulsified in saturated NaCl (~30 ml), and sodium bicarbonate added until gas evolution ceased. The aqueous solution was then extracted with 2 x 100 ml of ether, the latter extract dried and evaporated to yield a yellow syrup. Recrystallization from ligroin (b.p. 60-110°C) gave 340 mg of diacetylneomorphine, having a melting point of 117-125°C (reported melting point is 127-128°C). Analysis by tlc, vpc, and nmr indicated purity. This material was used as is for the subsequent conversion to norneomorphine.

C. Preparation of Norneomorphine

To 230 mg of diacetylneomorphine was added 5 ml of benzene and 0.15 ml of diethylazodicarboxylate. The resulting mixture was brought to reflux, under nitrogen, and refluxed for 3 hours at 110°C, and an additional hour at 115°C. The cooled solution was evaporated to near dryness in vacuo. The residue was taken up in 3 ml of NaOH, and the resulting solution refluxed at 125°C, under

nitrogen, for two hours. The pH of the cooled solution was brought to 8.6 with 5% H_3PO_4. The solution was extracted with 6 x 30 ml of $CHCl_3$/IPA (3:1), the organic extract dried over Na_2SO_4, evaporated to near dryness in vacuo, and then to dryness under high vacuum. This yielded 133 mg of a pale yellow solid, m.p. 120-150°C (preceded by bubbling).

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