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INTERMEDIATES IN THE BIOSYNTHETIC CONVERSION OF THEBAINE TO CODEINE IN *PAPAVER SOMNIFERUM*

Harriet Inez Parker
(Ph.D. Thesis)

March 1971

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INTERMEDIATES IN THE BIOSYNTHETIC CONVERSION OF THEBAINE TO CODEINE BY *PAPAVER SOMNIFERUM*

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Harriet Inez Parker

Lawrence Radiation Laboratory
University of California
Berkeley, California

March 1971

ABSTRACT

The biosynthesis of opium alkaloids has interested scientists for many years, both because of the complexity of structure of these compounds and because a greater understanding was sought regarding their importance *in vivo* in the hope that this understanding would also lend some insight into the reasons for the great pharmacological importance of these alkaloids. Many of the steps on the biosynthetic route to morphine have already been elucidated. The final steps have been shown to involve the conversion of thebaine to codeine, which is then demethylated to morphine. Since the conversion of thebaine to codeine involves two reactions, a demethylation and a reduction, at least one intermediate must be involved. If reduction occurs first, codeine methyl ether will be an intermediate in the conversion. On the other hand, if demethylation occurs first, codeinone will be the required intermediate.

The initial purpose of the research described in this paper was to determine which of these two paths was followed *in vivo*. Experimental evidence was obtained by three types of experiments: precursor feedings of the proposed intermediates; a search for each of the

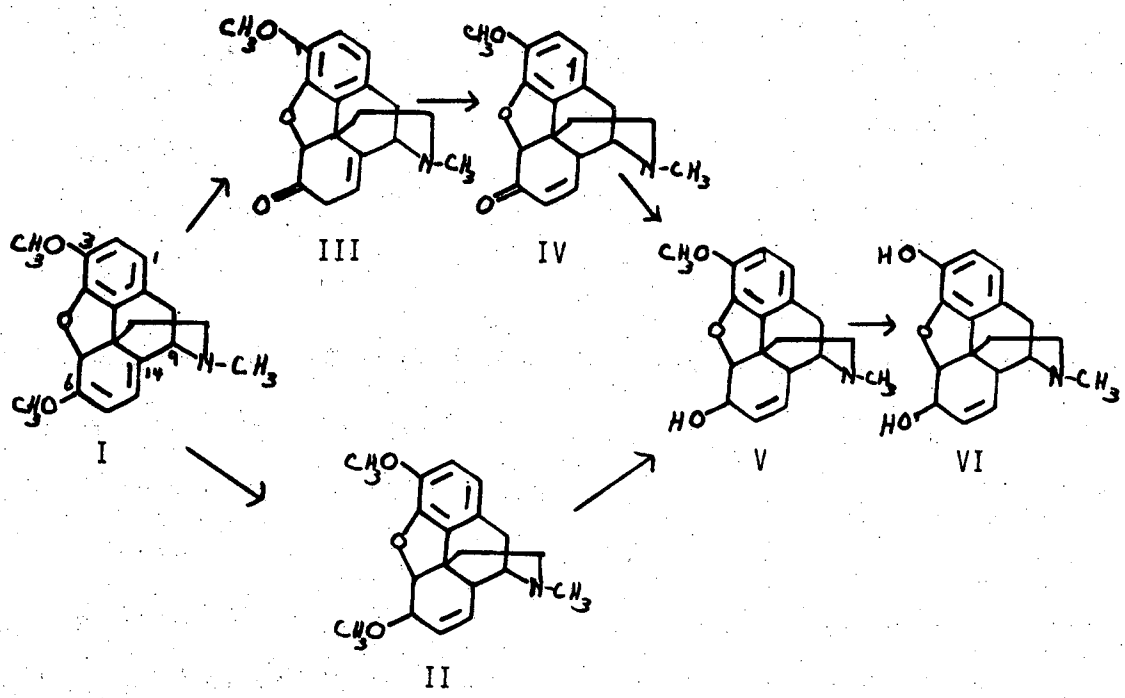
compounds in opium poppy plants (Papaver somniferum); and finally, in the case of codeinone, an exposure to $^{14}CO_2$ under "steady state" conditions (i.e., conditions which are identical with the normal conditions of growth -- except for the radioactive label -- and in which the specific activity of the $^{14}CO_2$ does not decrease during the exposure time), followed by a comparison of the specific activities of the isolated thebaine, codeinone, and codeine. Although the precursor feedings demonstrated that both codeine methyl ether and codeinone could be converted to codeine by the plants, no codeine methyl ether could be found in the plants. On the other hand, the presence of codeinone was detected, and after the "steady state" exposure its specific activity fell between those of thebaine and codeine, as would be expected if it were an intermediate in the conversion.

Once the role of codeinone had been established, the possible involvement of neopinone in the biosynthetic conversion of thebaine to codeinone was studied. A precursor feeding did indicate that neopinone could be converted to codeine by the plants. In addition, the presence of neopinone in the plants was detected. Therefore, it appears likely that the biosynthetic route from thebaine to codeine involves initial demethylation to neopinone, rearrangement to codeinone, and then reduction to codeine.

I. INTRODUCTION

The biosynthesis of opium alkaloids has interested scientists for many years, both because of the complexity of structure of these compounds and because a greater understanding was sought regarding their importance *in vivo* in the hope that this understanding would also lend some insight into the reasons for the great pharmacological importance of these alkaloids. Since good reviews have frequently appeared on the subject of the biosynthesis of morphine (the most recent such review is by Kirby[1]), only a brief discussion of the known sequence of steps will be presented in this thesis (Chapter II).

The final steps in the biosynthesis of morphine have been shown[2] to involve the conversion of thebaine (I) to codeine (V), which is then demethylated to morphine (VI). Since the conversion of thebaine (I) to codeine (V) involves two reactions, a demethylation and a reduction, at least one intermediate must be involved. If reduction occurs first, codeine methyl ether (II) will be an intermediate in the conversion. On the other hand, if demethylation occurs first, codeinone (IV) will be the required intermediate. The initial purpose of the research described in this paper was to determine which of these two paths was followed *in vivo*. After evidence was obtained which favored the role of codeinone (IV) in the conversion of thebaine to codeine, the potential involvement of neopinone (III) in the route from thebaine to codeinone was studied in order to gain some



understanding of the mechanism of this conversion. Evidence was obtained which did favor the involvement of neopinone in the biosynthesis of morphine. A detailed discussion of this work is part of this thesis.

In the course of studying a biosynthetic route, the methods (and assumptions) used for such a study have come under careful scrutiny. Some very important changes were made in these methods in order to reduce the kinds of misinformation to which biosynthetic studies are subject. Therefore, a discussion of biosynthetic methods and the changes which were made in some of these methods will also be presented in this thesis.

II. BIOSYNTHESIS OF MORPHINE

Although there are many compounds, such as amino acids and carbohydrates, which exist in all plants, the group of compounds known as alkaloids is not nearly as widespread. Alkaloids may be defined as organic compounds which are naturally occurring which contain nitrogen as an amino group (although synthetic analogues are often given this title as well). The nitrogen is usually present in a heterocyclic ring, and the structures of most alkaloids are quite complex, involving several fused rings.

The distribution of alkaloids in living organisms is quite broad, but nevertheless they do not exist in most species. Most alkaloids which are known today have been found in vascular plants, but alkaloids have also been found in fungi (for example, the ergot alkaloids), and there are also several alkaloids which have been isolated from animals.[4] Within the plant kingdom, alkaloids have been found in at least 38 families,[3] over 3000 different alkaloids having been isolated from about 4000 plant species.[4] Aside from the poppy family Papaveraceae, in which all species have been found to contain alkaloids, within most families only specific species or genera contain alkaloids. In general, when the relationship between genera is close, the structures of the alkaloids isolated from species within these genera are also quite similar.[3]

There are no plant organs in which alkaloids have not been found. However, in a given species, certain organs may be alkaloid-free -- for example, the opium poppy seeds are devoid of alkaloids. The places in which high concentrations of alkaloids usually occur are in: (a) active tissues and growing fruits; (b) surface tissues, such as the epidermal layer and the layers just below it; (c) vascular sheaths; and (d) latex vessels.[3]

The function of the alkaloids within plants is still a subject open for much speculation at this point. Since only approximately 15% of the known species contain alkaloids, and the structures of these alkaloids are quite diverse, it is likely that the role of these alkaloids is not universal (in fact, an alkaloid-containing plant, when grafted to a non-alkaloid-containing plant, will cease to produce alkaloids without showing any physiological abnormalities).[3] Some ideas which have been proposed for the function of the alkaloids are[3]: (a) involvement in nitrogen metabolism and detoxification; (b) protection from insects or other predators; (c) direct involvement in general metabolism, either as methyl carriers, or possibly as cofactors, or being linked to regulatory mechanisms in some manner; and (d) no metabolic function, their formation being merely a result of harmless mutation. At present, none of these roles have been firmly demonstrated, and much further study must be done in this field.

In the case of the opium poppy plants, the concentration of thebaine and other alkaloids varies greatly with the age of the plants. No unbound alkaloids have been found in the seeds. The presence of thebaine can barely be detected (a concentration of 1 μg/g fresh plant

weight) in 3-day-old seedlings, but then it rapidly accumulates and reaches its maximum concentration (125 μg/g fresh plant weight) in 5-day-old seedlings.[5] In older plants the concentration is considerably lower, and is around 3-4 μg/g fresh plant weight during the time the plant is blossoming and forming its seeds. On the other hand, codeine can be first detected in 5-day-old seedlings, but it does not reach its maximum concentration (125 μg/g fresh plant weight) until 60 days, after which its level decreases to approximately 40 μg/g fresh plant weight during plant blossoming. It is apparent that the rate of synthesis of the opium alkaloids is not the same throughout the life-cycle of the plant. This difference in rate is probably due to different levels of enzymes -- a determination which has not yet been done. The alterations in alkaloid concentration with the age of the plant also would tend to indicate that alkaloid synthesis is not a totally useless function for the plant -- i.e., that at certain times in the plant's growth the need for alkaloids is greater for some reason or another, and therefore more are formed.

Elucidation of the structures of the alkaloids and in vitro synthesis of these molecules has challanged chemists for years. Many alkaloids also have the added attraction of having important physiological effects when used by humans -- in fact, many of the plants from which alkaloids can be isolated were used by witch doctors in their treatments or were (and are) used in social ceremonies for psychological effects induced by the alkaloids -- or were used as poisons!

Opium has been known for thousands of years, and has been used in many societies, both for its medicinal effects and for the euphoric

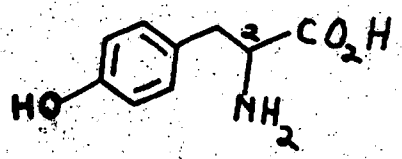
effects it produces when smoked. It is obtained from the immature seed capsules of the poppy Papaver somniferum (although other species within the Papaver genus contain some of the opium alkaloids, none contain morphine or codeine).[3] The capsules are slit, and a viscous, milky liquid (the latex) exudes. This liquid soon dries and darkens in the air, forming a dark, slightly sticky substance which can be scraped off the outside of the capsule. This is opium. The alkaloids of opium are also present throughout the plant at all stages of growth, except possibly as seedlings.[5] Most of the alkaloids are found in the latex, which occurs in latex tubes throughout the plant and are particularly numerous in the wall of the capsule.[3]

The chief alkaloid responsible for opium's medicinal and psychic effects is morphine (VI), which may constitute up to 20% of the opium.[6] Even today, morphine is the best pain reliever available. Codeine (V), which is also found in opium, acts as a milder analgesic, its effects being caused by in vivo demethylation to morphine in the patient.[7] Other opium alkaloids have also been found to have pharmacological activity,[8] and some, such as thebaine (I), have provided a good starting point for synthesis of related compounds. Although opium has been the source of many pharmacologically important compounds, most of these compounds are addicting. Therefore, the search for analogous compounds which are non-addicting still continues.

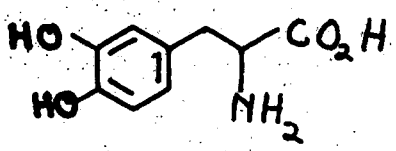
Both from the chemist's and from the pharmacologist's point of view, the structure and synthesis of each of the medicinally useful opium alkaloids has been of great interest. Work towards the elucidation of the structure of morphine started with its isolation from opium

by Sertürner in 1805,[9] and was not finished until two complete syntheses of morphine were achieved in the 1950's by Gates and Tschudi[10,11] and by Elad and Ginsburg,[12,13] and an X-ray analysis was reported by Mackay and Hodgkin.[14]

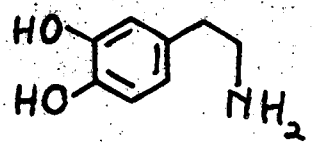
The biosynthesis of morphine has also been of interest for several years, even before its structure was completely known. As early as 1910, Winterstein and Trier[15] attempted to describe the biosynthesis of alkaloids as part of the general metabolism of amino acids. Then in 1917, Robinson[16] suggested that amino acids such as tyrosine (VII) might be involved in the biosynthesis of benzylisoquinoline alkaloids. It was Robinson who really laid the foundations of modern biogenetic theory through his structural correlations of many widely differing alkaloids. He based his discussions on known chemical reactions. For example, if dihydroxyphenylalanine (VIII) were, on the one hand, to decarboxylate to the amine (dopamine) (IX), and on the other, to oxidatively deaminate to the ketoacid (X), condensation between the amine and the carbonyl could occur, followed by cyclization and decarboxylation to a benzylisoquinoline alkaloid. One further suggestion which Robinson made at that time, based on an incorrect structure of morphine, was a suggestion which nevertheless has been substantiated by recent studies -- i.e., that certain benzylisoquinoline alkaloids are the biosynthetic precursors of morphine. In fact, a subsequent suggestion in 1925,[17] restated in his book in 1955[18] when the structure of morphine was known, pointed out that rearrangement of the skeleton of norlaudanosoline (XI) by rotation of ring A about the dotted line, as shown on the following page, yields a structure whose relationship to morphine (VI)



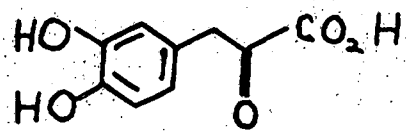
VII



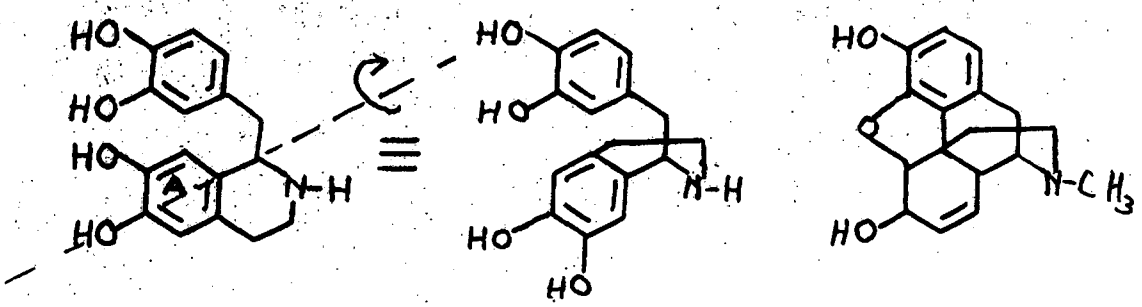
VIII



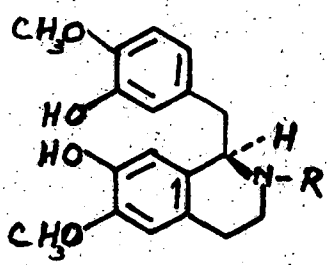
IX



X

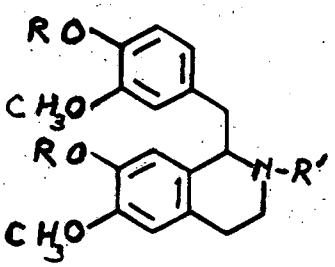


XI VI



XII R = CH_3

XV R = H



XIII R = CH_3, R' = H

XIV R = H, R' = CH_3

is immediately apparent. This early work led to the proposal for the structure of morphine which was finally shown to be correct several years later.

Very little progress was made in the actual study of the biosynthesis of alkaloids until discovery of radioactive isotopes. With the availability of a label, tracer studies could be conducted to detect compounds present in trace amounts, and feeding experiments could be conducted to determine whether a proposed intermediate of a biosynthetic conversion actually was involved in that sequence.

During the past decade, much work has been devoted to the study of the biosynthesis of opium alkaloids, particularly to that of morphine. Many of the steps in this sequence have been elucidated, although there are several questions which still remain to be answered, not the least of which are questions regarding the biological function of morphine and the other alkaloids in the plant.

The involvement of tyrosine (VII) or related compounds in the biosynthesis of morphine (VI), as proposed by Robinson,[18] was examined by several groups. Both Battersby[19] and Leete[20] fed tyrosine-2-^{14}C to Papaver somniferum plants and obtained incorporation of activity into morphine to an extent ranging from 0.017%[20] to 1.7%,[19] depending upon the feeding technique employed (Leete used a root feeding method, and Battersby injected the labelled compound directly into the immature seed capsule of the poppy plant). Degradation of the morphine isolated revealed that specific incorporation of label had occurred at C-9 and C-16, as would be expected if the biosynthetic route from tyrosine goes as predicted through a benzylisoquinoline intermediate,

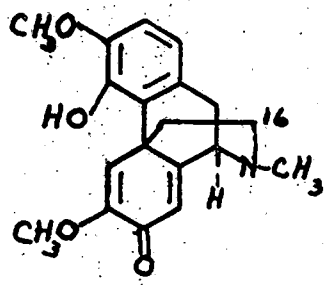
followed by cyclization to a morphine-type structure. In addition, a similar feeding[21] of 3,4-dihydroxyphenethylamine-1-^{14}C (dopamine (IX)) led to incorporation into morphine, with the label only at C-16, once again giving support to a biosynthetic route to morphine from compounds related to tyrosine.

Several benzylisoquinoline alkaloids were examined for their involvement in the biosynthesis of morphine. Norlaudanosoline (XI), labelled either at C-1 or at C-3, was found to have good incorporation into morphine (2.2 - 3.9%),[22] with the incorporated label specifically at C-9 or C-16, as would be expected. Various methylated forms of norlaudanosoline were also examined for incorporation. Rapoport et al.[2] had already demonstrated that morphine is derived from thebaine (I) by demethylation and reduction (see below). Therefore, the benzylisoquinoline precursor of thebaine would be expected to be methylated, probably in a very specific manner. The compound which has a methylation pattern agreeing with that of thebaine (I) is reticuline (XII). Upon being fed to the plant, reticuline was incorporated into the morphine-type alkaloids. In order to answer the objection that the incorporation of reticuline might have been due to prior demethylation of reticuline (XII) to norlaudanosoline (XI), the reticuline fed was specifically labelled in the N-methyl and O-methyl groups as well as at C-3.[24] The ratio of activity in the methyl groups to that at C-3 was determined. In the isolated morphine these same activity ratios were measured and found to be unchanged, thus indicating direct biosynthesis from reticuline. Also, it was found that a benzylisoquinoline with a different methylation pattern (for example, tetrahydropapaverine (XIII) or even

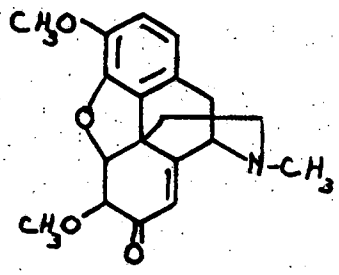
orientaline (XIV) -- and others were also tried) was not incorporated into the morphine-type alkaloids.[22,24] The methylation sequence leading from norlaudanosoline (XI) to reticuline (XII) must still be determined. Preliminary work[22] has shown that N-norreticuline (XV), when fed to the plants, does become converted to morphine.

To account for the transition from a benzylisoquinoline structure to a morphine-type structure, a method of oxidative cyclization of reticuline (XII) was proposed by Barton and Cohen.[25] They suggested a radical oxidation of the phenolic hydroxyl groups of reticuline to furnish two phenolate radicals which, by intra-molecular coupling, would yield a dienone (XVI). By analogy with their work on Pummerer's ketone and the cyclization following dienone formation observed there (XVII),[26] they originally proposed that formation of the oxide bridge would then lead to the enone (XVIII), which on reduction would give the enol (XIX), which in turn could be dehydrated to thebaine (I) and thence eventually to morphine. An alternate route was suggested by Battersby[27] and Ginsburg,[8] who proposed that the dienone (XVI) was reduced to the dienol (XX) rather than cyclized to the enone (XVIII). Cyclization of the dienol (XX) would lead directly to thebaine (I).

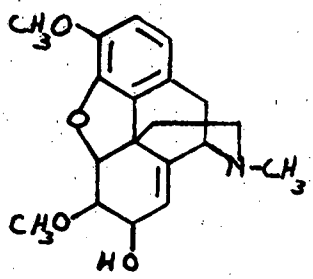
The dienone (XVI) was not a known compound at the time, but Barton *et al.*[28] were able to synthesize it. Shortly thereafter, a new alkaloid, salutaridine, was isolated from *Croton salutaris* and was shown to be identical with the dienone synthesized by Barton.[23] A study of the dienone salutaridine (XVI) in both acid and base demonstrated that its most stable form was the open one, and that ring closure to the enone (XVIII) could not be induced to occur "spontaneously." On the



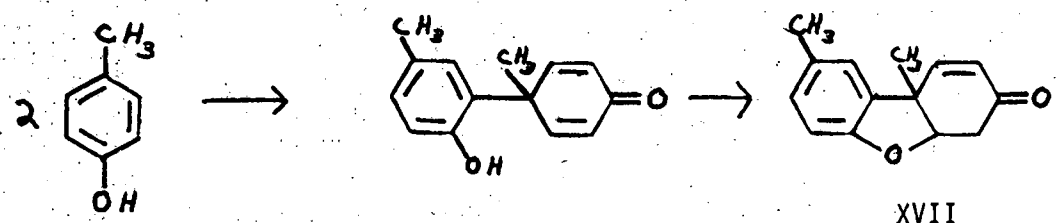
XVI



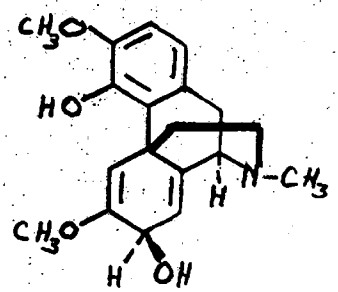
XVIII



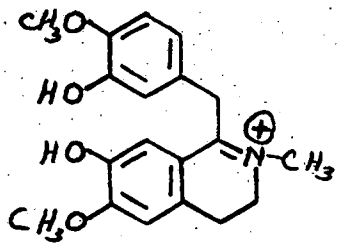
XIX



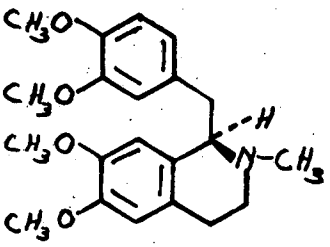
XVII



XX



XXI



XXII

other hand, salutaridine (XVI) was easily reduced with borohydride to salutaridinol (XX), which was found to cyclize quite readily to thebaine in aqueous acid.[28] This *in vitro* observation added further support to the proposed roles of salutaridine (XVI) and salutaridinol (XX) in the biosynthetic conversion of reticuline (XII) to thebaine (I).

Indeed, salutaridine (XVI), when fed to the plants, was found to be incorporated into the morphine-type alkaloids.[28] In addition, its presence was finally detected in trace amounts in *Papaver somniferum* plants.[28] The reduction of salutaridine mentioned above had led to two readily separable epimeric alcohols, salutaridinols-I and -II. Each of these alcohols was fed separately to the plants, and salutaridinol-I (XX) was found to be incorporated into the morphine-type alkaloids 15-20 times more readily than salutaridinol-II. A subsequent analysis of the stereochemistry at the alcohol function indicated that salutaridinol-I has the structure shown (XX).[29]

At this point, some other interesting stereochemical studies might be mentioned. Reticuline (XII) has one asymmetric center, at C-1, thus providing the opportunity for optical isomers. The stereochemistry about the corresponding asymmetric center of thebaine (I) (C-9) would predict that (-)-reticuline (XII) and not (+)-reticuline should be an effective precursor of thebaine. All studies described to this point had been with (±)-reticuline, so no stereochemical differentiation could have been determined. However, the enantiomers were resolved,[24] and then each compound was fed separately to poppy plants. The results were quite surprising indeed: both isomers were incorporated into thebaine! In fact, subsequent experiments were able to isolate

reticuline from Papaver somniferum plants and to show that both (+)- and (-)-reticuline occurred naturally in the plants, the ratio of the two varying with the plant's age.[30,31] A facile conversion of (+)- and (-)-reticuline into dehydroreticuline (XXI) was proposed to account for both isomers being incorporated into thebaine. Dehydroreticuline was then fed to the plants, and excellent incorporation of label into morphine was detected.[24]

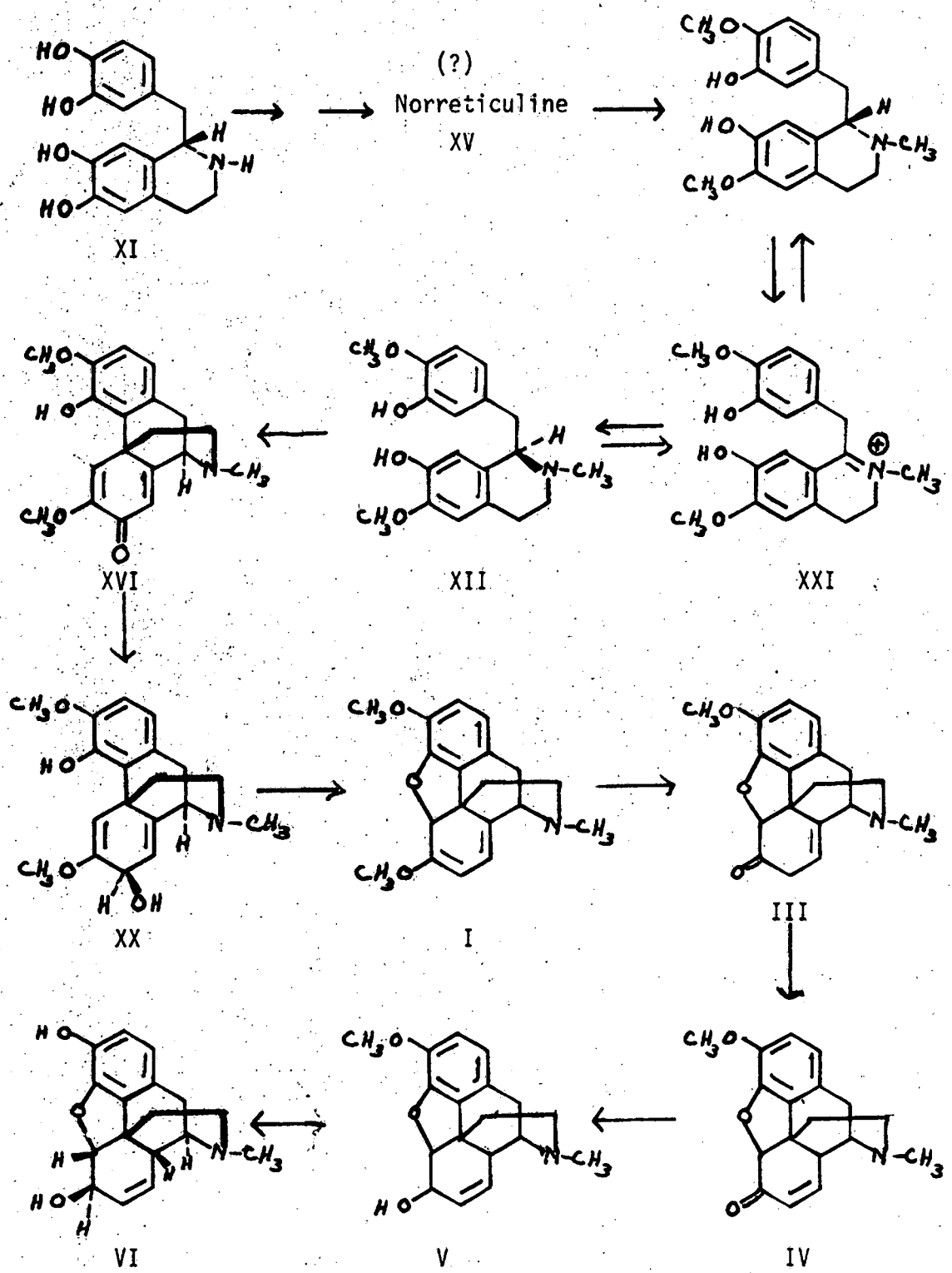
In terms of absolute stereochemistry, the interconversion of enantiomers is a step which must occur somewhere along the line in the biosynthesis of morphine. The absolute configuration of the morphine alkaloids has been established by chemical[32] and X-ray[33] evidence. The configuration about C-9 is the R configuration, whereas the absolute configuration of naturally-occurring amino acids is the S configuration (as related to glyceraldehyde). Battersby[24] has related the configuration of (+)-reticuline to that of (+)-laudanosine (XXII), whose absolute configuration has been established to be S.[34] Therefore, (+)-reticuline must be converted to (-)-reticuline (XII) before the formation of thebaine (I).

The concluding steps in the biosynthesis of morphine (VI) have been found to be a conversion of thebaine (I) to codeine (V) and finally to morphine (XI).[2,35] The sequence of these final steps was elucidated by two methods. Short- and long-term exposures to $^{14}CO_2$ were conducted and the relative specific activities of each of these alkaloids were determined.[35] A comparison of these specific activities agreed with a thebaine - codeine - morphine sequence. In addition, feeding experiments were done with each of these alkaloids.[2]

Incorporation from thebaine was found in both codeine and morphine, whereas incorporation from codeine was found only in morphine, and no incorporation into either of the other two alkaloids was detected when morphine was fed.

The complete biosynthetic scheme, as it is currently known from norlaudanosoline (XI) to morphine (VI) is presented on page 16. In this scheme, the involvement of codeinone (IV) and of neopinone (III) has also been indicated. Evidence with regard to the roles played by these compounds is the subject of the remainder of this thesis.

Scheme I: Biosynthesis of Morphine



III. TECHNIQUES USED FOR BIOSYNTHETIC STUDIES

Before the advent of readily-available radioactive isotopes, very little progress was made in studying the biosynthesis of natural products. *In vitro* experiments were conducted, and predictions about biochemical reactions could be made based on known chemical reactions. As was discussed in the previous section, Robinson's work[18] was quite significant in this regard. Predictions of the kind that he made helped to remove a certain "black box" aura from the study of chemical conversions *in vivo*. Although it is known that enzymes in living organisms can assist chemical conversions which are often very difficult, if not impossible, to induce on the chemist's laboratory bench, nevertheless the reactions and interactions involved are, in the last analysis, chemical and physical in nature and do not defy the fundamental laws of science.

On the other hand, it must be understood that reactions which may occur *in vitro* may not necessarily occur *in vivo*. As a trivial example of this, consider the codeine - morphine system. *In vivo*, codeine is demethylated to morphine, but the reverse reaction does not occur,[2] in spite of the fact that the plant system is known to contain agents which will methylate phenols, such as norlaudanosoline[22] (see Chapter II). On the other hand, both the methylation of morphine and the demethylation of codeine can be done *in vitro*.[36] Therefore, one thing which must always be kept in mind when studying or

predicting biosynthetic conversions is that *in vitro* reactions have, at best, a tenuous relationship to reactions which occur *in vivo*.

Before the availability of radiotracer techniques, very few *in vivo* experiments had been conducted to study the biosynthesis of alkaloids. Some attempts were reported in which large amounts of a proposed precursor of an alkaloid were fed to the plant in the hopes of being able to observe an increase in the pool size of the alkaloid.[37] For example, lysine was fed to an opium poppy, leading to increased amounts of morphine.[38] It is now known that lysine is not a precursor of morphine. The lysine fed must have stimulated the system which does lead to the production of morphine.[37] Now, using radiotracer techniques, an error of this variety can be avoided.

There are a number of types of *in vivo* experiments which have been used in studying a biosynthetic route. One method which has been used extensively with microorganisms is the use of mutants, which lack some enzyme necessary on the pathway and thus create a block. Another method which has so far been little used with plants (but definitely used with unicellular organisms) is that of radioactive competition experiments.[86] By far the most common method used to study biosynthesis of alkaloids in plants is that of precursor feedings -- *i.e.*, feeding a labelled compound which is thought to be a precursor of the alkaloid under investigation and looking for incorporation of label into the alkaloid. Another method, whose popularity is increasing in work with plants, is that of growth in a labelled environment: the organism under consideration is grown in an environment containing some labelled compound which that organism

will assimilate (this is not the same as a precursor feeding in that the organism is normally accustomed to assimilating that compound from its environment -- for instance, labelled sucrose may be used instead of ordinary sucrose in a medium for growing bacteria, or labelled carbon dioxide may be used instead of ordinary carbon dioxide in studies of photosynthetic organisms). In general, one type of experiment does not provide sufficient evidence to determine a biosynthetic route. However, if a potential precursor of an alkaloid is already known to exist in the organism, and a feeding of that compound, labelled, leads to incorporation of label into the alkaloid (particularly if the label is at a specific place and retains its specificity), then such a precursor feeding is usually felt to be sufficient to establish the role of that compound in the biosynthesis of the alkaloid. Much more conclusive evidence is obtained, however, if the organism can be grown for a short time in a labelled environment and the relative specific activities of the compounds of interest are examined.

The remainder of this chapter will be a careful examination of the kinds of radiotracer experiments which can be conducted when studying the biosynthesis of alkaloids in plants. Some of the methods which have been used in the past and which are still being used will be discussed, along with the kinds of information which can be obtained from these experiments and the dangers associated with interpretation of the data.

By far the biggest danger to which all <u>in vivo</u> experiments are liable is that the methods used to examine the organism under

investigation will themselves alter the organism, so that that which is observed is not actually an indication of the natural behavior of the organism. The organism (in this case, a plant) could actually be damaged during the study, or the existence of an unnatural environment (or an abnormally high concentration of some compound) may induce metabolic pathways which are normally of minor importance, or a non-specific enzyme may end up catalyzing a reaction which is foreign to the plant. The existence of these problems does not completely invalidate all biosynthetic studies, but it does mean that caution must be exercised in the design and interpretation of these experiments. From this point of view, the closer the experimental conditions can be to the natural ones for that organism, the more confidence the scientist can have that the data he obtains are true indications of what is normally happening in the organism.

Precursor feedings, by their very nature, can never completely circumvent the problem of an unnatural environment -- or of an abnormally high, locallized concentration of some compound. Many different techniques have been used to feed labelled compounds to plants. Kleinschmidt and Mothes used three techniques for feeding glucose[39,40] and tyrosine[40,41] to opium poppies. One method they employed was to spread a concentrated, aqueous solution of the labelled compound on the isolated leaves. The second method was to extract some of the latex from the plants, mix the labelled compound with the fresh latex, and then keep the mixture in a closed system for a given period of time. The third approach was to inject a concentrated, aqueous solution of the labelled compound directly into the immature seed

capsule.[42] Battersby et al.[19] also used the injection technique in most of their work, their justification for using this method being that they obtained higher incorporation of label into the alkaloids under consideration by using this method than by smearing the labelled compound on the leaves or by root feedings. In working with other kinds of plants, direct injection into the stem of the plant has been tried by Leete.[43] Leete has also used a wick feeding method,[37] in which cotton thread is threaded through the stem of the plant and then the ends of the thread immersed in a concentrated, aqueous solution of the labelled compound, which then moves up the thread and into the plant by "blotter action."

One objection to all of the feeding methods described so far (with the possible exception of injection into the capsule) is that they all damage the plant in some way. An even more severe objection is that the compound, when introduced to the plant, is in a non-physiological medium, and it is essentially forced into the plant at one highly-locallized spot, thus creating an abnormally high concentration of the compound at that point. It is to avoid such situations that the feeding experiments done in this laboratory are done by root feedings: the labelled compound is dissolved in the normal plant nutrient solution and allowed to be absorbed through the roots of the plant. Objections can also be raised to this method of feeding. There is always the possibility that microorganisms in the nutrient solution or on the root hairs modify the labelled compound before it becomes absorbed into the plant. Some workers have tried to eliminate this possibility by cutting off the old roots and allowing new roots

to develop while the plant is growing in a sterile solution, or else by washing the roots with a germicidal solution before feeding.[37] However, both of these methods entail exposing the plant to rather drastic conditions. In the case of the feedings which we have conducted, analysis of the nutrient solution after feeding and of an acid wash of the roots after feeding always indicated that the radioactivity which did not pass into the plant was still in the compound fed -- i.e., no other radioactive products were found in the nutrient or root wash, indicating that microbial action need not be a cause for great concern in the experiments done here. Another objection to root feedings is that the plant is not accustomed to absorbing the compound which is being fed through the roots. This, of course, is a problem inherent to all feeding experiments, and all that can be said is that in the root feeding method the plant is not forced to absorb the compound.

Precursor feedings can give important, but possibly misleading, data concerning a biosynthetic sequence. It is significant to know that a compound, when fed to a plant, can be converted to the alkaloid under consideration. However, the very fact that feeding experiments involve feeding the plant something which it is not accustomed to being fed can mean that the plant alters its metabolic behavior as a consequence. Therefore, conclusions based solely on such feedings must be regarded with some caution.

Growth of an organism in an isotopically-labelled environment can circumvent a number of the problems introduced by an unnatural environment. In the case of plants and other photosynthetic organisms,

this usually involves exposure to $^{14}CO_2$, a technique first used with opium poppies by McIntosh et al.[44] in 1950. There are a number of purposes for such experiments. On the most basic level, exposure experiments may be used as a tool to detect the presence of a certain compound in the plant. Often, due to the complexity of its structure, the mere fact that a compound can be detected in the plant lends a great deal of support to its possible involvement in a biosynthetic scheme. However, more definitive data can be obtained when the specific activity of each of several compounds from the proposed path is analyzed. For these studies to be valid, true "steady state" conditions must exist.

The requirements for ideal "steady state" are two-fold: (1) The plants must not experience any change in their environment when passing from normal conditions of growth to growth in an atmosphere containing $^{14}CO_2$. (2) The specific activity of the $^{14}CO_2$ must not decrease during the exposure. These requirements will be discussed in some detail below. For the sake of clarity in the following discussion, a system of nomenclature must be established. All references to "carbon dioxide" or "CO_2" refer to the entire quantity of that gas, labelled or unlabelled. If the discussion is specifically about the labelled gas, the designation "$^{14}CO_2$" will be used -- and if it is specifically about the unlabelled gas, then "$^{12}CO_2$" will be used.

In order to satisfy the first requirement for "steady state", factors such as pressure, lighting, carbon dioxide concentration, humidity, and nutrient must all be the same in the exposure chamber as they are in the normal greenhouse. Most of these conditions are

easily controlled. However, up until now careful control of the carbon dioxide concentration has not been possible. As a consequence, in most of the exposures conducted the carbon dioxide concentration has started out quite high (0.15 - 0.2%, whereas air-level CO_2 concentration is 0.04%) in order to insure having enough carbon dioxide for the length of the exposure time, and it has then been allowed to decrease steadily during the course of the exposure, sometimes to a concentration even lower than that of air-level. Although in some cases the $^{14}CO_2$ is added in spurts to prevent the concentration from getting too high, in no case has continuous control of the carbon dioxide concentration been possible. Such control is necessary, because it is dangerous to assume that plants (or any photosynthetic organism) will grow in exactly the same way or follow the same metabolic paths at high and low carbon dioxide concentrations. A major path at one concentration of carbon dioxide may be only a minor path at another. Therefore, if we are to determine the plant's true biosynthetic path to any given end-product, we must ensure that the plant is under completely natural conditions.

The need for the second requirement for "steady state" conditions (i.e., that the specific activity of the $^{14}CO_2$ must not decrease) may not be completely obvious. When $^{14}CO_2$ exposures of the "steady state" variety are used in the elucidation of biosynthetic pathways, the specific activities of several compounds from the proposed paths are compared. For example, if $A \rightarrow B \rightarrow C$, the specific activity of A will be higher than that of B, which in turn will be higher than that of C, if true "steady state" conditions apply and the specific activity of

the $^{14}CO_2$ has not decreased (see Figure 1a). However, if the specific activity of the $^{14}CO_2$ has decreased during the exposure, virtually any arrangement of the specific activities of the three compounds in question could be possible (see Figure 1b).[45,46] For example, B might even have a higher specific activity than either A or C (time X), instead of having its specific activity fall between those of A and C! It is to avoid such confusion that the second requirement is a very necessary one for "steady state" conditions.

During the course of the work described in this thesis, a system was developed in which plants can be exposed to $^{14}CO_2$ under true "steady state" conditions. The $^{14}CO_2$ is generated from $Ba^{14}CO_3$ and trapped in a slender brass cylinder of small volume (25 ml) (Figure 2) by immersing the cylinder in liquid nitrogen. The entire generation and trapping procedure take place on a vacuum line to insure efficiency of transfer. Since the $^{14}CO_2$ generated is of very high specific activity, it can often be diluted by the addition of a known amount of $^{12}CO_2$. Alternatively, if it is necessary to keep the $^{14}CO_2$ at a high specific activity, pressure can be built up in the chamber by the addition of nitrogen from a nitrogen cylinder. In either case, the final result is that at room temperature the $^{14}CO_2$ is in a cylinder under pressure, and hence the rate of flow out of the cylinder and into the exposure chamber can be regulated by means of a needle valve. Regulation is crucial, for in order to maintain normal air-level concentration of carbon dioxide in the exposure chamber carbon dioxide must constantly be added as the plants consume it. If a small number of plants is used, as is frequently the case, this rate is usually less than 1 ml/min.

Specific activities of compounds in a biosynthetic route:

a) under true "steady state" conditions of exposure to $^{14}CO_2$;

b) if the specific activity of the $^{14}CO_2$ decreases during the exposure.

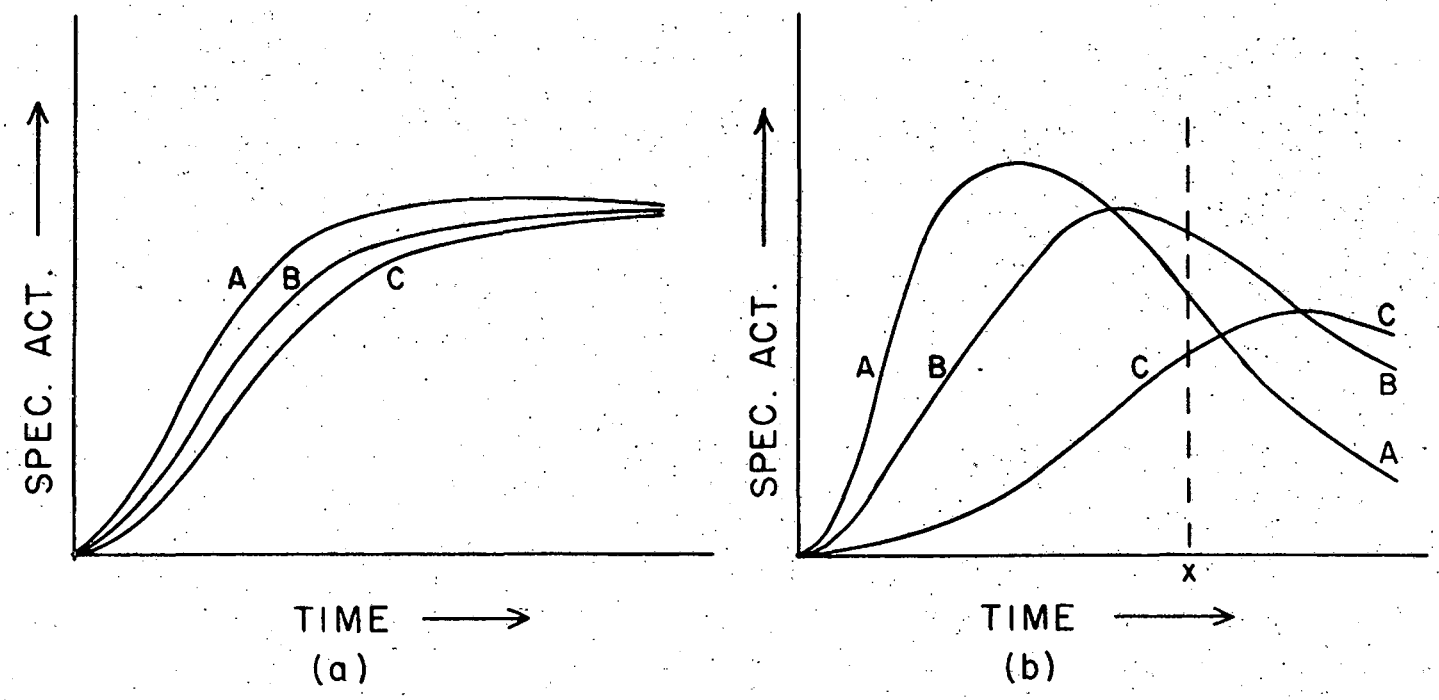


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Figure 1

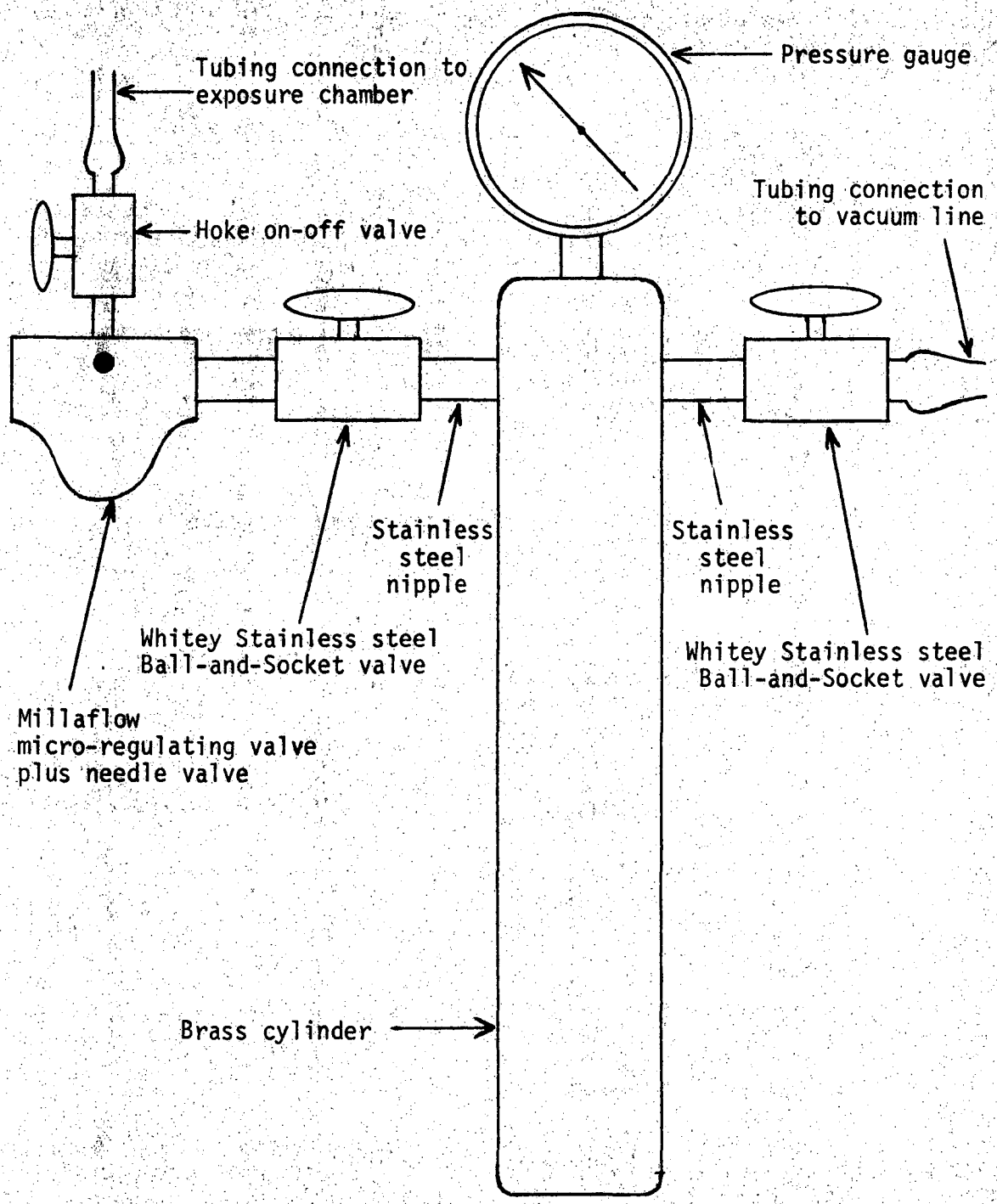


Figure 2 . Cylinder for the trapping and regulation of $^{14}CO_2$ for "steady state" exposures.

An exposure experiment is conducted in the following manner (see Figure 3 for a diagram of the exposure chamber). The air in the exposure chamber is freed of carbon dioxide by circulation through ascarite for several hours. Then $^{14}CO_2$ and $^{12}CO_2$ from cylinders are added to the chamber up to air-level concentrations of carbon dioxide. The reason for adding some $^{12}CO_2$ at the beginning of the experiment is that Weigl et al.[47] have shown that respiration of carbon dioxide does occur at the same time as photosynthesis. This respired carbon dioxide would, at least initially, have a specific activity much lower than that in the exposure chamber and hence would dilute the specific activity of the gas in the exposure chamber. In order to insure that the overall specific activity of the $^{14}CO_2$ in the chamber will not decrease during the exposure, the $^{14}CO_2$ added during the exposure has a higher specific activity than that originally present. To accomplish this original dilution, some $^{12}CO_2$ is mixed with the original $^{14}CO_2$ used to fill the chamber. All carbon dioxide added subsequently is from the $^{14}CO_2$ cylinder.

Once the exposure chamber has an air-level concentration of carbon dioxide (now ^{14}C-labelled), the plants are admitted via a dual-door system. The entire experiment is conducted in the light at approximately 75°C. $^{14}CO_2$ is admitted to the chamber from the cylinder as it is needed in order to maintain air-level concentrations of carbon dioxide in the chamber throughout the exposure time. Regulation of the $^{14}CO_2$ in the exposure chamber is monitored by circulating the air in the chamber through a sensitive CO_2 analyzer and through a vibrating reed electrometer.

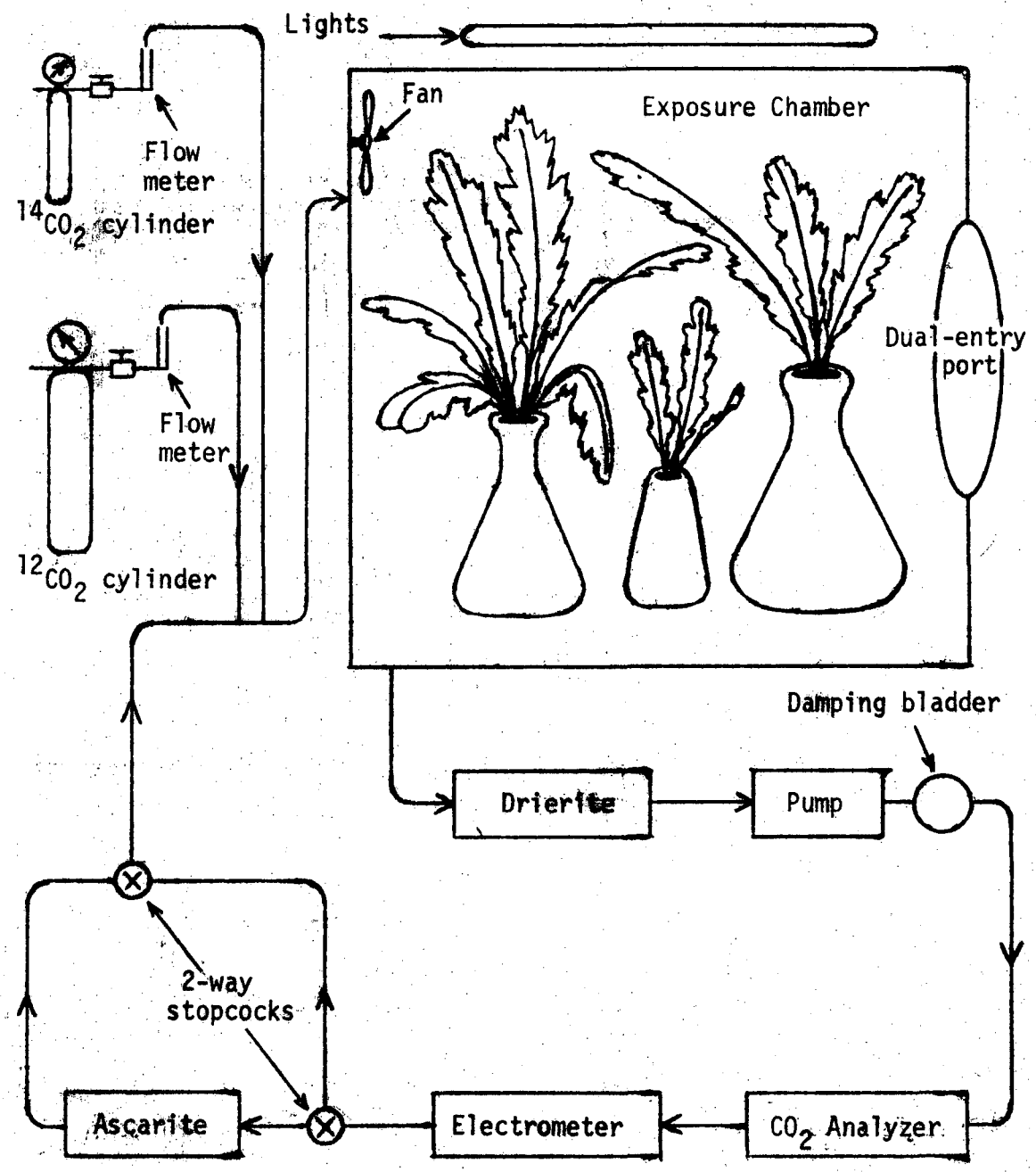
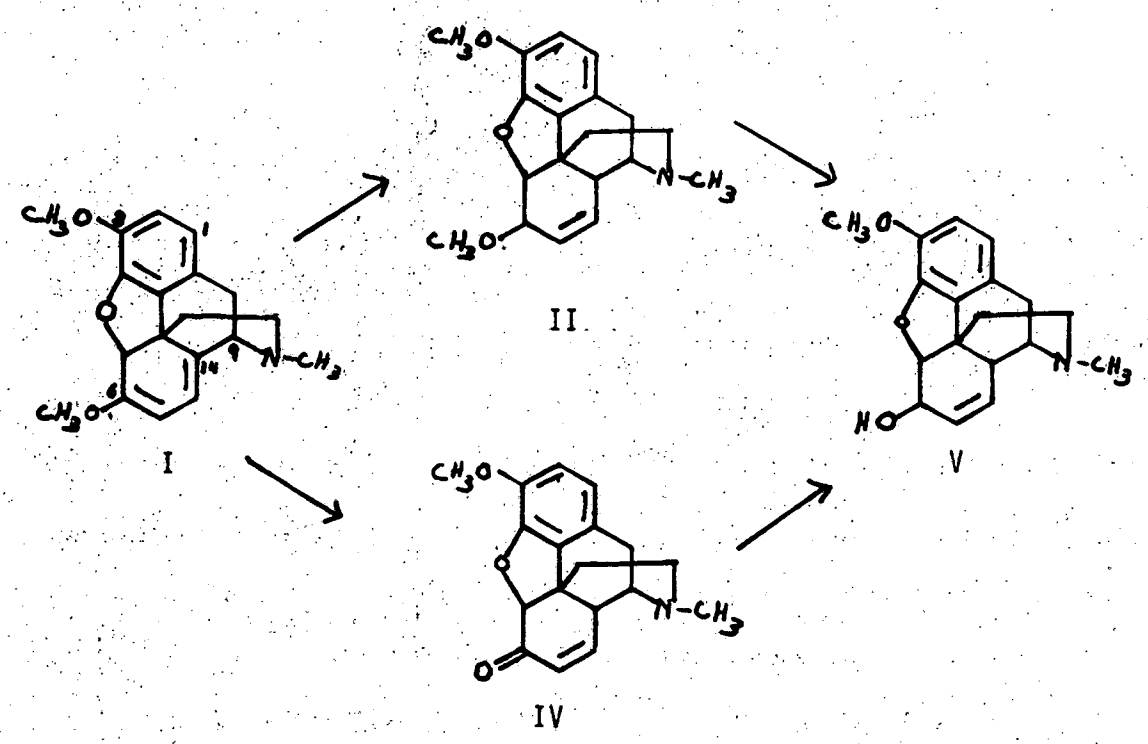


Figure 3. "Steady state" apparatus for $^{14}CO_2$ exposures.

In studying the biosynthesis of morphine we have used precursor feedings and exposures to $^{14}CO_2$ in order to obtain information. Since none of the compounds whose role we were investigating had ever been detected in opium poppies, our first experiment with each compound was to feed the ^{14}C-labelled compound to the plant and look for incorporation of radioactivity into codeine and morphine. When such incorporation was detected, then a more exacting search for the compound in the plant was conducted, followed by a comparison of specific activities as final evidence if sufficient amounts of the compound could be isolated from the plant material to make this last experiment meaningful.

IV. ROLE OF CODEINONE

The initial question in the study of the biosynthetic conversion of thebaine (I) to codeine (V) was to determine the sequence of the steps involved, these steps being demethylation and reduction. Regardless of the route, since two steps are involved there must be at least one intermediate, and there could be more. In order to determine which route is the one followed, the most likely intermediate from each route was chosen for investigation. If the route were to first involve reduction of thebaine, followed by demethylation, it appears that codeine methyl ether (II) would be involved in that route at some point. Even if the initial reduction were not a 1,4-reduction, subsequent rearrangement to codeine methyl ether would be necessary before demethylation could occur to yield codeine. For this reason, codeine methyl ether was chosen as the compound to study. Likewise, if the route were to first involve demethylation and then reduction, it would be difficult to conceive of such a route which did not involve codeinone (IV) at some point. Therefore, initial investigations were conducted using codeinone and codeine methyl ether. Once the basic route was established, other possible intermediates could also be investigated.



A. Codeine Methyl Ether

In order for this argument to be complete, the work with codeine methyl ether (II) must be included, although all of that work was done here by Dr. Gottfried Blaschke.[48,49] The first experiment conducted was a feeding experiment. The codeine methyl ether fed to the plants was nuclear-labelled -- i.e., only the ring carbons were labelled with ^{14}C; the N- and O-methyl groups were unlabelled. This precaution was taken to avoid seeing effects which were caused only by transmethylation. Stermitz and Rapoport[2] noticed a decrease in the O^3-methyl/ring ratio in codeine isolated after a ^{14}C-thebaine feeding. However, no alteration of the N-methyl/ring ratio was detected. To avoid any such peripheral questions, most of the feeding experiments

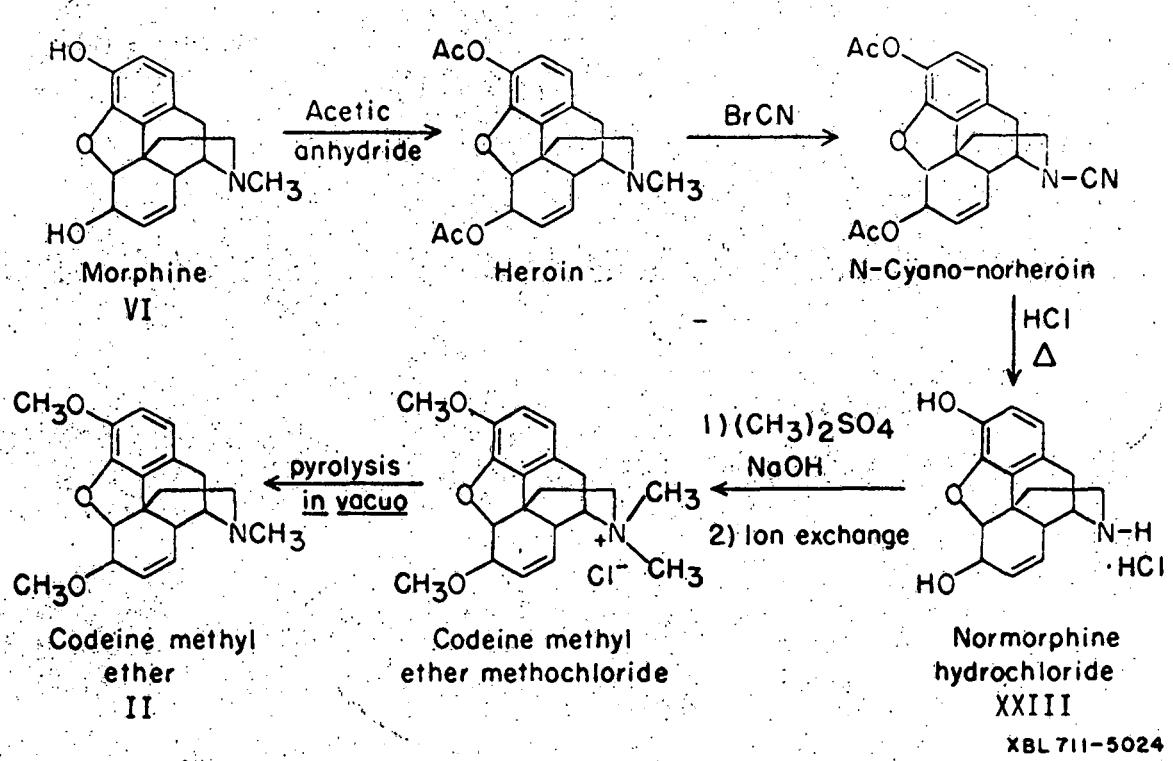
were conducted with nuclear-labelled compounds in order to ensure that any activity incorporated by codeine and morphine was from the actual compound fed and not merely from methyl groups (the one exception to nuclear-labelled feeding was the feeding of neopinone (III) -- see Chapter V).

Nuclear-labelled codeine methyl ether (II) was synthesized from randomly-labelled biosynthesized morphine (VI) (see Scheme II, page 34). The morphine was converted to normorphine (XXIII) using the von Braun reaction with cyanogen bromide.[50] Conversion to codeine methyl ether was then accomplished by methylation with dimethylsulfate, ion exchange and then pyrolysis.[51] The compound was fed to the poppy plants through the roots along with the nutrient solution. Feeding was allowed to proceed for 24 hours. Prior to work-up, carrier codeine methyl ether, codeine, and morphine were added to the plant mash. The non-phenolic and phenolic alkaloid fractions were obtained by the usual work-up procedure (see Chapter VII), and codeine was separated from codeine methyl ether by column chromatography. Further purification of all three compounds was done by sublimation and recrystallization to constant activity. The results of the feeding experiment are presented in Table I (page 35). Incorporation of activity into both codeine and morphine was detected. Similar results were also reported subsequently by Brochmann-Hanssen _et al_.[52]

Upon effecting a successful feeding experiment, the next step was to detect codeine methyl ether in opium poppy plants. There was one report in the literature[53] of the isolation of codeine methyl ether from raw opium (but the possibility of its being an artifact of

Scheme II

METHOD FOR SYNTHESIS OF NUCLEAR-LABELLED CODEINE METHYL ETHER



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Scheme III

METHOD FOR SYNTHESIS OF NUCLEAR-LABELLED CODEINE AND CODEINONE

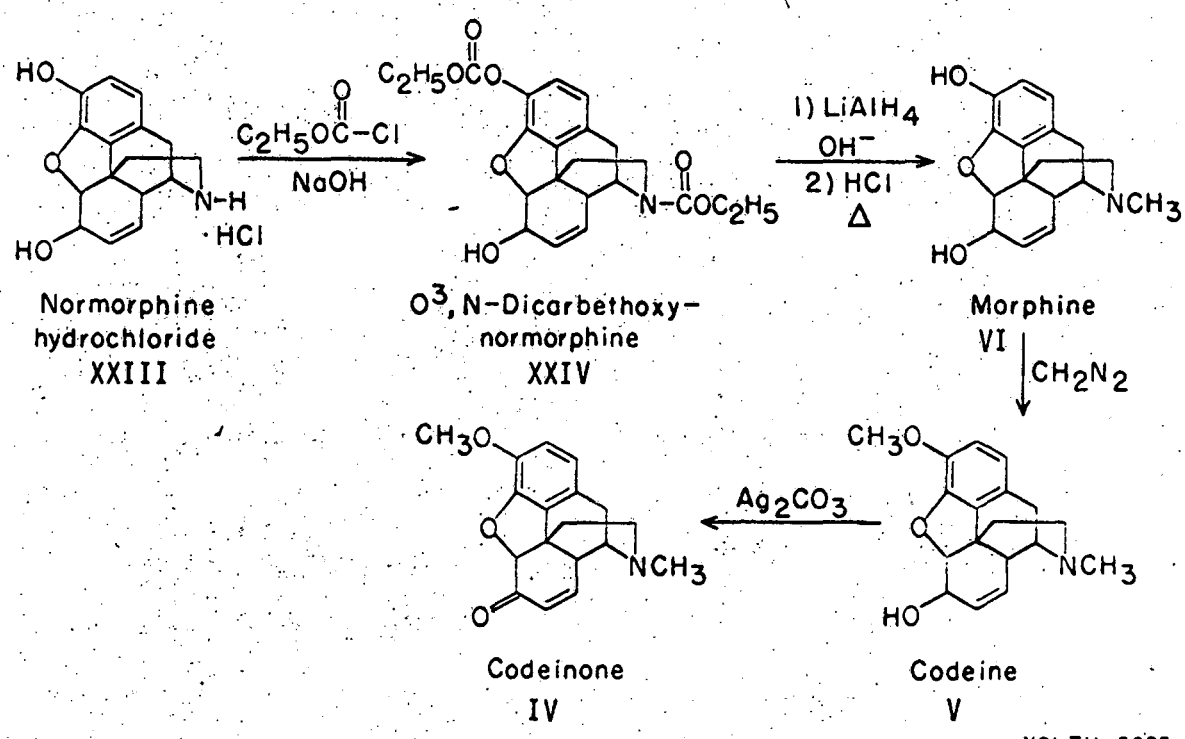

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Table I

^{14}C-Nuclear Labelled Alkaloid Feeding Experiments

Compound fed	Codeine methyl ether	Codeinone	Codeine
Activity fed (dpm)	74,000	380,000	130,400
Mass fed/100 g fresh plant (mg)	12.1	7.8	12
Incorp. into plants (dpm) (%)	52,500 (71)	309,000 (81)	79,100 (61)
% of incorp. activity in compounds isolated:			
Thebaine	---	0	---
Codeine	4.7	14.5	54
Morphine	3	2.8	13
Codeinone	---	14	0
Codeine methyl ether	52	---	---

Table II

Search for Codeine Methyl Ether and Codeinone in *Papaver somniferum* Plants after $^{14}CO_2$ Exposure

Compound sought	Compound analyzed	Carrier added (mg)	Specific activity of isolated compound (dpm/mg)	Total activity in plant (dpm)	% of Thebaine activity
Codeine methyl ether	Thebaine	0	2.8×10^7	1.9×10^6	
	Codeine	0	3.5×10^6	1.8×10^6	94
	Codeine methyl ether	33		324	<0.02
Codeinone	Thebaine	62.3	176,000	11×10^6	
	Codeine	60.0	20,000	1.2×10^6	10.9
	Codeinone (as dihydro-codeinone)	298	2,000	6×10^5	5.4

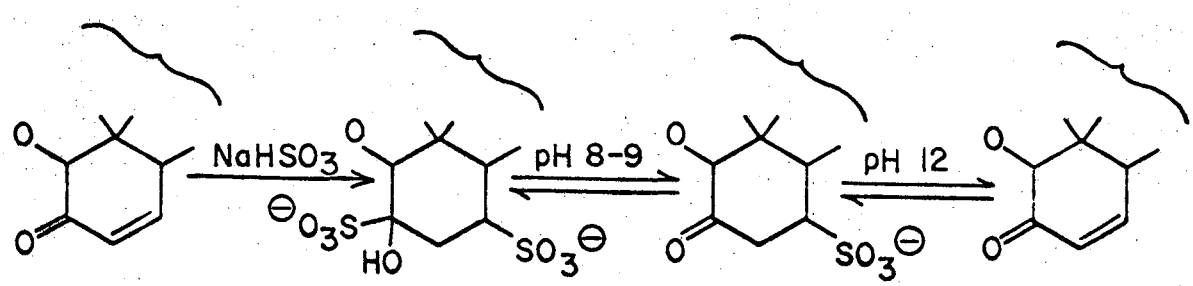
that isolation procedure was also mentioned), but no codeine methyl ether had ever been detected in the poppy plants themselves. The search for codeine methyl ether was conducted by first growing a plant in an environment containing $^{14}CO_2$ mixed with air. The plant was worked up for analysis after an exposure period of four hours. Column chromatography and vapor phase chromatography were used to separate codeine, codeine methyl ether, and thebaine. However, no codeine methyl ether could be detected by gas chromatography. In order to increase the sensitivity of the detection, carrier codeine methyl ether was added to the material trapped from VPC at the time corresponding to the retention time of codeine methyl ether (prior experiments had shown that codeine methyl ether could be trapped from that VPC to the extent of 40% and that it retained its identity). The trapped substance, mixed with the carrier, was purified to constant activity by TLC and recrystallizations. As can be seen in Table II (page 35), activity corresponding to only 0.02% of that found in the thebaine isolated in this experiment could be detected in the codeine methyl ether. This does not eliminate the possibility that codeine methyl ether is involved in the biosynthesis of codeine: its pool size may, in fact, be extremely low, or it may be enzyme-bound and hence not extracted by the extraction methods used. Before any conclusions can be drawn, the results of similar experiments with codeinone must be examined.

B. Codeinone

At this point, attention was turned to codeinone (IV) as a possible intermediate in the biosynthetic conversion of thebaine (I) to codeine (V). The results from experiments with codeine methyl ether had shown that P. somniferum can convert fed codeine methyl ether (II) to codeine (V) and thence to morphine (V). However, virtually no codeine methyl ether could be detected in the plants. A comparison with data obtained in a similar manner studying codeinone (IV) was felt to be necessary before any conclusions could be drawn.

Early in the work with codeinone it was discovered that the stability of codeinone is considerably lower than that of codeine, codeine methyl ether, or of thebaine. For this reason, the problems of isolation, purification, and analysis were somewhat more difficult to overcome. The first goal was to find a method to separate codeinone from the other non-phenolic alkaloids (specifically, thebaine and codeine). Chromatography did effect some separation, but both silica gel and alumina caused some decomposition of the codeinone.

It was finally decided to isolate codeinone by means of its bisulfite adduct. As an α,β-unsaturated ketone, codeinone should form a bis-adduct with bisulfite, and this has been demonstrated to be the



Codeinone

case.[54] The formation of this adduct is reversed at high pH (above pH 12), and free codeinone can then be obtained. Most of the morphine alkaloids have a pK of approximately 8. In order to isolate codeinone, the alkaloid mix was dissolved in benzene and shaken with 1 M bisulfate solution (pH 4). Due to the acidic pH, all of the alkaloids went into the aqueous phase. Adjustment of the pH to pH 9-10, followed by extraction with benzene, extracted all of the alkaloids except codeinone (it was shown that no codeinone was released at this point). The aqueous solution was then made quite basic and was shaken with chloroform for two days to extract all of the codeinone, which was slowly released from its bisulfite adduct.

Due to the instability of codeinone, it was decided that more effective purification and analysis could be accomplished if the isolated codeinone were converted to some other compound prior to further manipulations. Hydrogenation of codeinone over Pd/C produces dihydrocodeinone (XXV) in good yield.[55] Dihydrocodeinone is more stable than codeinone, therefore much more easy to manipulate. In addition, it has never been detected in opium poppies. Therefore, this was the compound used in the analysis of the results from the feeding experiment and in the initial search for codeinone in the plant, both of which experiments were done by G. Blaschke.[48,49]

As with the codeine methyl ether, nuclear-labelled codeinone for the feeding experiment was synthesized from randomly-labelled normorphine (XXIII) obtained from biosynthesized morphine (see Scheme II, page 34). The normorphine was N-methylated to morphine <u>via</u> O^3N-dicarbethoxynormorphine (XXIV) followed by reduction with lithium

aluminum hydride.[56] The morphine thus obtained was O^3-methylated to codeine using diazomethane, and finally oxidized with silver carbonate to codeinone (IV).[57]

Nuclear-labelled codeinone was fed to the plants by means of root feeding in nutrient solution. The feeding time was 24 hours, after which the plants were ground up with carrier codeinone, codeine, morphine, and thebaine and then worked up to isolate the alkaloid fractions. Codeinone was separated from codeine and thebaine in the non-phenolic alkaloid fraction by means of its bisulfite addition product, and was then converted to dihydrocodeinone for further purification. Codeine and thebaine were separated by means of column chromatography. All of the alkaloids were purified to constant activity by means of sublimation and recrystallization. The results from the feeding experiment are presented in Table I (page 35). Incorporation of activity into both codeine and morphine was detected, but no activity could be found in thebaine. Analogous results were reported subsequently by Battersby _et al_.[58,59]

As can be seen upon examination of Table I, both codeinone and codeine methyl ether are incorporated into codeine and morphine when they are fed to the plant. These data by themselves open up several possibilities but provide few conclusions. The only thing which can be stated with assurance is that the _Papaver somniferum_ plants can use both codeinone and codeine methyl ether when these compounds are administered to the plants. This opens up the possibility of dual pathways from thebaine to codeine. On the other hand, there is also the possibility that one of the pathways is aberrant: it is either induced

by the presence of a strange molecule, or conversion to codeine has occurred solely because of the presence of non-specific demethylating or reducing enzymes.

The first clue to help resolve this apparent dichotomy was obtained when a search was made for codeinone in the plant. No previous report could be found of the existence of codeinone in P. somniferum. In order to maximize sensitivity in our search, some poppy plants were grown in an environment containing $^{14}CO_2$ for four hours. Carrier codeinone, codeine, and thebaine were added to the plant material before work-up. After separation from codeine and thebaine by means of bisulfite, codeinone was converted to dihydrocodeinone for further purification. The amount of radioactivity found in each of the purified compounds is presented in Table II (page 35). For comparison, the results from the experiment in which codeine methyl ether was sought are also presented in the same table, although the actual experiments were somewhat different (in particular, no carrier was added to the codeine or to the thebaine). Although the amount of comparison which can be done between these two experiments is rather limited, one point which does stand out is the large amount of codeinone detected compared to the amount of codeine methyl ether found. These data, in combination with the results of the feeding experiments, give strong support to a biosynthetic route of thebaine going to codeine by way of codeinone.

C. Development of the "Steady State" System and its Use in the Study of the Role of Codeinone

Even more conclusive support for the role of codeinone in the biosynthesis of morphine was sought by means of a short-term $^{14}CO_2$ exposure, at the end of which the specific activities of thebaine, codeinone, and codeine would be compared. If the sequence occurs as expected and is thebaine → codeinone → codeine, then the specific activity of thebaine after a short-term exposure to $^{14}CO_2$ should be higher than the specific activity of codeinone, which in turn should be higher than the specific activity of codeine.[45]

Initially, the system used in these studies for the exposure of plants to $^{14}CO_2$ was a modification of the apparatus used by Bassham *et al.*[60] in their work with algae and photosynthetic bacteria. This system does maintain an atmosphere containing $^{14}CO_2$ instead of $^{12}CO_2$, but it provides very little opportunity for control. The $^{14}CO_2$ is introduced into the system by means of a "loop" into which it was originally trapped after generation from $Ba^{14}CO_3$. There is the opportunity to have two such loops in the system, but aside from that the admission of $^{14}CO_2$ to the system is an "all or nothing" affair. When working with a plant, this means that the system must start with a very high concentration (compared to normal air level) of carbon dioxide in order to have enough present for the duration of an exposure (two to four hours in this case). At the end of the exposure, the concentration of carbon dioxide in the system may even be significantly lower than that of air level. Needless to say, the use of such a system raises several questions regarding the validity of the data

thus obtained being used as a reflection of reactions which occur naturally in the plant. However, this was the only system available at the time for use with short exposures desiring high activity incorporation, so it was initially used for our biosynthetic studies. Due to experimental problems not related to the exposure procedure, the exposure experiment had to be repeated a couple of times. The results of one such exposure indicated some very abnormal pool sizes, and it was this experiment which was finally the "straw which broke the camel's back". Our prior qualms about the exposure system we were using were now shown to be quite justified. For this reason, a complete "steady state" system was developed. A thorough discussion of the requirements of such a system and a description of the system which we developed for our use is presented in Chapter III. During the development of this system, an even greater appreciation was obtained for the amount of luck which is assumed if non-"steady state" conditions are used to obtain meaningful data of the type sought from these exposure experiments (i.e., comparison of specific activities of isolated compounds as an indication of a biosynthetic sequence).

In order to compare specific activities of biosynthetic compounds after a short-term exposure of the plant to $^{14}CO_2$, no carrier compounds may be added to the work-up. Since the pool sizes of some of the compounds in the plant are very low, excellent separation techniques and highly sensitive methods of analysis must be used in order to study these compounds. In the case of the thebaine, codeinone, codeine sequence, the problem was particularly severe for codeinone: its pool size in the plant is very low, and it is somewhat unstable under many conditions.

As has been discussed, early work with codeinone (i.e., the feeding experiment and the initial search for codeinone in the plants) converted the isolated codeinone to dihydrocodeinone (XXV) before further analysis. The advantages of this procedure were two-fold: dihydrocodeinone is more stable than codeinone and analysis for it can be much more sensitive, and also it is not known to be a naturally-occurring alkaloid. It was originally felt that dihydrocodeinone could also be used for the analysis of codeinone isolated from the "steady state" exposure. However, this was found not to be the case. Dihydrocodeinone is prepared from codeinone by hydrogenation over Pd/C.[55] The yield is good and the side-products few and low in yield. However, it was found that if the reaction was scaled down to a scale of 50 mg or less, the yield of dihydrocodeinone was greatly decreased, and the impurities in the product greatly increased. A change of catalyst to $Pd/BaSO_4$ did not help, and a complete change (for instance, to Adams catalyst) completely altered the course of the reaction, yielding a mixture of dihydrothebainols as the product.[61] Since the amount of codeinone expected from the plant material was on the microgram scale, analysis of it as dihydrocodeinone was not feasible. Another derivative was sought.

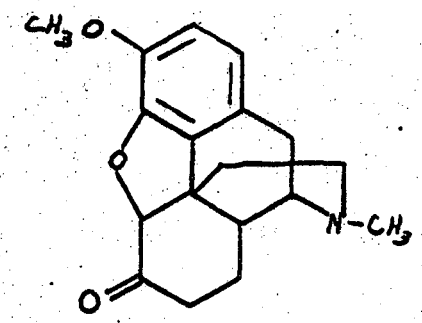
The next compound considered for use in the analysis of codeinone was codeine (V). Borohydride reduction of codeinone produces codeine in quantitative yield.[62] The disadvantage of using codeine was that codeine is a known plant alkaloid -- in fact, the "steady state" experiment was also going to isolate codeine for analysis. However, it was felt that if the bisulfite procedure could completely separate

codeinone and codeine, then the subsequent analysis of the codeinone as codeine would be quite valid. However, trial runs of the bisulfite procedure using ^{14}C-codeine revealed that as much as 2-3% of the codeine was carried along with the codeinone, although the reason for this cannot be explained. Therefore, the use of codeine was felt to be a rather unsatisfactory method for the analysis of codeinone.

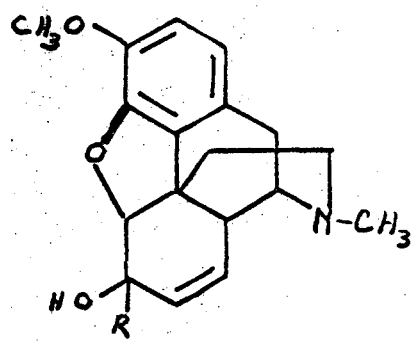
There is one further reaction of codeinone which is very clean and quantitative: reaction with methyllithium to produce 6-methylcodeine (XXVI).[63] We found that use of excess methyllithium produced no side effects, and the reaction could be run with quantitative yield even with very small quantities of codeinone. Also, 6-methylcodeine is not known to occur naturally in poppy plants. However, in the end 6-methylcodeine was not used in the analysis of the results from the "steady state" exposure. Mass determinations of the compounds of interest from the "steady state" exposure were done by gas chromatography using a flame ionization detector. Sensitivities in the range of 50-100 nanograms were obtained for the alkaloids. However, the retention time for 6-methylcodeine was always exceptionally close to that of codeine on the good columns tried (i.e., the ones which gave good sensitivity and good separation of codeine and thebaine). Acylation of 6-methylcodeine and of codeine[64] was tried as a means to separate these compounds further on VPC, but still the resulting peaks were too close together.

It was felt that increasing the molecular weight of the codeinone derivative might increase its retention time, thus making analysis easier. Other lithium reagents have been used with dihydrocodeinone,[65]

but only methyllithium was ever reported to have been used with codeinone.[63] Initially, we tried reaction with n-butyllithium. However, multiple products were obtained, at least three of which were in sizable yield. Reaction with phenyllithium was tried next. 6-Phenylcodeine (XXVII) was obtained in quantitative yield (as one compound), and it had a retention time on gas chromatography which was much longer than those of thebaine or codeine or of any alkaloid found in the non-phenolic alkaloid fraction isolated from the poppy plants. Therefore, 6-phenylcodeine was used for the analysis of codeinone from the "steady state" exposure.



XXV



XXVI R = CH_3

XXVII R = C_6H_6

On work-up of preliminary "steady state" exposures, a very small amount of thebaine was isolated after the bisulfite treatment which removed codeinone from the other alkaloids. It was found that the thebaine was not carried over and subsequently extracted with the codeine. Instead, it remained in the aqueous solution, even at high

pH (subsequent investigation by Dr. Bjeldanes in this laboratory has shown that, in the presence of oxygen and bisulfite, thebaine becomes converted to demethylsalutaridine (XXVIII)).[66] To avoid this extensive loss of thebaine, instead of using 1 M $NaHSO_3$ in the bisulfite procedure, a bisulfite-sulfite buffer at pH 7 was used. Very little thebaine went into the aqueous phase, whereas the codeinone bisulfite adduct was still readily formed. In this manner, most of the thebaine could be preserved for analysis, and at the same time codienone could be separated from the other alkaloids.

The final "steady state" exposure could now be done. The plants were kept in the light under "steady state" conditions in the exposure chamber for three hours before removal and work-up. After isolation of the non-phenolic alkaloids, codeinone was separated from the other compounds by means of its bisulfite adduct, formed in a pH 7 bisulfite-sulfite buffer solution. The isolated codeinone was then converted to 6-phenylcodeine (XXVII) for subsequent analysis. The masses of all three compounds were determined on gas chromatography. Aliquots of the samples were also trapped and then counted by liquid scintillation counting to obtain an activity determination. (The trapping efficiency was first determined for each compound. In the case of thebaine, some decomposition occurs on VPC, but no loss of label occurred -- this was determined by trapping standard samples of ^{14}C-thebaine.) The specific activities of the compounds could be calculated from these data and are presented in Table III (page 47).

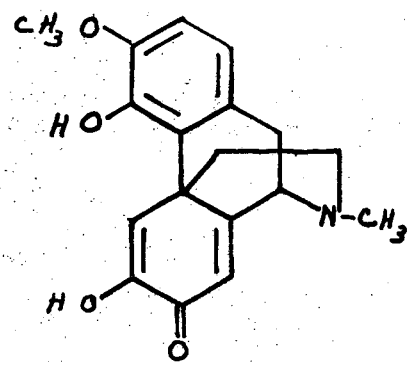
One further determination needed to be done before the results from the "steady state" exposure could be unambiguously interpreted.

Table III

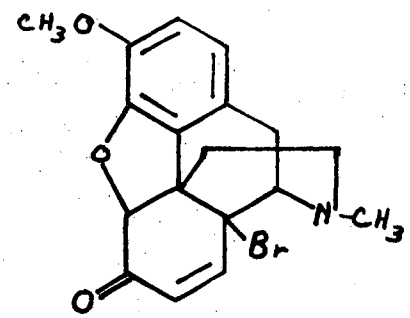
$^{14}CO_2$-"Steady State" Exposure of Poppy Plants

Compound	Specific activity (dpm/μM)	Total alkaloid isolated/ 100 g fresh plant (μg)
Thebaine	24×10^7	85
Thebaine (minus 6-OCH_3)	18×10^7	
Codeinone (as 6-phenylcodeine)	16×10^7	0.2
Codeine	6×10^7	135

The 6-OCH_3 group of thebaine (I) does not exist in either codeinone (IV) or codeine (V). An accurate comparison of the specific activities of the three compounds isolated from the exposure must be done only on the carbon atoms which all three compounds have. In order to determine the amount of activity in the thebaine which was due to the 6-OCH_3 group, the following procedure was used: A portion of the non-phenolic alkaloid mixture was mixed with unlabelled thebaine and submitted to column chromatography. The isolated thebaine was purified to constant activity by means of sublimation and recrystallization. This sample was then reacted with N-bromosuccinimide to produce 14-bromocodeinone (XXIX).[67]



XVIII



XXIX

The 14-bromocodeinone was purified to constant activity, and its specific activity compared to that of thebaine, indicating that 24% of the specific activity of thebaine had been in the 6-OCH_3 group which was lost in the conversion to 14-bromocodeinone. Going back to the data in Table III, the value for the specific activity of the thebaine was corrected to eliminate the amount of activity contributed by the 6-OCH_3 group.

It can be seen from Table III that the relative specific activities of thebaine, codeinone, and codeine follow the pattern which would be expected for a biosynthetic sequence of thebaine → codeinone → codeine -- i.e., the specific activity of thebaine is higher than that of codeinone, which is higher than that of codeine. In addition, the specific activity of codeinone is very close to that of thebaine, which would be expected to be the case for a compound of small pool size.

The specific activity of the 6-OCH_3 of thebaine may be compared with the specific activity of the $^{14}CO_2$ in the exposure chamber. The specific activity of the $^{14}CO_2$ is only known within 10-15%, as no exact determination was conducted. However, the specific activity of the 6-OCH_3 of the thebaine equals that of the $^{14}CO_2$, within this experimental error, a fact which would tend to indicate that the methyl group is put on a very immediate precursor of thebaine (or that the pool sizes of the immediate precursors are very small), and that the turnover of thebaine is quite rapid -- or else that the methyl group is very labile. (However, Barton et al.[23] found no exchange of methyl groups in the conversion of reticuline (XII) to thebaine, so this last possibility appears unlikely.)

One further question exists with regard to the role of codeinone in the conversion of thebaine to codeine, and that is the possibility of an equilibrium existing between thebaine and codeinone or between codeinone and codeine (it is already known that the overall conversion of thebaine to codeine is not reversible[2]). Upon feeding labelled codeinone to the plant, no radioactivity was found in the thebaine (Table I, page 35), thus indicating that the conversion of thebaine to codeinone is irreversible. In addition, nuclear-labelled codeine was fed to some poppy plants, and no radioactivity could be detected in the codeinone (Table I). Battersby et al.[58,59] also could detect no evidence of any conversion of codeine back to codeinone.

The incorporation of codeine methyl ether, reported in Table I, now becomes an interesting case. It is still possible that codeine methyl ether is a true intermediate in the conversion of thebaine to

codeine, but occurs in a very small pool. However, this is rather doubtful, as no codeine methyl ether could be detected down to a limit of 0.02% of the thebaine present -- whereas codeinone was shown to have a pool size as much as 5% of that of thebaine. Another possibility is that codeine methyl ether is converted back to thebaine, which is then converted to codeine. The work of Brochmann-Hanssen et al.[52] provides evidence against this alternative, as they could detect no activity in thebaine after they fed labelled codeine methyl ether to the plants. It would appear, then, that the conversion of codeine methyl ether to codeine is an aberrant path, probably caused by demethylation by a non-specific demethylating enzyme.

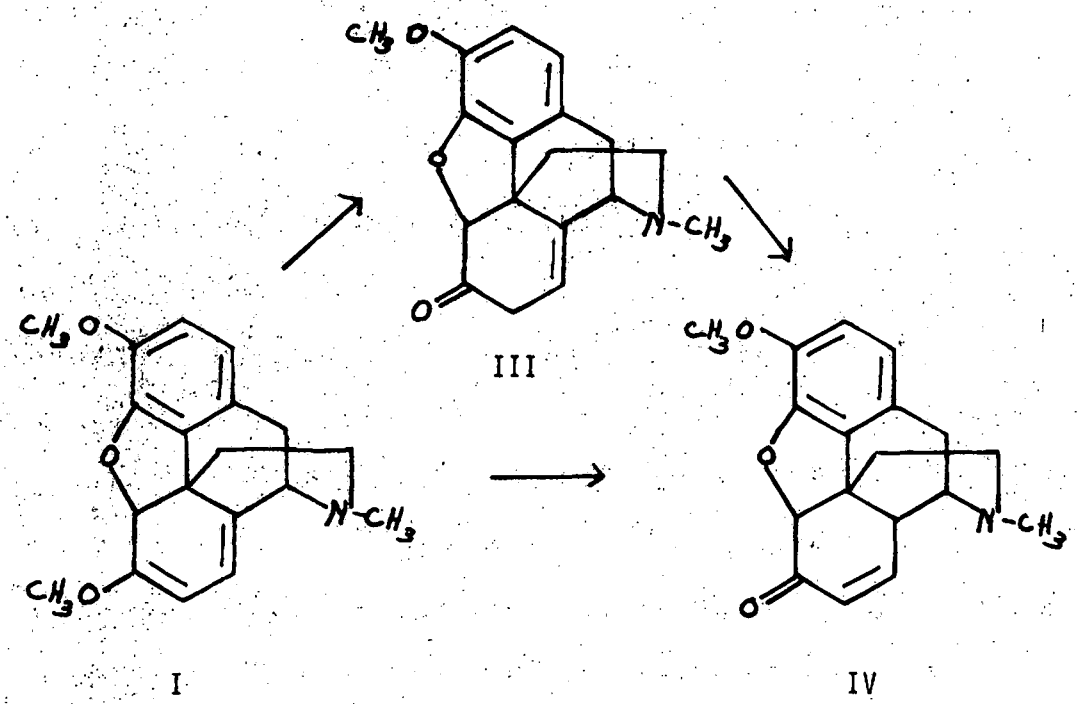
The data presented in this chapter should serve as good evidence in support of a biosynthetic sequence of thebaine → codeinone → codeine. In other words, demethylation of thebaine occurs before reduction. At this point, the main sequence of steps involved in the conversion of thebaine to codeine has been determined. Now a more detailed examination of these steps would be of interest. The second step -- i.e., reduction of codeinone to codeine appears to be a fairly straightforward reduction of a ketone to an alcohol. However, the formation of codeinone from thebaine may involve the intermediacy of neopinone (III), with subsequent rearrangement to codeinone. The possible role of neopinone in the conversion of thebaine to codeinone will be investigated in the following chapter.

V. ROLE OF NEOPINONE

In the preceding chapter, the basic route by which thebaine (I) is converted to codeine (V) in *Papaver somniferum* plants was described. Evidence was presented to support the role of codeinone (IV) in this conversion. Although codeine methyl ether (II), when fed to the plants, was converted to codeine, this compound could not be found to be naturally occurring to the limits of the detection methods used. Therefore, it was concluded that the most likely route by which thebaine was converted to codeine was by initial demethylation of codeinone, followed by reduction of codeinone to produce codeine.

The formation of codeinone from thebaine became of interest at this point. Thebaine is the enol ether of both codeinone (IV) and neopinone (III). Codeinone, being an α,β-unsaturated ketone, is thermodynamically more stable than neopinone. However, this fact does not eliminate the possibility that initial demethylation of thebaine may produce neopinone first, which then rearranges to the more stable codeinone. The possibility that this could be the case would also help to explain a (partial) loss of tritium label at C-7 which was noted by Barton *et al.*[23] in the conversion of thebaine to codeine.

The synthesis of neopinone is reported by Conroy,[67] and uses thebaine as a starting point. Thebaine can be converted to 14-bromocodeinone (XXIX) by reaction with N-bromosuccinimide. Hydrogenation



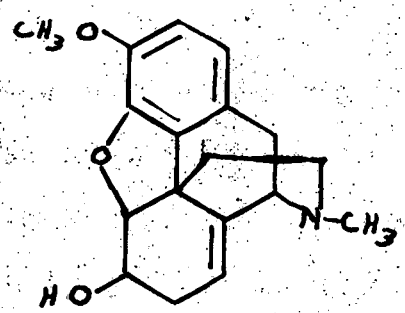
of 14-bromocodeinone leads to different products, depending upon the conditions of the reaction.[68] However, in a neutral medium (90% chloroform, 10% methanol), with 10% Pd/C as the catalyst, 14-bromocodeinone slowly absorbs one mole of hydrogen, yielding neopinone as the product. After filtering off the catalyst, the neopinone exists in solution as the hydrobromide salt. Free neopinone can be liberated by shaking with a solution containing an equivalent amount of sodium carbonate or bicarbonate. Utmost care must be taken in this procedure, as a small amount of codeinone is formed quite readily.

This contamination by codeinone presented a major problem in the biosynthetic studies. Almost every sample of neopinone prepared had some codeinone in it, usually to the extent of 15-20%. The drive to obtain a compound in its absolutely pure state can, at a certain

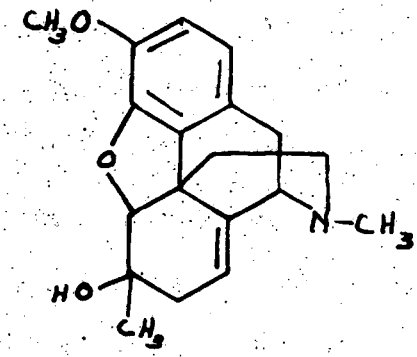
point, become merely an aesthetic one, particularly if that compound can be derivatized and the purification of the derivatives is an easy task. For most biosynthetic studies with neopinone, analysis could (and would) be done by means of derivatives. The sole reason (aside from the aesthetic) for the efforts described below to obtain absolutely pure neopinone was for a feeding experiment. It was particularly desirable not to have codeinone present with the neopinone during the feeding experiment, as the biosynthetic incorporation of codeinone into codeine and morphine had already been demonstrated.

Confirmation that the contaminant in neopinone was indeed codeinone was obtained from a number of sources. Reaction of neopinone with borohydride resulted in a product of neopine (XXX) containing up to 20% codeine. These two compounds could be readily separated and identified. In addition, reaction with methyllithium, which produced a number of unidentified products and also gave a fair yield of 6-methylneopine (XXXI), produced 6-methylcodeine (XXVI) in a yield of approximately 20%. Infar-red spectroscopy of neopinone showed a major carbonyl absorption, due to an unconjugated carbonyl, at 1735 cm^{-1}, and also a smaller absorption, due to a conjugated carbonyl, at 1679 cm^{-1}. Thin layer chromatography on formamide-impregnated cellulose[69] indicated the presence of two spots, Rf 0.47 and 0.30 (Rf for codeinone: 0.47), and the bottom spot disappeared when the neopinone was converted to codeinone (see following page).

Confirmatory evidence for the presence of codeinone was also provided by NMR. The NMR of both compounds has been reported by



XXX



XXXI

Okuda et al.,[70] (but on careful examination of their published spectrum of neopinone, we feel that their sample of neopinone also had some codeinone contaminant). There are several regions in the NMR spectrum in which codeinone and neopinone have characteristic absorptions. Probably the most instantly apparent is the absorption of the 7-H of codeinone (two doublets, 6.06 ppm) and the absorption of the 8-H of neopinone (two doublets, 5.44 ppm). However, since these absorptions are quartets, they are broad and are not very good regions for pinpointing impurities. A region which is far more useful is that of the 5-H, a sharp singlet at 4.69 ppm for codeinone and 5.00 ppm for neopinone. In addition, upon obtaining spectra of mixtures of codeinone and neopinone we noticed that the absorption of the 3-OCH_3 can be used, as it appears at 3.85 ppm for codeinone and at 3.91 ppm for neopinone -- just enough separation so that a good spectrum of a mixture shows two well-resolved peaks. (However, at low concentration

of codeinone in the neopinone this region is difficult to work with, as ringing from the neopinone absorption often interferes.) The most useful area for quantitative determination is that for the 5-H. The spectrum of neopinone in almost every case showed a small absorption (approx. 15%) at 4.69 ppm. In addition, several mixtures containing a known amount of codeinone added to the crude neopinone were studied, and these spectra also indicated that the neopinone contained codeinone to the extent of 15-20%.

Several different approaches were used to try to obtain codeinone-free neopinone. Conroy[67] reported that neopinone could be made to crystallize as a chloroform adduct and codeinone did not form this adduct. This method was tried by us, and although a slight decrease in the codeinone content of the neopinone was observed (as seen by NMR or derivative formation), the method never really got rid of the codeinone.

Various types of chromatography were attempted. Karusz and Rüll[68] reported that alumina converted neopinone to codeinone, and this observation was confirmed here. In addition, silica gel also converted most, if not all of the neopinone to codeinone (as well as to some other, unidentified products). Paper chromatography was also considered. Brochmann-Hanssen[71] had had success in distinguishing codeine and neopine (XXX) by the use of paper chromatography with paper buffered with pH 6.5 McIllvaine buffer.[72] However, a good separation of codeinone and neopinone could not be obtained using the solvent systems he had used. Paper chromatography is a very time-consuming procedure, and the load-limit of the paper is so small as to make it

rather impractical for preparative work, so little time was spent looking for other solvent systems (in retrospect, it also looks as though the process of elution from the buffered paper and subsequent separation from the citric acid in the buffer would have led to isomerization of some of the neopinone anyway).

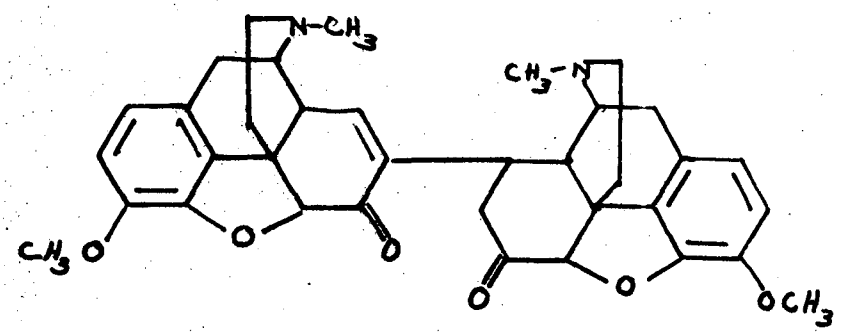
Cellulose chromatography seemed to hold forth quite a bit of hope. Teichert et al.[69] were reported to have had some success separating several opium alkaloids by chromatography on formamide-impregnated cellulose. Using their solvent system, chromatography of neopinone showed two spots, Rf 0.47 and 0.30. Rf for codeinone was 0.47. By means of preparative chromatography, the two spots from neopinone were isolated. Elution with methanol also eluted the formamide, but complete elution could not be obtained with chloroform. The methanol eluate then had to be evaporated, and the resulting oil taken up in chloroform and water. The chloroform was washed several times with water to remove all of the formamide. The two products thus isolated showed, on rechromatography, that they each retained their identity. NMR of the material obtained from the top spot was a very crude spectrum of codeinone. NMR of the material from the bottom spot, however, showed that neopinone was present only to a very small extent -- numerous unidentified absorptions were also present. It would appear then that neopinone was either altered by the chromatography itself or by the isolation procedure. In any case, the final product was far less pure than the starting material.

Since chromatographic procedures were not too successful in removing codeinone from neopinone, attention was turned to the bisulfite

adduct of codeinone[54] (see discussion on page 37). As a ketone, neopinone would be expected to form a bisulfite adduct at the carbonyl -- but this adduct formation should be reversed easily in mild base (pH 8-9). As has already been discussed, the bis-bisulfite adduct formed with codeinone does not release codeinone in mild base; pH greater than 12 is required for the release of codeinone, and even then it occurs very slowly. Neopinone was subjected to the bisulfite treatment, using either 1 M $NaHSO_3$ of a pH 7 bisulfite-sulfite buffer. After the adduct had formed, the solution was cooled on an ice-bath, and the pH was carefully raised to pH 8.6 using ice-cold saturated sodium carbonate solution. The neopinone extracted from this solution was shown by NMR and by conversion to 6-methylneopine (plus 6-methylcodeine) to contain some codeinone. Even when the pH adjustment was done with a pH 9 borate buffer,[73] the isolated product contained some codeinone. When the precautions described were not taken in the pH adjustment, the isolated product contained much higher quantitites of codeinone.

From this work, it would appear that neopinone is rather unstable in aqueous solution, particularly in solution which is even mildly basic. This observation was confirmed by placing neopinone in a pH 9 solution and allowing it to stay there for varying lengths of time, then extracting and taking an NMR. At first, the amount of codeinone increased. However, after an hour the appearance of a third product could be detected by cellulose TLC. After 24 hours, this product was the major substance, and was shown to be identical with the dimer (XXXII) reported by Conroy,[67] which is formed by neopinone in basic

solution by a Michael-type addition to codeinone and subsequent rearrangement.



XXXII

This concern about obtaining codeinone-free neopinone, as already mentioned, was aimed at trying to find a method to obtain pure neopinone for a feeding experiment, to determine whether neopinone could be converted to codeine and morphine by P. somniferum. At this point it was decided to check the stability of neopinone in the nutrient solution used during root feedings. Neopinone was placed in the nutrient solution for varying lengths of time. At the end of the specified time, the pH of the solution was raised to pH 8.6 using ice-cold saturated sodium carbonate solution (admittedly, this procedure to raise the pH caused some conversion to codeinone, as seen from the bisulfite work described above, but no other method for analysis was available), and the neopinone was then extracted. NMR of the isolated neopinone indicated that slow conversion to codeinone was taking place in the nutrient. After four hours, a 5-10% increase in the amount of codeinone present could be detected,

and the amount was greater after longer periods of time. It was apparent that neopinone was not converted to codeinone as fast in nutrient solution as it was in pH 9 solution, but nevertheless conversion did occur slowly.

Keeping the instability of neopinone in mind, a feeding experiment was finally designed in the following manner. Three feedings were conducted in parallel, on plants of the same age and size. Six plants were fed ^{14}C-neopinone, which had been prepared from biosynthetic ^{14}C-thebaine. Six other plants were fed approximately the same mass of ^{14}C-codeinone. Finally, some other plants were fed a mass of ^{14}C-codeinone corresponding to 15-20% of the neopinone fed (in other words, in the last feeding the codeinone fed corresponded to the amount of codeinone in the neopinone fed). All three feedings were conducted for only four hours, at the end of which time the plants were allowed to grow for another 24 hours in pure nutrient solution. Prior to work-up, carrier codeinone, codeine, and morphine were added to the plants from the codeinone feedings, and carrier neopinone, codeine, and morphine were added to the plants from the neopinone feeding. The non-phenolic and phenolic alkaloid fractions were isolated by the usual procedure. In the case of the two codeinone feedings, codeinone was separated from codeine by means of the bisulfite procedure and was then converted to 6-methylceopine (XXXI) for further purification. In the case of the neopinone feeding, the neopinone was converted to codeinone by chromatography on alumina,[68] and the codeinone was then separated from the codeine by the bisulfite procedure and then converted to 6-methylcodeine.

Purifications of all compounds were accomplished by varying kinds of TLC and by sublimation. The results from these feedings are presented in Table IV, page 61.

From the data presented in Table IV, it can be seen that the incorporated activity from neopinone into codeine and morphine was much greater than could be attributed to a 20% codeinone impurity. Since it has been demonstrated in this paper that neopinone becomes slowly converted to codeinone in aqueous solution, it could always be argued that the neopinone, once incorporated into the plant, continued to undergo such conversion, and the codeinone thus formed was converted to codeine -- i.e., that the conversion of neopinone to codeinone was not part of a natural, biosynthetic route, but merely occurred because of the instability of neopinone.

The next step was to try to detect the presence of neopinone in *Papaver somniferum* plants. In order to hope to do this, a derivative of neopinone was sought which could only come from neopinone. Two derivatives were considered: neopine, and 6-methylneopine. Although no neopine has been isolated from poppy plants, the possibility of its existing there undetected is present -- in fact, such a possibility would become rather large if neopinone could be shown to be in the plant, as it could be converted to neopine by simple reduction in the same manner as codeinone is converted to codeine in the plant. To avoid any such questions, it was decided to look for neopinone in the plant by converting it to 6-methylneopine. In order to be sure that no interference occurred from other compounds, ^{14}C-thebaine, ^{14}C-codeine, and ^{14}C-codeinone were all treated with methyllithium

Table IV

Four-hour Feedings of ^{14}C-Neopinone and ^{14}C-Codeinone

Compound fed	Neopinone	Codeinone (large)	Codeinone (small)
Activity fed (dpm)	88,800	240,000	101,160
Mass fed/100 g fresh plant (mg)	80	106	14
Incorp. into plants (dpm) (%)	43,170 (48.6)	138,080 (57.5)	78,820 (72)
%of incorp. activity in compounds isolated:			
Codeinone (or neopinone)	19	2.3	2.5
Codeine	8.0	16	17
Morphine	8.7	4.7	3.4

Table V

Search for Codeinone and Neopinone in *Papaver somniferum* Plants after $^{14}CO_2$ Exposure

Compound sought	Compound analyzed	Carrier added (mg)	Specific activity of isolated compound (dpm/mg)	Total activity in plant (dpm)	% of Thebaine activity
Codeinone	Thebaine	62.3	176,000	11×10^6	
	Codeine	60.0	20,000	1.2×10^6	10.9
	Codeinone (as dihydro-codeinone)	298	2,000	6×10^5	5.4
Neopinone	Thebaine	58	2.1×10^6	12×10^7	
	Codeine	50	1.5×10^6	7.5×10^7	62
	Neopinone (as 6-methyl-neopine)	240	3.1×10^4	7.4×10^6	6.1

and the products subjected to the same purification procedures as would be used to isolate 6-methylneopine. No activity from these compounds was carried over with 6-methylneopine (thebaine does react a small amount with methyllithium, but not in a way to interfere; codeine comes through the procedure as pure codeine, and codeinone, of course, is converted to 6-methylcodeine, which can be separated from 6-methylneopine by alumina TLC).

In order to search for neopinone in the poppy plants, the plants were grown for two days in an environment containing $^{14}CO_2$. At the end of the exposure period, carrier neopinone, codeine, and thebaine were added to the plant material prior to work-up. Upon isolation of the non-phenolic alkaloid fraction, the entire fraction was treated with methyllithium in order to convert the neopinone to 6-methylneopine. Thebaine, codeine, 6-methylneopine, and 6-methylcodeine (which was also present in sizeable quantity due to conversion of neopinone to codeinone during the work-up) were separated by means of alumina TLC, using two different solvent systems. Thebaine, codeine, and 6-methylneopine were then purified to constant activity by sublimation followed by several different kinds of TLC. The results are presented in Table V, page 61. (For convenient comparison, the results from the search for codeinone are also presented here again.)

This last experiment has demonstrated that neopinone in fact does exist in Papaver somniferum plants. The earlier questions regarding the feeding experiment can now be answered. It is known that neopinone rearranges to codeinone in aqueous solution. That

this can happen inside the plant as well as in vitro is no great surprise, and a feeding experiment which shows that neopinone can be converted to codeine in the plant has no particular significance except to show that there is nothing in the plant which prevents the isomerization of neopinone to codeinone (which can then proceed to codeine). Now that neopinone has actually been found to be a naturally-occurring alkaloid, it is quite reasonable to suppose that naturally-produced neopinone is converted to codeinone and thence to codeine.

A rough comparison of the apparent pool sizes of neopinone and codeinone can be made from Table V. It would appear that these two compounds have approximately the same pool size. However, since neopinone is known to be quite unstable, and readily rearranges to codeinone -- even during the work-up procedure -- a significant amount of the codeinone reported to have been found in the plants may have actually occurred as neopinone which was changed to codeinone by the work-up procedure. In fact, the point could be raised that no codeinone exists in the plant -- i.e., that all of the codeinone which has been isolated from plants came from rearrangement of neopinone during the work-up procedure. Two points would speak against this suggestion. One is that codeinone fed to the plants is converted to codeine and morphine.[48,58,59] (However, we also know that codeine methyl ether fed to the plants is converted to codeine and morphine,[48,52] yet it cannot be found in the plants.[48]) The other point is a mechanistic one: it is very difficult to conceive of a route by which neopinone could be reduced to codeine

without prior rearrangement to codeinone. Preliminary reduction to neopine, followed by rearrangement of neopine to codeine, is, of course, a possibility, but not a very likely one, particularly since the rearrangement of neopinone to codeinone is known to occur very readily, whereas a similar rearrangement of neopine to codeine has never been observed. Therefore we feel reasonably safe in concluding that thebaine is converted to codeine by way of neopinone and codeinone, although nothing can be said at this point about the relative pool sizes of these two intermediates.

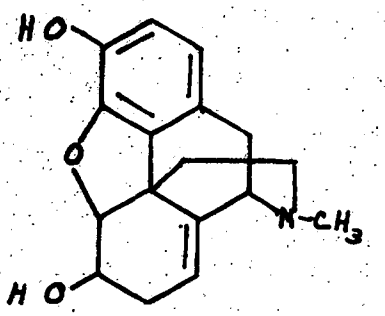
VI. CONCLUSION

The work presented in this thesis has provided evidence for a biosynthetic route for thebaine going to codeine *via* neopinone and codeinone. Although codeine methyl ether was shown by G. Blaschke to be converted to codeine *in vivo*, no codeine methyl ether could be found naturally occurring in *Papaver somniferum* plants.[48] On the other hand, both codeinone and neopinone were found in the plants, and *in vivo* conversion to codeine and morphine was also demonstrated. In the case of codeinone, a study of its specific activity in relation to those of codeine and thebaine after a "steady state" exposure to $^{14}CO_2$ also supported the role of codeinone as an intermediate in the conversion of thebaine to codeine.

The involvement of neopinone in the biosynthetic conversion of thebaine to codeine may help to explain a partial loss of tritium label at C-7 observed by Barton *et al.*[23] in the transformation of dual-labelled thebaine to codeine. A 14% loss of tritium was observed. Since the label was not completely lost or completely retained, there are two possibilities for the role of neopinone. One possibility is that proton addition to thebaine in the formation of neopinone and subsequent removal in the rearrangement to codeinone is not completely stereospecific but does have a steric preference. The other possibility is that there are two paths from thebaine to codeinone: 85% of the codeinone is formed directly from thebaine

demethylation, while 15% is formed by prior conversion to neopinone. At present, no answer can be provided for this question.

Now that the occurrence of neopinone has been demonstrated in Papaver somniferum plants, the possibility of neopine being present is also quite likely. Neopine has been isolated from opium itself,[74] but no report has been made of neopine occurring naturally in the plant. A further extension of this search would also be a search for neomorphine (XXXIII), formed by demethylation of neopine.



XXXIII

Now that many of the steps in the biosynthesis of morphine have been elucidated, a search for the enzymes involved in these steps would definitely be in order. Once these enzymes have been isolated, their specificity would be of great interest, particularly with regard to using the plant as a "factory" in order to make morphine analogues, as has been done in the case of nicotine.[87] In addition, if enzyme studies can be conducted, a better idea can be obtained concerning the rate of synthesis of the morphine alkaloids, their turnover rates, and possibly even their ultimate fate within the plant.

Subsequent work could also study the order of methylation of the precursors of thebaine. Rapoport *et al.*[2] have proposed that the morphine alkaloids may play a role as methyl carriers or transmethylating agents, and this idea could be pursued further. In addition, the metabolic fate of morphine in the plant could be determined. Further studies in these lines may help clarify the role of the morphine alkaloids in *Papaver somniferum* plants and may also help give a clearer understanding of alkaloid chemistry, both *in vitro* and *in vivo*.

VII. DESCRIPTION OF EXPERIMENTAL WORK

A. Starting Materials

Thebaine, codeine, and morphine were obtained in crystalline form from Mallincrodt Chemical Works. Codeinone was prepared from codeine by oxidation with silver carbonate as described by Rapoport and Reist.[57] Neopinone was prepared according to the method of Conroy.[67] The plants used in this work were from seed of *Papaver somniferum* L., var. *alba*, U.S.D.A. No. 40, grown at Somerton, Arizona in 1950.

B. Bisulfite Treatments of Codeinone

1. 1 M $NaHSO_3$

Fifty mg. codeinone plus 20 mg. codeine were dissolved in 10 ml. benzene and placed in an erlenmeyer flask with 10 ml. freshly-prepared 1 M $NaHSO_3$. The flask was flushed with nitrogen, stoppered, and shaken on a mechanical shaker for half an hour, after which time the benzene layer was removed. Analysis of the contents of this layer indicated that all of the alkaloids had been removed. The pH of the aqueous solution was then raised to pH 8.5, and the solution was extracted several times with benzene to remove the codeine. No codeinone was liberated, as seen by TLC (silica gel, solvent system of $CHCl_3$ - CH_3OH : 3/1 plus 0.02% NH_4OH. *Note:* These conditions of TLC were the ones always used unless otherwise indicated). Subsequent

experiments using ^{14}C-codeinone under the same conditions also showed no release of activity at this pH. The pH of the aqueous solution was then raised to pH 12-14. The basic solution plus 10 ml. chloroform were shaken in the dark (to avoid decomposition accelerated by light)[75] on a mechanical shaker for 48 hours (in a stoppered flask flushed with nitrogen). During this time, the chloroform layer was changed after 2, 4, 8, and 24 hours, and finally removed after 48 hours. Each organic extract was immediately dried over sodium sulfate, filtered, and evaporated to dryness, but the products from all of the extracts were combined. Yield of codeinone was approx. 55%, but this substance was shown on TLC to have some polymeric material in it. Purification could be accomplished at this stage by sublimation and recrystallization, but generally in these studies derivatization was first effected, followed by the easier purification of the derivative.

An attempt was made to improve the final extraction of codeinone from the aqueous solution by continuous extraction with methylene chloride. However, a lower yield (27%) was obtained, and the amount of polymer (as seen by TLC) in this product was much higher.

2. pH 7 Bisulfite

A pH 7 bisulfite-sulfite buffer was made in the manner described by Rapoport and Lovell,[54] and consisted of a 1:3 mixture of 1 M $NaHSO_3$ and 1 M Na_2SO_3 (with final pH adjustment with one or the other of these solutions as needed). A treatment similar to that described above was given to codeinone. It was found that codeinone took a

longer period of time to form the bisulfite adduct at this pH. Therefore, the initial shaking time was increased to one hour. Under these conditions, no codeinone was liberated when the pH was raised to pH 8.5, indicating that it had all formed the adduct (this was also checked using ^{14}C-codeinone). Recovery of codeinone was accomplished in the same manner as described above.

C. Problems with the Bisulfite Procedure as a Method for Separating Codeinone from Other Non-phenolic Alkaloids

1. Loss of Thebaine

Originally, 1 M $NaHSO_3$ was used in the separation of codeinone from the other non-phenolic alkaloids. However, at times rather extensive loss of thebaine was observed from this treatment. Therefore, an investigation was initiated to determine where this loss occurred and how to avoid it. Thebaine is known to undergo several reactions in acid, depending on the conditions.[8,36,76] Our initial investigation was to see if the loss of thebaine was due to the low pH (~4) of the 1 M $NaHSO_3$ solution. When this was shown not to be the case, then pH 7 bisulfite was tried for the extraction scheme, and the loss of thebaine was greatly reduced. Since codeinone could still be effectively separated from the other alkaloids with the pH 7 treatment, a change to this pH was made in the extraction. Some further work as to the identity of the thebaine-bisulfite product was conducted, and finally Bjeldanes[66] was able to show that the product was demethylsalutaridine (XXVIII). Its formation could be avoided by working in the absence of oxygen. The results for codeinone

extraction using pH 7 bisulfite are described on the preceding pages. The details of the thebaine work are presented below.

a) Comparison of thebaine behavior in phosphate and bisulfite solutions. Ten ml. thebaine stock solution (approx. 5 mg. thebaine/100 ml. benzene) were shaken on a mechanical shaker for one hour with 10 ml. phosphate buffer at pH 2.3 (1 N H_3PO_4 + NaH_2PO_4). The benzene layer was removed, the pH of the aqueous layer taken to pH 9.7 with 1 N NaOH, and the solution was extracted with benzene. This benzene solution was evaporated, and the residue analyzed quantitatively by UV (methanol solvent).

Thebaine from the same stock solution was similarly shaken with 1 M $NaHSO_3$ (pH ~4), taken to pH 9.7, extracted, and the benzene extract analyzed. The pH of the aqueous solution was then taken to pH 12.5, and the solution was shaken for four hours on a mechanical shaker with benzene, changing the benzene layer twice during this period. UV analysis: λ_{max} = 285 mμ

Sample	Absorbance	% Recovery of Thebaine
Standard	0.572	
After phosphate treatment	0.565	99
After bisulfite treatment		
pH 9.7 extract	0.205	35
pH 12.5 extract	0	0

b) Treatment of thebaine with bisulfite at pH 4 and at pH 7. Ten ml thebaine stock solution (approx. 10 mg/100 ml.) were shaken with 10 ml pH 7 bisulfite-sulfite buffer solution on a mechanical

shaker for half an hour or for one hour. The benzene layer was removed and worked up for UV analysis. The pH of the aqueous solution was taken to pH 9.5 - 10, and the solution was re-extracted with benzene. Exactly the same procedure was followed using 1 M $NaHSO_3$ (pH ~4).

UV analysis: λ_{max} = 285 mμ

Sample	Absorbance	% Recovery	% Total Recovery
A. pH 7 Bisulfite			
Standard	1.52		
1/2 hr shaking			
pH 7 extract	1.04	68	
pH 10 extract	0.26	17	85
1 hr extract			
pH 7 extract	1.02	67	
pH 10 extract	0.19	12	79
B. 1 M $NaHSO_3$			
Standard	1.47		
1/2 hr shaking			
pH 4 extract	0	0	
pH 10 extract	0.34	23	23

2. Carry-over of Codeine with Codeinone in the Bisulfite

Before it was decided to use lithium reagents to provide derivatives of codeinone useful for further purification and analysis, the conversion of codeinone to codeine[62] had been considered. However, early in this work the suspicion arose that there was a small carry-

over of codeine with the codeinone in the bisulfite procedure. Such a carry-over would automatically invalidate any data obtained about codeinone if the isolated codeinone were converted to codeine for analysis. To check this, 100 mg. (143,000 dpm) ^{14}C-codeine plus 230 mg. codeinone (non-labelled) were carried through the plant work-up procedure and then carried through the bisulfite procedure. No codeine could be seen by TLC in the codeinone isolated, but the Rf values for the two compounds were very close (codeine: Rf 0.52, codeinone: Rf 0.60). However, the codeinone had an activity count corresponding to 2.7% of the activity originally added. This activity could only come from codeine. In fact, a subsequent experiment converted the isolated codeinone to 6-methylcodeine (XXVI),[63] purification of which eliminated the radioactivity carry-over. Similar tests for carry-over by thebaine indicated that there was no difficulty in this line. The reason for the codeine carry-over cannot be explained, as calculations based on distribution coefficients predict a clear separation.

D. Hydrogenation of Codeinone

1. Using 10% Pd/C

The conversion of codeinone to dihydrocodeinone (XXV) by hydrogenation over 10% Pd/C has been described by Mannich and Loewenheim,[55] and we can confirm their results. TLC of the product indicated that the major product was dihydrocodeinone, but the presence of at least two other substances to the extent of 20% of the total yield could also be detected. An increase in the amount of catalyst increased

the amount and number of side-products. In handling the codeinone isolated from plant material, a very small amount of codeinone would be expected -- such that it would be difficult to calculate and use an exact ratio of catalyst. A derivative of codeinone was sought whose formation would be independent of the amount of reagent or catalyst used.

2. Using 5% $Pd/BaSO_4$

It was hoped that a change of catalyst might eliminate the side-products observed in the reduction of codeinone. Use of a Pt-containing catalyst completely altered the product.[61] Therefore, another Pd-containing catalyst was tried. Using 5% $Pd/BaSO_4$ (18 mg. catalyst + 34 mg. codeinone in 5 ml. absolute ethanol, hydrogenated at room temperature under 1 atm. H_2), the uptake of hydrogen was slower than with 10% Pd/C, but the isolated dihydrocodeinone still contained up to 20% other products (none of which could be identified as dihydrocodeine, either).

E. Reaction of Codeinone with Lithium Reagents

1. Reaction with Methyllithium

The reaction of codeinone with methyllithium to yield 6-methylcodeine (XXVI) was conducted according to the procedure of Findlay and Small,[63] and a quantitative yield of product was obtained.

2. Reaction with n-Butyllithium

a) Codeinone plus n-butyllithium. (i) Three hundred mg. codeinone were dissolved in toluene and stirred at 0°C under nitrogen. Four ml. n-butyllithium solution (1.6 M in n-hexane) were added, and

the reaction mixture was stirred for one hour. (In all cases, the presence of excess lithium reagent at the end of the reaction was confirmed by the Gilman test with Michler's ketone.[77]) After this time, the reaction mixture was deactivated with ice-water, and the aqueous layer was extracted with toluene. The dried toluene was evaporated to dryness. TLC revealed a complex mixture: a good deal of unreacted codeinone, a long smear below codeinone, and at least three spots with Rfs higher than codeinone.

Experimental conditions were varied as follows: (ii) Same as (i) except at room temperature instead of 0°C. (iii) Solvent for codeinone was 20% ethyl ether, 80% toluene; reaction was run at room temperature for one hour. (iv) Same as (iii) except for two hours. In all cases the product was a complex mixture of compounds, including codeinone. VPC indicated three major products, corresponding to the three compounds of higher Rf seen on TLC. Attempts to separate these three compounds by column chromatography (silica gel Woelm, $CHCl_3/CH_3OH$ = 5/1 + 4 drops NH_4OH per 100 ml) led to the isolation of the largest component (the one with the highest Rf), but the other two compounds remained together. Mass spec. analysis of the single isolated compound indicated a parent peak of 355 (probably 6-butylcodeine, MW 355). Mass spec. analysis of the fraction containing the other two compounds indicated a "parent peak" of 413, which is the same as the molecular weight of a dibutylcodeine. Further identification has not been done at this time.

<u>b) Dihydrocodeinone plus n-butyllithium</u>. Since the reaction of codeinone with n-butyllithium indicated that we were obtaining

multiple products (some of which were probably due to α,β-unsaturated nature of the carbonyl) while at the same time we were able to isolate at least 20% unreacted starting material, we were interested in observing the behavior of dihydrocodeinone with n-butyllithium. It would be quite surprising if we were to obtain multiple products with dihydrocodeinone, but a similar lack of reactivity as that shown by codeinone might be expected. Small and Rapoport[65] did not report any second product or reisolation of any starting material on reaction of dihydrocodeinone with n-amyllithium, but they had obtained an oil, and lack of access to TLC may have failed to reveal the presence of other compounds.

Eight mg. dihydrocodeinone were dissolved in toluene-ether (4:1) and stirred at room temperature under nitrogen for two hours with 2 ml. n-butyllithium solution (1.6 M in n-hexane). TLC of the product indicated the complete absence of dihydrocodeinone and the presence of one spot, of higher Rf than dihydrocodeinone.

The behavior of codeinone with n-butyllithium appears to be rather unusual. One further surprise came when we discovered that codeinone reacts quantitatively with phenyllithium to yield one product: 6-phenylcodeine (see below). It therefore appears that steric factors are not the sole reason to account for codeinone's behavior toward lithium reagents. Further investigation in this line could prove very interesting.

3. Reaction with Phenyllithium

One gram codeinone was dissolved in 40 ml. toluene and reacted at 0°C for one hour with phenyllithium solution, with periodic

monitoring with the Gilman test.[77] At the end of the reaction time the mixture was deactivated with ice-water. The aqueous layer was acidified and extracted several times with toluene to remove the biphenyl present from the phenyllithium reagent. The acid solution was then made basic and extracted several times with chloroform. The organic extract was dried, filtered, and evaporated to dryness. Yield: 1.1 g. (90%). TLC indicated the complete absence of codeinone (Rf 0.53) and the presence of only one product (Rf 0.81). Efforts to crystallize the product were unsuccessful. However, drying overnight at 45° on a vacuum line (200-300 μ) produced a glass. A powder from this glass slowly softened and fluidized between 57° and 70°C. Analysis: Calc. C 76.77%, H 6.71%, N 3.73%; Found: C 76.62%, H 6.58%, N 3.70%. Mass spec. revealed a parent peak at 375 (MW of 6-phenylcodeine (XXVII): 375). UV: λ_{max} = 385 mμ, log ε = 3.185. NMR was similar to that of codeine[70,78] with the absence of the 6-H (4.2 ppm) and the presence of a multiplet for the phenyl group (7.3 ppm). VPC (for complete description, see later) gave a single peak at 21 minutes, a time at which no non-phenolic alkaloid isolated from poppy plants gave a peak.

F. Acetyl Derivatives of Alkaloids

Another attempt to obtain a derivative of codeinone which was quantitative and which would produce a peak on VPC distinct from those of other alkaloids and also one which was very sensitive was to try acylating various existing derivatives of codeinone. On-column acylation of codeine and morphine was tried by Anders and

Mannering,[64] and was very successful in the case of morphine but quite unsuccessful in the case of codeine. We tried co-injection of a sample of codeine with acetic anhydride, but obtained a new peak to the extent of only 5% -- the remainder of the effluent from the VPC was still codeine. Similar results were obtained in the case of 6-methylcodeine (XXVI). Therefore, it was decided to acylate first and then VPC.

Twelve mg. 6-methylcodeine were placed in a flask with 2 ml. acetic anhydride. The reaction mixture was allowed to reflux under nitrogen. The reaction was followed by periodically taking a 2 μl. sample and injecting it into the VPC. After eight hours, the reaction was complete.

VPC: 6-methylcodeine: 4 min. 15 sec.
6-acetyl-6-methylcodeine: 6 min. 45 sec.
(thebaine: 6 min. 45 sec.)

In a similar manner, 9 mg. codeine were refluxed 2 hours with acetic anhydride, the reaction being followed by VPC.

VPC: codeine: 4 min. 45 sec.
6-acetylcodeine: 6 min. 45 sec.

All of the VPC peaks described above are very sensitive, and the acylation reactions were quantitative. TLC of 6-acetyl-6-methylcodeine and 6-acetylcodeine showed that the two could be separated by TLC.

G. Vapor Phase Chromatography of Opium Alkaloids

The first attempt to separate opium alkaloids by means of VPC was reported in 1960 by Lloyd *et al.*[79] Since that time, many

studies have been conducted in order to reduce tailing and increase sensitivity. A good discussion of some of the precautions necessary is found in an article by McMartin and Street.[80] The system which we have ended up using has been developed as a result of long, slow development. A description of the present system follows.

The use of a glass column is definitely necessary. Comparison with a metal column, using identical packing material, showed that far less tailing and much higher sensitivity was available with a glass column. The column packing material must also be carefully prepared so as to reduce the number of polar sites on the solid support. Either Gas-Chrom Q or Aeropak 30 (100/120 M) can be used as the solid support -- both have been DMCS treated and very carefully acid- and base-washed. Use of some kind of silicone rubber has been found to be most successful for the stationary liquid. Recently, several silicone rubbers have become available which are stable at temperatures above 350°C. Of these, OV-1 and OV-17 have both been used with some success.

The column preparation procedure is as follows. The 6 mm. glass tubing for the column is rinsed thoroughly with water, then with acetone-methanol (2:1), and then with chloroform. It is then allowed to dry thoroughly in a stream of nitrogen. The packing material is then prepared according to the method of Kruppa et. al.[81] Twenty g. Aeropak 30 (100/120 M) are slowly added with stirring (but no grinding against the sides of the beaker) to a solution of 1.5 g. OV-1 in 100 ml. $CHCl_3$. The mixture is then allowed to sit for 5-10 minutes. At the end of this time, any particles of solid support floating on the chloroform are removed. By a process of very gently

stirring, the Aeropak is then mixed up with the supernatant, and the resultant slurry is poured into a filter funnel and the excess chloroform removed. Calculations based on the amount of chloroform recovered can give an approximate estimate of the amount of coating which has occurred. In this case, it is usually around 3%. The Aeropak is sucked reasonably dry on the filter funnel. It is then placed in an Applied Sciences Fluidizer, in which a warm stream of nitrogen suspends the particles and removes all excess solvent. A final drying is done in a large, flat dish in the oven at 100°C. The prepared packing material is placed in the glass VPC column by means of suction accompanied by gentle tapping. Curing of the column is accomplished by first placing the column in the VPC oven and heating the oven to 300°C for two hours. No gas passes through the column during this time. The temperature is then lowered to 250°C, carrier gas (argon) is turned on to approximately 60 ml/min, and the column is allowed to cure in this manner for 24 hours. Using a column prepared in this manner, we have been able to obtain good resolution of the alkaloids and sensitivities in the range of 70 nanograms for codeine and 200 nanograms for thebaine. VPC conditions used for the alkaloids were: column temp., 210°C; flash heater and detector temp., 250°C; carrier gas and flow rate, 60 ml/min argon.

1. Trapping of Compounds from VPC

In order to determine the specific activities of the compounds isolated from the "steady state" exposure, a mass determination was done by means of VPC. After this, the column was fixed so that the

effluent could be trapped in a capillary tube containing fine glass beads. The efficiency of trapping of 6-phenylcodeine was determined by trapping a sample of standard which had been injected, and then re-injecting this trapped material (with the column once again attached to the flame ionization detector) to determine the amount recovered. Approximately 80% recovery was obtained. In the case of codeine and thebaine, known ^{14}C-standards of these compounds were available. These standards were injected and then trapped. Liquid scintillation counting of the trapped material showed approximately 80% recovery in both cases. Martin[82] has reported that thebaine decomposes on VPC. He used a metal column for his work. Whether or not this occurs using a glass column is, at this point, not important, since we now know that we can isolate most of the carbon from thebaine by trapping (80% recovery), and for a specific activity determination that is what is necessary.

H. Standard Work-up Procedure to Isolate Alkaloids from *Papaver Somniferum*[82]

The procedure described here is effective for 50-100 g. fresh plant material. The plants are frozen in liquid nitrogen, chopped up, and then ground in a Waring blendor. At this point, if carrier compounds are to be added, they are added -- dissolved in butanol-benzene (1:1). One hundred ml. butanol-benzene (1:1) and 25 ml. 10% sodium carbonate solution are added to the plant material, and the entire mixture is ground in the blendor for 10 minutes. More butanol-benzene is then removed, and fresh butanol-benzene added,

with further mixing for 5 minutes in the blendor. Four or five such butanol-benzene extractions are conducted. The combined organic extracts are then extracted three or four times with pH 2.5 phosphate solution, made from 1 N H_3PO_4 and NaH_2PO_4 crystals. The pH of the aqueous extract is then adjusted to pH 13-14 using conc. NaOH solution, and the non-phenolic alkaloids extracted with chloroform. If the determination of morphine is also necessary, the pH is readjusted to pH 8.6, and the phenolic alkaloids extracted with chloroform. (Note: Subsequent to this work, R. Miller[83] has developed better methods for extracting the phenolic alkaloids, as low recoveries are obtained by the methods described here.)

I. $^{14}CO_2$ "Steady State" Exposure of *Papaver Somniferum*

Nine poppy plants, 64 days old, each weighing approximately 30 g., were exposed to "steady state" conditions of $^{14}CO_2$ (see Chapter II) in the light for three hours. The $^{14}CO_2$ in the cylinder had a specific activity of 0.2 mC/ml. (STP). The level of carbon dioxide in the exposure chamber was maintained at 0.04% throughout the time of the exposure. A total of 215 ml. $^{14}CO_2$ (43 mC.) were used. Of this, 70 ml. (14 mC.) were used to fill the exposure chamber; hence that much was also left in the chamber at the end of the exposure, meaning that 145 ml. (29 mC.) $^{14}CO_2$ went into the plants.

At the end of the three hour exposure time the plants were removed from the chamber and immediately frozen in liquid nitrogen. The usual work-up procedure was followed to isolate the non-phenolic alkaloids (no carrier alkaloids were added, either during work-up or

analysis). The crude non-phenolic alkaloid mix was taken through the bisulfite extraction procedure to separate codeinone from the other alkaloids. The codeinone in turn was treated with phenyllithium to produce 6-phenylcodeine (XXVII). Mass analysis of each of the alkaloids was done by VPC, with the column at 210°C for the analysis of codeine (4 min. 45 sec) and thebaine (7 min) and at 235°C for the analysis of 6-phenylcodeine (9 min. 15 sec). Radioactivity of each of the alkaloids was measured by trapping each peak after VPC (after first determining the trapping efficiency for each alkaloid), eluting with liquid scintillation solution, and counting by liquid scintillation counting. The results are presented in Table III on page 47.

J. Determination of Percent ^{14}C Activity in the 6-OCH_3 Group of Thebaine after the "Steady State" Exposure

One gram thebaine and 0.5 g. codeine were added as carrier alkaloids to the sample containing these two alkaloids obtained from the "steady state" exposure. The alkaloids were separated by means of column chromatography, and the thebaine was purified by sublimation and then recrystallization to constant activity (from methanol). The specific activity of the thebaine thus obtained was 40,400 dpm/μM. The procedure of Conroy[67] was used to convert a portion of this thebaine to 14-bromocodeinone (XXIX) using N-bromosuccinimide. The only deviation from his procedure was that acetone was the sole solvent for the reaction (instead of 2:1 acetone-water), this change in solvent being found necessary in order to obtain complete reaction

of thebaine on this small scale. Precipitation of the product was accomplished by the addition of water at the end of the reaction. The product was purified by recrystallization from benzene-ligroin and prep.-TLC. Its specific activity at the end of this purification was 30,800 dpm/μM, or 76% of the starting thebaine. Therefore, 24% of the activity in the thebaine from the "steady state" exposure was in the 6-OCH_3 group. Taking this fact into account, the corrected value for the thebaine specific activity in Table III is 18×10^7 dpm/μM.

K. Feeding of Nuclear-Labelled ^{14}C-Codeine

Nuclear-labelled ^{14}C-codeine, with a specific activity of 50,200 dpm/mg, was obtained from G. Blaschke during his synthesis of nuclear-labelled codeinone.[48] Four plants of Papaver somniferum, 43 days old, 6 g. each, were hydroponically fed 3 mg. (130,400 dpm) ^{14}C-nuclear-labelled codeine. The plants were placed in four vials, and a solution of the ^{14}C-codeine in a mixture of 0.3 ml. 0.1 M H_3PO_4 and 4 ml. Hoagland's nutrient solution[84] was divided equally between the plants. After one minute, an additional 1 ml. nutrient solution was added to each plant, these additions being repeated every ten minutes for thirty minutes. After this period, the level of nutrient was maintained at 8-10 ml. for each plant for forty-eight hours. The plants were then removed, their roots washed with 0.1 M H_3PO_4 and then with distilled water. A count of the radioactivity in these washes as well as in the nutrient solution remaining in each vial at the end of the feeding revealed the presence of 51,240 dpm codeine (and only codeine,

as seen by TLC) not incorporated into the plants, indicating that 79,000 dpm were incorporated into the plants. All future calculations will be based on this figure of the amount incorporated.

"Cold" codeine, codeinone, and morphine were added as carriers in the work-up of the plants. Morphine was separated with the phenolic alkaloids and was purified by a couple of different kinds of TLC and then sublimation. Codeinone was isolated by means of its bisulfite addition product and was then reacted with methyllithium to obtain 6-methylcodeine, which was then purified by prep.-TLC, sublimation, and recrystallization. Codeine was purified by column chromatography, sublimation, and recrystallization. The results are presented in Table I, page 35.

L. Chromatography of Neopinone

Krausz and Rüll[68] reported that alumina chromatography of neopinone converted it to codeinone. We confirmed this observation, analyzing the product by NMR and IR. In addition, we found that silica gel chromatography caused significant conversion to codeinone. For this reason, neither of these systems could be used for the analytical or preparative chromatography of neopinone. On the VPC the retention times of codeinone and neopinone are the same (and both with very low sensitivity), so VPC cannot be used for analysis.

Brochmann-Hanssen *et al.*[71] reported a paper chromatographic system which was a good method to distinguish codeine and neopine. It was decided to try this system for the separation of codeinone and neopinone. The papers (Whatman No. 1) were buffered with pH 6.5 McIlvaine buffer,[72] made by mixing 710 ml. 0.2 M NaH_2PO_4 with 290 ml.

0.1 M citric acid. The solvent used for the paper chromatography of the alkaloids was water-saturated n-butanol. The following Rf values were observed:

Codeine	0.7
Neopine	0.5
Codeinone	0.8
Neopinone	0.7
6-Methylcodeine	0.8
6-Methylneopine	0.6
Dihydrocodeinone	0.7
14-Bromocodeinone	0.9

It was apparent that neopinone and codeinone could be separated (though not well) by this method. However, it is a slow method and is not particularly amenable to preparative work due to the low load capacity of the paper and the problems associated with elution and subsequent separation from the citric acid used in the buffer. Therefore, a more convenient system was sought.

Since one of the disadvantages of paper chromatography was time, cellulose TLC was tried next. Teichert et al.[69] have used formamide-impregnated cellulose plates for the chromatography of some opium alkaloids. Using their solvent system of benzene - n-heptane - chloroform - diethylamine (60:50:10:0.2), the following Rf values were obtained:

Codeine	0.35
Neopine	0.20
Codeinone	0.47

Neopinone	0.30
6-Methylcodeine	0.65
6-Methylneopine	0.53

Thus, cellulose TLC provided a fairly rapid method for distinguishing neopinone and codeinone. It was also investigated for preparative work, as a possible method for separating codeinone and neopinone. Analytical plates were the "instant", plastic-backed plates available from Brinkmann. Preparative plates were made using a slurry of 42 g. Machery Nagel Cellulose Powder MN 300 G in 160 ml. water, which was sufficient for coating five plates with a 1 mm. thick layer. The plates were allowed to stand at room temperature for at least 24 hours before using. One hour before use they were immersed in a solution of acetone-formamide (4:1) and then allowed to dry in the open air.

For preparative work, the maximum load per plate was 10 mg. The preparative separation of neopinone and codeinone was attempted, using a sufficient amount of material and plates so the NMRs could be run of the eluted products. After chromatography, the region corresponding to a particular compound was scraped into a Millipore apparatus equipped with a Millipore Solvinert filter (pore size 0.25 μ). Chloroform was ineffective for elution, so methanol had to be used. In the process, the methanol also eluted the formamide from the plate. The filtrate after elution was evaporated down until it was an oil. It was then dissolved in chloroform and in water. The chloroform layer was extracted several times with water to remove all of the formamide, and then finally the chloroform was

dried over sodium sulfate, filtered, and evaporated to dryness. Rechromatography on cellulose TLC of the two spots obtained from a crude noepinone sample (i.e., neopinone and codeinone) showed that each spot retained its integrity -- except that occassionally chromatography of the bottom spot isolated (neopinone) showed a faint presence of codeinone. IR of the top spot isolated showed a strong carbonyl absorption at 1680 cm^{-1}, and NMR corresponded to that of codeinone. IR of the bottom spot, however, showed two carbonyl absorptions, at 1735 cm^{-1} and at 1680 cm^{-1}. NMR also indicated the neopinone was not the only substance present. Some possible contamination by codeinone might have been present, but there were other impurities as well -- although cellulose TLC of the material from the bottom spot had shown only one spot, with a faint spot for codeinone. Also, silica gel TLC of the material from this bottom spot showed at least four substances to be present, whereas silica gel TLC of the starting nelpinone only showed one spot with a slight tail. It was obvious that cellulose TLC could not be used for the purification of neopinone, as it caused extensive deterioration of the neopinone.

M. Reaction of Neopinone with Borohydride

Neopinone could be reduced with sodium borohydride in methanol to yield neopine, as described by Conroy.[67] Both paper chromatography and cellulose TLC of the product indicated that the product contained approximately 20% codeine (estimated by spot size), which would have been produced by borohydride action on the codeinone

contaminant in the ceopinone.[62] NMR of the product also showed the presence of codeine in the neopine, the most sensitive area for this determination being the absorption due to the 5β-H (a soublet at 4.85 ppm for codeine and at 4.64 for neopine).[70,78]

N. Preparation of 6-Methylneopine

1.2 g. crude neopinone (known to contain approximately 20% codeinone) was dissolved in 20 ml. dry toluene and reacted at room temperature (under nitrogen) with methyllithium solution for half an hour. At the end of this time, the solution was deactivated with ice-water. The aqueous solution (which was strongly basic) was extracted several times with toluene, which was then dried over sodium sulfate and evaporated to dryness. The yield at this point was only 0.6 g. A large amount of material remaining in the aqueous solution could be extracted at pH 8.6, but not at a lower pH, indicating that it was probably phenolic in nature. The non-phenolic product was purified by alumina TLC. A solvent system of cyclohexane-chloroform-acetone (8:2:6) gave a moderately good separation of 6-methylneopine (Rf 0.35) and 6-methylcodeine (Rf 0.25). (Other Rf values with this system were: codeine, 0.10; thebaine, 0.40). The 6-methylneopine thus obtained was checked by AlTLC to be sure it was pure, and then it was sublimed at 65°C and 40 μ pressure. The product was rather glassy in nature but turned crystalline after several days. MP 106-8°C. Analysis: Calc. C 72,82%, H 7.40%, N 4.47%; Found: C 73.65%, H 7.26%, N 4.27%. Mass spec. showed a parent peak at 313 (MW of 6-methylneopine: 313). UV:

λ_{max} = 283 mμ, log ε = 3.21. NMR was similar to that of neopine,[70,78] with the absence of the 6-H (4.23 ppm) and an absorption for the 6-CH_3 at 1.34 ppm. In addition, the 5β-H was changed to a singlet and appeared at 4.37 ppm.

O. Bisulfite Treatment of Neopinone

Neopinone was taken through the usual bisulfite treatment, using pH 7 bisulfite. pH of the aqueous solution was adjusted to pH 8.6 using ice-cold sat. Na_2CO_2 solution or using a pH 9 borate buffer.[73] The solution at pH 8.6 was then immediately extracted with chloroform to recover the neopinone. However, NMR of the product indicated the presence of codeinone to the extent of 10-15% (i.e., not quite as much as was in the starting material). Conversion of this product to 6-methylneopine (plus approximately 15% 6-methylcodeine, as seen by alumina TLC and by NMR) also confirmed that codeinone was present in the neopinone isolated from the bisulfite procedure. Codeinone present at the beginning of the bisulfite treatment would not have been released at pH 8.6 (this fact was demonstrated earlier in this experimental section). Therefore, the only source of the codeinone must have been the neopinone, some of which became isomerized to codeinone during the pH adjustment to pH 8.6.

P. Stability of Neopinone at pH 9

Three 100 mg. samples of neopinone were treated as follows: each sample was dissolved in chloroform and then extracted into a

pH 2.5 phosphate solution. The pH of the aqueous solution was then adjusted to pH 9 using 1 M Na_2CO_3. One sample was immediately extracted with chloroform. Another sample was allowed to stand at room temperature for one hour before chloroform extraction. The third sample stood 24 hours before extraction. NMR analysis showed a definite increase in the amount of codeinone present after one hour. The spectrum of the sample isolated after 24 hours corresponded to that of a dimer reported by Conroy,[67] formed by neopinone in basic solution (see below).

Q. Neopinone Dimer (XXXII)

The neopinone dimer reported by Conroy[67] was prepared according to his method by dissolving 2 g. neopinone in 20 ml. benzene and shaking this solution for one hour in a flask flushed with nitrogen with a solution of 1 g. KOH in 15 ml. water. The benzene layer was then removed, and the aqueous solution was extracted twice more with benzene. The combined organic extracts were dried over sodium sulfate, filtered, and evaporated to dryness. The residue was dissolved in a small amount of warm ethyl acetate, whereupon crystals formed almost immediately. IR showed two carbonyls, one at 1735 cm^{-1} and one at 1679 cm^{-1}. Characteristic NMR absorptions: aromatic protons at 6.6 ppm, three peaks, integrating for 2:1:1 protons; broad singlet at 6.30 ppm, integrating for one proton, ascribed to the 8-H of the codeinone part of the dimer; two sharp singlets, one proton each, at 4.63 and 4.57 ppm, ascribed to the 5β-H of the codeinone and the dihydrocodeinone parts of the dimer; a sharp singlet

at 3.86 ppm, integrating for 6 protons, ascribed to the 3-OCH_3 groups of the two parts of the dimer. The sample decomposed on heating, and this may explain the difficulty in obtaining a mass spectrum. Two mass spectra were taken, one of which gave a mass peak at 592, and the other gave a mass peak at 593. The MW of the dimer is calculated to be 594.

R. Stability of Neopinone in the Poppy Nutrient Solution

Since it had already been demonstrated that neopinone was isomerized in basic solution (pH 9), the behavior of neopinone in nutrient solution was checked before a feeding experiment was conducted. Four 100 mg. samples were treated as follows: each was dissolved in a minimum amount of 0.1 M H_3PO_4, and then 100 ml. Hoagland's nutrient solution was added. Samples were kept in flasks which were partially darkened, but open to the air, for varying lengths of time (5-10 minutes, one hour, four hours, and 24 hours). At the end of each time period the solution was made ice-cold and the pH was adjusted to pH 8.6 using ice-cold sat. Na_2CO_3. The solution was then extracted with chloroform, dried, and evaporated to dryness. NMR analysis of the products from the four extractings indicated that during four hours only as much as 5% more codeinone had been formed by the neopinone. In fact, much neopinone was still present after 24 hours.

S. Preparation of ^{14}C-Neopinone

^{14}C-Neopinone was prepared according to the method of Conroy,[67] starting with ^{14}C-thebaine obtained from a biosynthesis in $^{14}CO_2$.

The thebaine was reacted with N-bromosuccinimide to form 14-bromocodeinone, which was then hydrogenated at 10 psi H_2 for six hours dissolved in chloroform containing 10% methanol and mixed with 10% Pd/C. Determination of purity was done by cellulose TLC and by NMR, which indicated that the sample was approximately 15-20% codeinone and 80-85% neopinone. The specific activity was 3,700 dpm/mg.

T. Feeding of ^{14}C-Neopinone and Comparative Feedings of ^{14}C-Codeinone

Since the ^{14}C-neopinone we had obtained contained up to 20% codeinone, we decided to conduct three feedings simultaneously and in parallel: (1) Approximately 100,000 dpm neopinone (~30 mg) was fed to six 58-day-old plants, each plant weighing 5 g. (2) Fed 30 mg. ^{14}C-codeinone (nuclear-labelled -- synthesized by G. Blaschke[48]) to six plants, the same age and weight as above. (3) Fed 20% of 30 mg.-- i.e., 6 mg of ^{14}C-codeinone (nuclear labelled). This feeding was only done to three plants (same age and weight as above), so only 3 mg. were used.

Note: The above numbers are very approximate. Exact details will be presented below.

1. General Feeding Procedure

The plants had all been in hydroponic solution for four days prior to the feeding experiment. On the day of the feeding, they were transferred individually to darkened 25-ml. erlenmeyer flasks. The sample to be fed (^{14}C-neopinone or ^{14}C-codeinone) was dissolved in 0.5 ml. 0.1 M H_3PO_4 and then in 12 ml. nutrient (half these values for the third feeding described above). Two ml. were fed to each

plant. Aliquots were also taken for counting. Small amounts of nutrient were added to each flask over a period of four hours. At this point, all of the nutrient was removed from the flasks and the roots of each plant were washed with fresh nutrient. The flasks were then filled with fresh nutrient, and the plants were allowed to grow until the next day. At this point the plants were removed, the roots washed with acid, and then the plants were frozen in liquid nitrogen and worked up for analysis.

2. Neopinone Feeding

A total of 88,800 dpm (24 mg) were fed to the six plants. Of this, 45,630 dpm were recovered in the nutrient and root washes (a combined total, both after four hours and at the end of the experiment). This material was shown on cellulose TLC to consist of neopinone and codeinone. Activity incorporated into the plants was 43,170 dpm. The following cold carriers were added during the work-up: morphine, codeine, and neopinone. The usual work-up procedure was followed to isolate the non-phenolic and phenolic alkaloid fractions. Purification of the morphine in the phenolic fraction was done using sublimation and prep.-TLC, using two different solvent systems. The non-phenolic fraction was placed on several alumina TLC plates in order to convert the neopinone to codeinone. The codeinone was then isolated *via* the bisulfite procedure and was subsequently converted to 6-methylcodeine, which was then purified by sublimation, prep.-TLC, and VPC. The codeine remaining in the non-phenolic fraction after the removal of codeinone was purified by sublimation, prep.-TLC on silica gel and on alumina. The final

results are presented in Table IV, page 61.

3. Large Codeinone Feeding

A total of 240,000 dpm (32 mg) were fed to the six plants. The recovery from the nutrient and root washes was 101,900 dpm, and this was shown by TLC to still be in the codeinone. Therefore, the activity incorporated into the plants was 138,000 dpm. Morphine, codeine, and codeinone were added as carriers in the work-up. The usual work-up procedure was used to isolate the phenolic and non-phenolic alkaloid fractions, and the individual alkaloids of interest were purified to constant activity using sublimation, prep.-TLC and VPC (if necessary). The final results are in Table IV.

4. Small Codeinone Feeding

A total of 101,160 dpm (2.1 mg) was fed to the three plants. The recovery from the nutrient and root washes was 28,340 dpm, and this was shown by TLC to be codeinone. The activity incorporated into the plants was 72,820 dpm. Morphine, codeine, and codeinone were added as carriers in the work-up. Work-up and purification of each of the alkaloids were done as usual. The final results are in Table IV.

U. Reaction of Methyllithium with Non-phenolic Opium Alkaloids

Samples of ^{14}C-labelled thebaine, codeinone, and codeine were subjected to the same reaction conditions used to convert neopinone to 6-methylneopine. Codeine was recovered unchanged, codeinone was converted to 6-methylcodeine, and thebaine was largely recovered

unchanged, but several other compounds were also seen on alumina TLC. Each alkaloid, after methyllithium treatment, was subjected to the same alumina chromatography procedure which would be used to purify 6-methylneopine, and the region on the plate which would correspond to 6-methylneopine was eluted and analyzed for any contamination of radioactivity from any of these other alkaloids. After two prep.-alumina TLC purifications one 0.5% of the starting activity from thebaine (none in the runs with codeinone and codeine) could be detected in the region corresponding to 6-methylneopine on the plate.

V. Search for Neopinone in the Poppy Plant

Ten poppy plants (60 days old, weighing approximately 15 g each) were grown under "steady state" conditions for two days while being administered 100 mC $^{14}CO_2$. Harvest was done after 48 hours in the exposure chamber. The following cold carriers were added during work-up: 50 mg. codeine, 58 mg. thebaine, and 300 mg. neopinone (consisting of 240 mg. neopinone and 60 mg. codeinone, as determined by NMR). After the usual isolation of the non-phenolic fraction, this entire fraction was dissolved in toluene and treated with methyllithium solution for half an hour at room temperature. Initial separation of the alkaloids was done by alumina TLC using the same solvent system as described in the preparation of 6-methylneopine. Three bands were obtained: codeine, 6-methylneopine, and 6-methylcodeine plus thebaine (these latter two co-chromatographed on the prep. plates although they can be separated with this solvent

system on the "instant" (Brinkmann) analytical plates). The 6-methylneopine was re-chromatographed on the alumina plates to ensure complete separation from 6-methylcodeine and other compounds. Thebaine and 6-methylcodeine (produced from the codeinone in the neopinone added as carriers as well as extensive conversion of neopinone to codeinone during work-up from the plant material) were separated from each other using a solvent system of benzene-acetone-chloroform (70:15:15) shaken with a solution of 3.5% NH_4OH in water.[85] Each of the alkaloids was subsequently purified to constant activity by using different kinds of AlTLC and silica gel TLC and sublimation. The final results are presented in Table V, page 61.

ACKNOWLEDGEMENTS

There are so many people who have provided me with help and encouragement while I have been doing the work for this thesis that it would be impossible to list all of them. I have a feeling of a great deal of gratitude towards all of them. There are a few people I would like to formally thank because of the special assistance they have given.

There is no doubt in my mind that Dr. Henry Rapoport is one of the key reasons I survived graduate school. His scientific guidance has, of course, been of great help and has taught me many things, both with regards to basic facts and with regards to the approach to scientific research. However, my deepest gratitude to him is for his support -- when the research was not going well and, even more significantly, when I was having my own doubts about continuing as a graduate student. For this acceptance and support I shall be eternally grateful.

Many thanks should also go to my colleagues in Dr. Rapoport's group. In particular, I would like to thank Robert Miller, Dr. Bradford Mundy, Dr. Urs Eppenberger, Dr. Gottfried Blaschke, and Dr. Melvin Rueppel for the assistance they have given me in innumerable ways.

Thanks also to Ed Heilstead of LRL Health Chemistry for his calm and capable handling of all problems with radioactivity -- and

particularly for his assistance in the development of the new "steady state" system.

Much gratitude also to Wallace Erwin, whose incredible patience and abundant assistance during my frequent struggles with the gas chromatograph-flow counter were greatly appreciated.

These are just a few names among the many who need to be thanked -- those in the Laboratory of Chemical Biodynamics, in the Chemistry Department, in the shops, and in the Lawrence Radiation Laboratory. So many people have helped me in one way or another, and I wish I could thank them all be name.

However, the technical assistance received was only a small fraction of the support given me while I was a graduate student. Of chief importance was the emotional support given by friends. Without their encouragement and understanding I know I could never have stuck it out this far. My thanks to all of them.

It should also be mentioned that I received a Predoctoral Fellowship from the National Institutes of Health from 1966 to 1970 as support while I was a graduate student.

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TECHNICAL INFORMATION DIVISION
LAWRENCE RADIATION LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720