Studies on the biosynthesis of the morphine alkaloids

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Studies on the Biosynthesis of the Morphine Alkaloids

A Thesis Submitted to
Loughborough University of Technology

by

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In partial fulfilment for the Requirements for the Degree of
Doctor of Philosophy.

December 1971
SUMMARY

Earlier work on the biosynthesis of morphine in the opium poppy, *Papaver somniferum*, is reviewed in Chapter I.

A variety of unnatural codeine precursors, closely related structurally to codeine and labelled with $^3$H at C-2, have been prepared and administered to *P. somniferum* plants. Their *in vivo* $\beta$-O-demethylation to give the corresponding morphine compounds was investigated using radiodilution techniques. Each of the feeding experiments utilised $[N$-methyl-$^{14}$C]codeine as an internal standard and the efficiency of the unnatural $\beta$-O-demethylations was compared with that observed for the natural conversion of codeine into morphine. Dihydrodesoxycodeine was more efficiently converted into dihydrodesoxymorphine than was codeine into morphine. The least efficiently demethylated unnatural precursor of the series was 1-bromocodeine. The efficient metabolism of dihydrodesoxycodeine suggested that the presence of a secondary alcohol group at C-6 was not important for the enzymic demethylation. However demethylation of dihydrocodeine occurred but was more efficient than for isocodeine.

As part of this investigation, a new, high yield (69%) preparation of isocodeine from codeine via codeine toluene-p-sulphonate was developed. Codeine methyl ether was also fed to *P. somniferum* plants and shown to be converted into morphine methyl ether, and into codeine and morphine. Biological methylation of $[N$-methyl-$^{14}$C]codeine, to give codeine methyl ether, was also observed, suggesting that codeine methyl ether is a natural alkaloid of *P. somniferum*. When $[2-^3$H,N-methyl-$^{14}$C]codeine was
fed to the plants the $^{3}\text{H}/^{13}\text{C}$ ratios of the isolated codeine and morphine were slightly larger and significantly different (statistically) from that of the codeine precursor. This may be due to some de-N-methylation occurring during metabolism.

The rearrangement of thebaine and its metho-salts in concentrated hydrochloric acid and trifluoroacetic acid has been investigated and shown to involve red oxonium ion intermediates. Quaternary salts of 7,8-dehydromethacetanone have been isolated and characterised.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor G.W. Kirby for his friendly interest and guidance throughout the course of this work. I also thank Professor Kirby for the privilege of working in his laboratories.

I thank my parents for their interest and encouragement during my time at University.

I acknowledge financial support provided by the Science Research Council.
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Chapter I

Review of the Literature on
Morphine Biosynthesis.
Investigations and speculation on the biosynthetic mechanisms for alkaloid formation in plants have been of great interest to organic chemists for the last fifty years. The experience and knowledge gained in the study of plant biosynthesis has contributed a great deal to our understanding of the chemistry in living systems, and has been further extended most recently in the study of the biosynthetic mechanisms of micro-organisms.

One of the first methods used to investigate the biosynthesis of a given alkaloid, was to study its synthesis in vitro by bringing together possible highly reactive precursors under pseudo-physiological conditions. This procedure very often produced the correct alkaloid, but was open to the criticism that the in vitro conditions did not parallel exactly with those found in the plants. With the increased availability and experience of simple organic molecules containing isotopic atoms (normally $^3$H and $^{14}$C both of which have conveniently long half lives) the most significant and rewarding method for the investigation of alkaloid biosynthesis was developed. Here radioactively labelled compounds which, usually from consideration of structural relationships, were thought to be likely precursors of the alkaloid were fed directly to the plant. Isolation of the alkaloid and its radiochemical assay were then used to produce evidence for biosynthetic pathways.

Although the in vitro synthetic experiments were incapable of proving a biosynthetic pathway, or even the involvement of a given compound as a precursor, they have certainly suggested directions for research using the radiotracer techniques.
One example, on which a great deal of work has been carried out, and which is of direct concern here, is the biosynthesis of the morphine alkaloids in the opium poppy, *Papaver somniferum*. A very detailed knowledge of the biosynthetic pathway to morphine has been gained from tracer experiments, and probably more is known than for any other group of alkaloids.

Cultivation of the plant *Papaver somniferum* dates back to the stone ages, and the use of opium, the dried exudate of the unripe seed capsule, as a medicine is mentioned in "Ebers Papyrus" which is thought to have been written in 1550 B.C. Morphine was first isolated from opium as a pure crystalline material in 1805 by Sertürner. Morphine also occurs in the blue poppy *Fructus papaveris*, *Papaver orientale*, and *Papaver spinosum*.

The correct structure for morphine was first postulated by Gulland and Robinson in 1923, and its validity has since been established by two independent syntheses of the molecule, and by X-ray analysis of morphine hydroiodide. The complex structure of morphine (1) raises the interesting problem of how it is constructed in the opium poppy. The problem was first discussed by Gulland and Robinson, who, in 1925, put forward a very important structural proposal. They suggested that if the bonds indicated by x and y in the morphine molecule (1a; R=Me) are broken, and the tetrahydroisoquinoline residue rotated about the axis indicated, the carbon skeleton obtained (2; R=H) resembles that of an unsubstituted 1-benzylisoquinoline system (3; R=R'=H). Robinson further postulated that morphine
was formed in the plant by some sort of oxidative ring closure of the l-benzylisoquinoline system, thus establishing a theoretical relationship between morphine and the benzylisoquinolines. More recently, a further important proposal came from Barton and Cohen\textsuperscript{11} who, on the basis of earlier work with Pummerer’s ketone\textsuperscript{12}, suggested that the C-12-C-13 bond of morphine was formed by phenol oxidative coupling of a suitably protected l-benzylisoquinoline. They suggested that the base reticuline (3a; \( R=\text{Me}, \ R^1=\text{H} \)) underwent a one-electron oxidation to generate a diradical which, by coupling, would yield the dienone (4). The fifth ring could then be formed as indicated and, finally, adjustment of the oxidation level of the base (5) would afford the hydrophenanthrene alkaloids of opium.

\*(Number refers to structures found only in this chapter)

1-Benzylisoquinolines (3) are well known, and several have been isolated from the opium poppy, e.g. reticuline itself (3; \( R=\text{Me}, \ R^1=\text{H} \)). It had been suggested in 1910 by Winterstein and Trier\textsuperscript{13} that 1-benzylisoquinolines, e.g. norlaudanosoline (3; \( R=\text{R}^1=\text{H} \)), were themselves derived by the combination of two \( \text{C}_6-\text{C}_2 \) units (Ar-C-C). The two units they suggested were 3,4-dihydroxyphenylacetaldehyde (6) and 3,4-dihydroxyphenethylamine (7) (dopamine), since a Mannich condensation of the two \textit{in vitro} provided a biogenetically modelled synthesis of the benzyltetrahydroisoquinoline skeleton. Both 3,4-dihydroxyphenylacetaldehyde and 3,4-dihydroxyphenethylamine were thought to be derivable from 3,4-dihydroxyphenylalanine (dopa) (8). Further, it was thought
that dopa arose in plants, as it does in animal tissues, by hydroxylation of the essential amino acid tyrosine (9), which has itself been detected in the opium poppy. Thus a great deal of sound speculation and theoretical knowledge was available, and the problem of morphine biosynthesis was ripe for experimental investigation.

The first experimental results, designed to test the general hypothesis, were reported by Battersby and, independently, by Leete. Both groups of workers fed *Papaver somniferum* plants with DL-\([2-\text{C}^{14}]\text{tyrosine}\) (9), and after about a two week period for metabolism isolated the morphine, which was found to be radioactive. The activity was retained even after rigorous purification, and represented a 0.7 to 1.7% incorporation (Battersby) and 0.01 to 0.02% (Leete) of the precursor into morphine.

According to the original theory the radiocarbon originating from \([2-\text{C}^{14}]\text{tyrosine}\) should reside only at the C-9 and C-16 positions in the morphine molecule (1). The most satisfying chemical degradative work on the radioactive morphine was carried out by Battersby. He showed that approximately half the total activity was at position C-16 while the other half resided at position C-9. The isolation of C-16 was carried out by methylation of the active morphine to give codeine methyl ether methiodide (11). Hofmann elimination cleaved the nitrogen to C-9 bond and the methine was hydrogenated to yield tetrahydrocodeinemethine methyl ether (12). Further Hofmann degredation gave inactive trimethylamine and the morphenol derivative (13) which was
degraded by cleavage of the derived cyclo. Thus formaldehyde was obtained containing C-16 of the morphine. The remainder of the original morphine molecule was isolated as the oxime (14), and the relative activities were determined. Isolation of the carbon atom at position-9 involved conversion of the radioactive morphine into acetylmethylmorphol (15), which was then oxidised with chromium trioxide to the quinone (16). The quinone (16) was oxidised with peracetic acid to give the diphenic acid (17) as the major product. Treatment of the acid (17) with concentrated sulphuric acid gave the coumarin (18). Hydrolysis of the coumarin (18) followed by methylation released the required carboxyl group to give the acid (19). Decarboxylation gave carbon dioxide representing C-9 of the radioactive morphine. The carbon dioxide was collected as barium carbonate and the activity determined. The remaining part of the molecule (20) was found to be radio-inactive. Thus tyrosine had been incorporated into morphine without scrambling of the radiocarbon label.

Both Battersby and Leete also fed DL-[2-14C] phenylalanine (10) and observed incorporation into morphine, but to a much smaller extent than that for DL-[2-14C] tyrosine. This was not thought to be unreasonable since, although phenylalanine is a known precursor for tyrosine, the efficiency of conversion of the former into the latter is known to be low in higher plants.

Parallel experiments in which 3,4-dihydroxyphen[1-14C] ethylamine was fed to the poppy plants were reported by
Battersby and separately by Loeto. Both observed incorporation of radioactivity into morphine, and degradation by the methods already described showed that the radiocarbon resided only at position C-16 of the molecule (1). These results suggest that two different units are used by the plant to build the opium alkaloids, and that one of them is likely to be dopamine (7) obtainable from dopa by decarboxylation. The other C<sub>6</sub>-C<sub>2</sub> unit may be derived from a C<sub>6</sub>-C<sub>3</sub> intermediate (Ar-C-C-CO<sub>2</sub>H). Probably the most favoured candidate being 3,4-dihydroxyphenylpyruvic acid, although proof of this is still lacking. It has been shown recently however that α-keto acids are involved in the biosynthesis of the tetrahydroisoquinoline alkaloids, anhalamine (21) and anhalonidine (22), in peyote cactus (Lophophora williamsii). 3-Hydroxy-4,5-dimethoxyphenethylamine (23), known to serve as an excellent precursor for both (21) and (22), reacts readily with pyruvic acid and glyoxylic acid to give the respective amino acids (24) and (25). Fresh slices of peyote were used to show that the enzymes present were capable of decarboxylating both pycoruvic acid (24) and peyoxylic acid (25). The yields of [1<sup>14</sup>C] carbon dioxide obtained in these experiments, using the racemates of the carboxyl-labelled acids (24) and (25), suggested that decarboxylation was stereo-specific. Further, [1-<sup>14</sup>C]pycoruvic acid and [1,9-<sup>14</sup>C]peyoxylic acid were administered in separate experiments to intact peyote cacti. The anhalonidine (22) isolated from the cacti fed with [1-<sup>14</sup>C]pycoruvic acid was radioactive, and all the activity
was shown to reside at C-1. Similarly, radioactive anhalamine (21) was isolated from the cacti fed with [1,9-\(^{14}\)C]peyoxylc acid, and degradation showed the radio-label was confined to C-1 as expected.

In the experiments already described, the conversion of the labelled precursor into morphine was fairly low (ca. 1%); this is not surprising and certainly did not invalidate the results. It may well be that the precursor is ineffectively absorbed by the plant, or it may be converted into substances which are unrelated to the observed end products. The difference in incorporation of tyrosine into morphine observed by Battersby\(^{16}\) (ca. 1%) and Leete (ca. 0.02%) can be explained by the different feeding methods employed. Leete introduced the precursor via the inorganic nutrient solution in which the poppies were growing, while Battersby utilised the more direct method of injection into the seed capsules. The latter seems the most successful method, and overcomes some of the absorption problems.

Although most of the degradative work described so far was carried out on morphine, the workers also observed conversion of tyrosine into the other two major alkaloids of the opium poppy, codeine (26) and thebaine (27). These two terminal alkaloids contain, respectively, one and two methyl groups more than morphine, and it was not certain which of the alkaloids was the pregenitor of the others in the series. The commonly held concept\(^{24}\) was that O-methylated alkaloids were the metabolic end products; thus the series would be morphine, codeine, thebaine. However, if the original
proposal of Barton and Cohen\textsuperscript{11} was correct the fully methylated alkaloid thebaine should have been the first in the series, followed by codeine and morphine. Initial evidence was provided by rate studies carried out by Battersby\textsuperscript{25} and also Rapoport\textsuperscript{26}. The approach adopted by Battersby was to feed a total of forty five plants with \( \left[ 2^{-14} \text{C} \right] \) tyrosine. Batches of five plants were harvested at given times ranging from 4h. to 14 days, and the total activities of the isolated thebaine, codeine, and morphine determined for each batch. The results showed an initial rapid incorporation of activity into thebaine followed, over a longer period of time, by a rise in the codeine activity. This was followed by a steady fall in the activities of thebaine and codeine relative to a rapid increase in the activity of the morphine. The results suggested that the biosynthetic pathway went from thebaine, through codeine, to morphine the end product of the synthesis. Rapoport on the other hand, exposed mature opium poppies to \( \left[ 14 \text{C} \right] \) carbon dioxide atmosphere for selected periods of time. The thebaine, codeine, and morphine isolated were randomly labelled with carbon-14. Comparison of the total activity present in each of the three alkaloids, for each of the exposure experiments suggested the biosynthetic pathway was thebaine \( \rightarrow \) codeine \( \rightarrow \) morphine. Further work by Rapoport\textsuperscript{27,28} provided the most convincing evidence that the above pathway was correct. Samples of thebaine, codeine, and morphine, generally labelled with carbon-14, were fed separately to plants by root absorption. The plant fed with
thebaine showed incorporation of radioactivity into both codeine and morphine, while the plant fed with codeine showed incorporation of radioactivity only into morphine and not thebaine. Morphine itself was not converted into either of the other two alkaloids. Thus this section of the biosynthetic pathway was shown to involve successive Q-demethylation in going from thebaine to morphine, giving further weight to the proposals of Barton and Cohen.11

The first step29,30,31, to investigate the biosynthetic intermediates between tyrosine (or suitable C6-C2 units) and thebaine, was to feed to the opium poppies (\(\text{[}^\text{14C}\text{]}\)) norlaudanosoline (28\(\text{[}3\text{]}\text{[}^\text{14C}\text{]}\) \(\text{R=H}\)) and (\(\text{[}^\text{14C}\text{]}\)) norlaudanosoline (28\(\text{[}3\text{]}\text{[}^\text{14C}\text{]}\) \(\text{R=H}\)), since it was reasoned that this was the 1-benzylisoquinoline most likely to be formed first12,32 in the plant. Both the labelled 1-benzylisoquinoline precursors were incorporated into morphine with efficiencies of 3.9% and 2.2% respectively. These values were higher than those observed for (\(\text{[}^\text{14C}\text{]}\)) tyrosine fed under similar conditions (\(\text{C.F.}\), 0.66-1.7%). This was in keeping with norlaudanosoline's standing closer than tyrosine to the final alkaloids. The morphine isolated from the plants fed with (\(\text{[}^\text{14C}\text{]}\)) norlaudanosoline was degraded by way of the Q-acetylmethylmorphol (15) already described. This showed, as expected, that the radiocarbon was located only at position C-9 in the morphine molecule (1). From the plants fed with (\(\text{[}^\text{14C}\text{]}\)) norlaudanosoline, the isolated radioactive thebaine (27) was degraded by conversion first into thebenine (29) by treatment with aqueous acid. Complete Q and N-methylation of (29) followed by Hofmann degradation gave the
vinylphenanthrene (30), C-16 of thebaine was hence isolated as formaldehyde containing, as expected, all the radioactivity. Thus, the relatively high incorporation of (+)-norlaudanosoline into thebaine, codeine, and morphine, without randomisation of the labels, made it highly probable that this 1-benzylisoquinoline lay on the direct biosynthetic pathway.

With thebaine firmly identified as the first of the hydrophenanthrene alkaloids to be formed, it was not unreasonable to conclude that norlaudanosoline was suitably methylated before being converted into thebaine. According to previous suggestions 11 reticuline (28; R\textsubscript{1}=H, R\textsubscript{2}=Me) would be the required base. Aqueous solutions of (+)-[\textsuperscript{14}C]nor-reticuline (28; R\textsubscript{1}=Me, R\textsubscript{2}=H) and (\textsuperscript{+})-[\textsuperscript{14}C]reticuline (28; R\textsubscript{1}=H, R\textsubscript{2}=Me) were injected separately into P. somniferum plants, and it was found that these precursors were incorporated efficiently into the hydrophenanthrene alkaloids. Both (\textsuperscript{+})-[\textsuperscript{14}C]reticuline (7.3% incorporation into morphine) and (\textsuperscript{+})-[\textsuperscript{14}C]nor-reticuline (3.6% incorporation into morphine) gave higher incorporation into morphine than did (\textsuperscript{+})-[\textsuperscript{14}C]norlaudanosoline (2.2%), without randomisation of the labels. Independent evidence for the conversion of (\textsuperscript{+})-reticuline into morphine was obtained by Barton and co-workers 33; (\textsuperscript{+})-[\textsuperscript{14}C, N-methyl]reticuline (28; R\textsubscript{1}=H, R\textsubscript{2}=Me) was incorporated into morphine again without randomisation of the labels.

Although it had been shown that reticuline was converted
into the hydrophananthrene alkaloids, it could be argued that
Q-demethylation occurred in the plant to give a simpler
benzylisoquinoline which was then converted to morphine by
some other route. The possibility was rejected by feeding
the plants quintuply labelled (2')-reticuline (28; R = H,
R = R = Me), with 14C in both methoxyl groups, in the
N-methyl group and at carbon C-3, and with tritium at
position C-3. The short term feeding experiment gave radio-
active thebaine, in which it was shown that no
Q-demethylation or N-demethylation had occurred during
biosynthesis.

Further tracer experiments illustrated the
necessity of both the presence and position of the phenolic
hydroxyl groups for the natural metabolism of reticuline.
The necessity for two phenolic hydroxyl groups was shown by
the fact that (2')-[3-14C]tetrahydropapaverine (28; R = R = Me,
R = H) was not incorporated into morphine to any significant
extent.

Reticuline has three structural isomers, the bases
(31), (32), and (33). The positional requirement for the
two phenolic hydroxyl groups was demonstrated by feeding
(2')-[1-3H, 3-14C]protosinomenine (31), (2')-[1-3H]orientaline
(32), and the (2')-[1-3H]benzylisoquinoline (33) to
P. somniferum plants under normal conditions. Only the
incorporation of activity into morphine was investigated,
and was found to be negligible. There is the possibility
that these precursors (31), (32), (33) could be converted
into alkaloids unrelated to morphine. For example
orientaline (32) could be converted into isothbeaine (34) in *P. somniferum*. It is known\(^5^6\) that orientaline is the precursor of isothbeaine, and that thebaine occurs together
with isothbeaine in *Papaver orientale*. It has however been shown that, (\(+\))\(^{14}\text{C}\)reticuline (28; \(R^1=H\), \(R=R^2=Me\)) was incorporated\(^37\) into thebaine in *P. orientale*, but the isothbeaine isolated was radioactive. In parallel experiments it was shown that (\(+\))\(^{14}\text{C}\)orientaline (32) was converted\(^37\) by *P. orientale* into isothbeaine but not into thebaine. Reticuline seems to be the only \(N\)-methylated, diphenolic benzylisoquinoline precursor of morphine in *P. somniferum*, also the enzyme systems operating in *P. somniferum* and *P. orientale* are highly specific in their action.

Although norlaudanosoline serves\(^2^9\) as a good precursor for morphine, it may not necessarily be one of the natural biosynthetic intermediates. Methylation to give the required reticuline may occur prior to isoquinoline formation. This possibility seems unlikely, since Francis\(^2^8\) observed that 4-hydroxy-3-methoxyphen[1\(^-{14}\text{C}\)]ethylanine (35) was not incorporated into morphine. Thus here again the enzyme systems carrying out the chemical changes are behaving in a very specific manner. No investigation of conversion of 4-hydroxy-3-methoxyphen[1\(^-{14}\text{C}\)]ethylanine (35) into the base (36) was made.

The work with benzylisoquinoline described so far was carried out using racemates. However, it would be expected that the enzymes carrying out the particular
chemical changes would be stereospecific, and that only one of the two optical isomers would act as a direct substrate. The use of resolved quadruply labelled precursors, carrying tritium at the asymmetric centre, was very informative about the detailed role of reticuline in the biosynthesis of the morphine alkaloids.

S-(+)-Reticuline \((37; R^1=H, R=R^2=Me)\) was first isolated from *Anona reticulata* and has the illustrated absolute configuration. The absolute configuration of the morphine alkaloids \((1, 26, 27)\) is well established, and it is R-(-)-reticuline \((38; R^1=H, R=R^2=Me)\) which should be the precursor for thebaine \((27)\), codeine \((26)\), and morphine \((1)\).

Separate, singly-labelled samples of (+)-reticuline \((37; R^1=H, R=R^2=Me)\) and (-)-reticuline \((38; R^1=H, R=R^2=Me)\) were prepared. Appropriate quantities of each were mixed to give effectively quadruply labelled precursors with \(^{14}\text{C}\) in the skeleton at C-3, in the 4-methoxyl group, and in the \(N\)-methyl group, and with tritium at C-1. The quadruply labelled precursors, (+)-reticuline and (-)-reticuline were fed by injection to separate *P. somniferum* plants. Good incorporation of (-)-reticuline into the hydrophenanthrene alkaloids was observed; but degradation of the isolated thebaine showed that there was only a 30-40% retention of tritium activity. Surprisingly there was also good incorporation of (+)-reticuline into the hydrophenanthrene alkaloids, but only a 13-18% retention of tritium activity was observed. The incorporation of (+)-reticuline, together with the loss of tritium activity from both the (+)- and (-)-isomers could
only be rationalised by conversion via the intermediate 1,2-dehydroreticuline (39; R\(^1\)=H, R\(^2\)=Me). This was shown to be the case\(^\text{35}\), since when \([3-\text{\(^{14}\)C}]1,2\text{-dehydroreticuline}\) was fed to the plants, the highest ever incorporation of a benzylisoquinoline into morphine (10.5%) was observed.

\[ R-(+)-\text{norlaudanosoline} \quad (38; \quad R=R^1=R^2=H) \]

corresponds in absolute configuration with morphine (1). However

\[ S-(-)-[3-\text{\(^{14}\)C}]\text{norlaudanosoline} \quad (37; \quad R=R^1=R^2=H) \]

was incorporated (6.7%) more efficiently into morphine than was the enantiomer \[ R-(+)-[3-\text{\(^{14}\)C}]\text{norlaudanosoline} \quad (38; \quad R=R^1=R^2=H) \]

(0.45%), therefore suggesting that there was not ready interconversion of the enantiomers by oxidation-reduction at this point in the biosynthetic pathway. This was supported by feeding\(^\text{38}\) \([3-\text{\(^{14}\)C}]1,2\text{-dehydronorlaudanosoline}\)

\[ (39; \quad R=R^1=R^2=H) \]

which showed very poor incorporation into morphine (0.07%). Nevertheless the morphine isolated from plants fed with \([1-\text{\(^{3}\)H}, 3-\text{\(^{14}\)C}]\text{norlaudanosoline} \quad (28; \quad R=R^1=R^2=H) \)

contained less than 10% of the original tritium present, and this loss must therefore occur at a later stage in the biosynthesis. It was also shown\(^\text{38}\) that \([3-\text{\(^{14}\)C}]1,2\text{-dehydro nor-\text{reticuline}}\)

\[ (39; \quad R^1=R^2=H, \quad R=\text{Me}) \]

was incorporated significantly, presumably through conversion either into 1,2-dehydroreticuline (39; R\(^1\)=H, R\(^2\)=Me) by N-methylation, or into nor-\text{reticuline} (28; R\(^1\)=R\(^2\)=H, R=Me) by reduction.

\text{Nor-reticuline} had been shown previously\(^\text{31}\) to act as a precursor for morphine, and this was confirmed by feeding \([1-\text{\(^{3}\)H}, 3-\text{\(^{14}\)C}]\text{nor-reticuline} \quad (28; \quad R^1=R^2=H, \quad R=\text{Me}) \) to the plants. Incorporation similar to that of before was observed,
SCHEME 1.

SCHEME 2.
and 82% of the tritium present at the asymmetric centre was lost during biosynthesis. In similar experiments

\[ \text{(+)}-[1-^3\text{H}, 3-^{14}\text{C}, \text{N-methyl}^{14}\text{C}]\text{laudanosoline (28; } R=R^1=\text{H}, R^2=\text{Me}) \], another possible intermediate between

S-(-)-norlaudanosoline and R-(-)-reticuline, showed good incorporation into morphine with 88% loss of tritium from position C-1. It appears that, in the conversion of S-(-)-norlaudanosoline into R-(-)-reticuline, necessary for the formation of morphine, the enzyme system is not as highly specific as had been observed at other stages in the biosynthesis. For example, R-(+)-norlaudanosoline showed a minimum incorporation into morphine, as was the case with 4-hydroxy-3-methoxyphenethylamine and the structural isomers of reticuline, (31), (32), and (33). So long as methylation takes place at the correct positions, and occurs after formation of the initial benzylisoquinoline, it does not appear to make any difference whether N-methylation precedes or follows O-methylation.

The loss of tritium from the asymmetric centre of the precursors nor-reticuline and laudanosoline, could be explained by an oxidation-reduction process on nor-reticuline (as Scheme 1) and on laudanosoline (Scheme 2). On the other hand both precursors could first be methylated to give reticuline, followed by the production of R-(-)-reticuline, with loss of tritium from C-1, via 1,2-dehydroreticuline. The last proposal seems the most likely, since the opium poppy has been shown to contain approximately 60% of S-(+)-reticuline and 40% of R-(-)-reticuline \(^{41}\). Separate experiments using
radiodilution analysis was in agreement, and showed that the S-(+)-form predominated. The use of resolved laudanosoline and nor-reticuline as precursors is likely to show more precisely the exact nature of the partially methylated intermediates in the natural system. The oxidation-reduction equilibrium which appears to exist between S(+) and R(-)-reticuline and 1,2-dehydroreticuline, could in theory involve the enamine (40) as a fourth partner. This was shown not to be the case by feeding both (+)- and (-)-[9-3H, 3-14C]reticuline. It might be interesting to see if the enamine (40) is itself metabolised by the plant to give morphine, since it differs only slightly from reticuline, but is methylated at all the correct positions.

Further evidence for the involvement of reticuline was provided by Rapoport on the basis of [14C]carbon dioxide exposure experiments. Comparison of the relative specific activities of the isolated reticuline and thebaine gave additional support for reticuline being the required benzylisoquinoline precursor of the hydrophenanthrene alkaloids.

As outlined earlier, the original proposals considered that R-(-)reticuline (38; R'H, R=R'Me) underwent phenol oxidative coupling via the resonance stabilised diradical (41), to give the then unknown dienone (4), which, after isomerisation to (5), was reduced and dehydrated to yield thebaine (27). The dienone (42; R=H) was synthesised by reduction of thebaine (27), using sodium in liquid ammonia to give dihydrothebaine (43). The phenol (43) was acetylated, and oxidised to give (42; R=OAc), which gave the required
dienone (42; R=H) on mild alkaline hydrolysis. A dienone alkaloid subsequently isolated from Croton salutaris, and called salutaridine, was found to be identical with (42; R=H). Using synthetic 9-R(+)-salutaridine (42; R=H) as a carrier alkaloid for in vitro experiments it was possible to show that tritium labelled (β)-reticuline and R-(-)-reticuline, but not S-(+)-reticuline, were converted into the dienone33,45 (42; R=H). The reaction conditions were those known to promote phenol oxidative coupling. The next stage in the research was to test salutaridine (42; R=H) as a biosynthetic intermediate for the hydrophenanthrene alkaloids. This was carried out as a collaborative effort by groups at Imperial College and the University of Liverpool.

\[ \text{[1-}^3\text{H]salutaridine and [16-}^{14}\text{C]salutaridine (both prepared from thebaia) were fed separately to } \text{P. somniferum plants. Very efficient incorporation into thebaia, codeine, and morphine was observed, the total incorporation values being as high as 15%. The thebaia isolated from the plants fed with [1-}^3\text{H]salutaridine was converted, after dilution with inactive material, into salutaridine (42; R=H).} \]

Bromination of (42; R=H) at the C-1 position gave inactive 1-bromosalutaridiné, showing that no scrambling of the radiolabel had occurred during biosynthesis. Thus 9-R-(+)salutaridine was established as the first biosynthetic intermediate to be formed after the oxidative coupling of R-(-)-reticuline. Moreover, by feeding \text{P. somniferum plants (β)-[3-}^{14}\text{C]norlaudanosol}
(28; \(R=\text{R}^1=\text{R}^2=\text{H}\)) and also (2)-[2-\(^{14}\text{C}\)]tyrosine, and diluting the extracted alkaloids with radioinactive salutaridine (42; \(R=\text{H}\)), it was possible to demonstrate that radioactive salutaridine had been formed. This overcame any objections that salutaridine was not a natural alkaloid of the opium poppy.

It was found that salutaridine (4) did not ring close under acidic or basic conditions to give the enone (5). It was reasoned that the open form (4) was thermodynamically the more stable, suggesting that in vivo closure of the benzofuran ring, to give thebaine, required a separate biochemical step. Modification of the originally proposed biosynthetic pathway was necessary, and subsequent suggestions \(^{46,47}\) involved reduction of salutaridine to the dienol (44), which could then undergo cyclisation as indicated to give thebaine. Chemical support for this modified route was provided by in vitro studies. Reduction of salutaridine with sodium borohydride gave two epimeric alcohols \(^{33,34}\) called salutaridinols I and II (44), differing only in the stereochemistry of the alcoholic group at C-7. Both these dienols gave thebaine on very mild treatment at pH3-4. In the work carried out at Imperial College samples of \([1,7-\text{H}]\)salutaridinols I and II were fed separately to poppies, while the Liverpool University group prepared and fed \([3-\text{H}, 16-\text{C}]\)salutaridinols I and II. Both groups found that salutaridinol-I was incorporated far more efficiently into thebaine (10 to 30 times) than was salutaridinol-II, and that there was no loss of tritium
from position C-7. The retention of tritium at position C-7 showed that salutaridinol-I was metabolised directly to give thebaine without prior oxidation to salutaridine \((42; R=H)\). Salutaridinol-I was also incorporated very efficiently into codeine and morphine, however, significant tritium loss from C-7 occurred during the conversion of thebaine to codeine.

Thus the results support the biosynthetic pathway:

Salutaridine \((42; R=H)\) $\rightarrow$ salutaridinol-I \((45)\) $\rightarrow$

thebaine etc., and high enzyme specificity, in the metabolism of only salutaridinol-I, was again demonstrated.

It was later shown\(^4\)\(^5\) that salutaridinol-I has the configuration indicated by the partial structure \((45)\), the alcohol group being on the same side of the molecule as the nitrogen bridge. This was thought to be surprising, since, if the cyclisation indicated by \((46)\) involving a one step \(\text{SN}_2\) displacement occurs, then it would be salutaridinol-II \((46)\) which would have the correct configuration. It was reasoned that an additional step was involved in the biochemical process. Two possibilities were put forward, although no experimental evidence for these has been reported. The first involves \(\text{SN}_2\) displacement of the hydroxyl (or a phosphate ester) at C-7 in salutaridinol-I \((45)\) by an enzyme functional group, giving an enzyme bound intermediate with the inverted configuration necessary for an \(\text{SN}_2\) ring closure. The second possibility requires a preliminary allylic rearrangement to give the isomer \((47)\), which then has the correct configuration for direct \(\text{SN}_2\) displacement at position C-5.

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We may now consider the last stage of the biosynthesis, namely, the conversion of thebaine (27) into codeine (26). The formation of codeine from thebaine must involve at least one intermediate, since both reduction and demethylation are occurring. The nature of this intermediate will depend on which of these two processes occurs first. The most favoured mechanism postulated\textsuperscript{11,34,46} that demethylation occurred first, to give neopinone (48) thence codeinone (49) and codeine. This mechanism might explain the loss of tritium from C-7 which occurred during conversion of thebaine into codeine during the salutaridinol experiments\textsuperscript{34}. Further, it had been shown that sodium borohydride reduction of codeinone gave codeine as the only product.\textsuperscript{48} The second postulated\textsuperscript{49} mechanism involved reduction of thebaine as the first step to give codeine methyl ether (50), followed by demethylation to yield codeine. Some evidence for this mechanism was provided by Brockmann-Hanssen\textsuperscript{50}, who showed that codeine methyl ether was present in the mother liquors produced during the extraction and purification of morphine from opium. The possibility that codeine methyl ether was formed as an artifact in the mother liquors, perhaps by reduction of thebaine, was eliminated by extraction of a sample of opium powder. The presence of codeine methyl ether was then indicated by gas and thin layer chromatography. This last mechanism could also account for the loss of tritium from C-7 of thebaine during conversion to codeine by postulating the further intermediate neopine methyl ether (51).
In order to gain evidence for the existence of either of these two intermediates (49) and (50), Rapoport\textsuperscript{51} carried out two series of experiments. In the first, 60 day old \textit{P. somniferum} plants were exposed (for 4h) to an atmosphere of $^{14}$C carbon dioxide. The non-phenolic alkaloids were isolated and the specific activities determined using a gas chromatograph-flow counter system. Codeine methyl ether could not be detected by either mass or radioactivity. However codeinone was detected and its specific activity lay between those of the isolated thebaine and codeine.

In the second series of experiments codeine methyl ether (50) and codeinone (49) which were generally labelled with $^{14}$C were fed separately to the plants. The phenolic and non-phenolic alkaloids were isolated, and this time radioinactive codeine and morphine were added to act as carriers. The specific activities were determined by scintillation counting and showed that codeine methyl ether had been converted into codeine to the extent of 4.7% and into morphine (3%), but that 51.5% was isolated as unmetabolised codeine methyl ether. In the codeinone feeding experiment, an incorporation into codeine of 14.5% and morphine of 2.8% was observed, and 14% of the codeinone was recovered. On the basis of the $^{14}$CO\textsubscript{2} exposure experiments, and the high conversion of codeinone into codeine, Rapoport suggested that codeinone is the required intermediate in the conversion of thebaine into codeine. The details shown by Rapoport indicate however, that codeine methyl ether is incorporated as efficiently into morphine as is codeinone. Further evidence for codeinone
being the required intermediate was provided by Battersby\textsuperscript{52,53}. \( [2-^3\text{H}] \)Codeinone was fed to \textit{P. somniferum} and showed good incorporation into morphine (18.4\%) and codeine (4.8\%). Battersby\textsuperscript{53} also prepared \( [2-^3\text{H}] \)codeine and \( [6-^3\text{H}] \)codeine, the two were mixed to give effectively \( [2,6-^3\text{H}] \)codeine in which the activity ratio of the labels was known. \( [2,6-^3\text{H}] \)Codeine was fed to the plants and the morphine and codeine isolated. The tritium at C-2 in the morphine was removed by exchange and the C-2 to C-6 tritium ratio calculated. The codeine was oxidised to codeinone and the ratio determined. It was found that the tritium ratios in the isolated alkaloids did not differ significantly from those in the precursor. Thus no codeine to codeinone conversion occurs prior to the formation of morphine.

Rapoport's\textsuperscript{51} conversion (4.7\%) of codeine methyl ether into codeine in \textit{P. somniferum} was confirmed independently by Brockmann-Hanssen\textsuperscript{49}. \( [3\text{-methoxy-}^{14}\text{C}] \)Codeine methyl ether was prepared, and fed to five opium poppies. The non-phenolic alkaloids were isolated and the codeine purified by chromatography, and crystallised to constant specific activity. The incorporation of codeine methyl ether into codeine was 2.5\%, and Zeisel Q-demethylation of the codeine showed that all the activity resided in the Q-methyl group. It was thought that too large an amount of precursor had been used for too few plants (56mg, 0.24lmCi for 5 plants), and this had resulted in a swamping of the biosynthetic pool. A second feeding experiment was carried out (using 5mg of precursor and 10
plants), and this resulted in a codeine methyl ether to
codeine conversion of 15.8%. The conclusion that the
authors drew from these results was that the opium poppy
possesses the ability to demethylate codeine methyl ether
to give codeine, but that this does not prove that the ether
is a natural intermediate formed during normal biosynthesis.
The workers failed to prove the presence of codeine methyl
ether in _P. somniferum_, by feeding (±)-\[^{13}\text{C}\]reticuline and
using radioactive codeine methyl ether as a carrier. This
part of the biosynthesis is still open to question, and will
be discussed later in connection with our own work.
However it does seem that here is another example where the
enzyme system, involved in demethylation, is not all that
specific.

Thus a great deal is understood about the way in
which morphine is built up in the plant. But the mechanism of
formation of the initial benzylisoquinoline, and of the
thebaine to codeine conversion is still open to some doubt.
One point is fairly certain however, that the enzyme
systems carrying out the various chemical changes, with only
two exceptions, are highly specific for the formation of
morphine.
Chapter II.

Metabolism of Unnatural Precursors

in Papaver somniferum.
Introduction

Ideally, in the study of any biosynthetic pathway only the natural precursor should be incorporated efficiently into the natural product. The general case is illustrated below in Scheme A in which the natural precursor (X) is converted into the natural product (Y).

\[ \begin{align*}
X & \rightarrow Y \\
X' & \rightarrow Y'
\end{align*} \]

Scheme A

However, examples have been reported\textsuperscript{50,51,54} of the incorporation of an unnatural precursor into a natural product, represented generally in Scheme A by an (X') to (Y) conversion. Two examples will serve to illustrate this latter type of incorporation. The first has already been discussed in some detail and is associated with morphine biosynthesis. Codeine methyl ether (6; \( R_1 = R_2 = \text{Me} \)) (complete proof for its natural occurrence in the plant is still lacking), represented in Scheme A by (X'), is converted by \textit{P.somniferum} into the natural alkaloid (Y) morphine (6; \( R_1 = R_2 = \text{H} \)). The second example was described by Leets\textsuperscript{54} in connection with the biosynthesis of Nicotine. Here the unnatural precursor [\( 6' - \text{N-methyl,2-}^{14}\text{C} \)ornithine (1)\textsuperscript{6} (X'), when fed to \textit{Nicotiana tabacum} plants, was incorporated into the natural alkaloid nicotine (5; \( R=\text{H} \))-(Y). Degradation showed that only the N-methyl and 2' carbon atoms of the nicotine (5; \( R=\text{H} \)) were radioactive.

\[ \begin{align*}
\text{Codeine methyl ether (6; } R_1 = R_2 = \text{Me} \text{)} & \rightarrow \text{Morphine (6; } R_1 = R_2 = \text{H} \) \\
\text{Nicotine (5; } R=\text{H} \) & \rightarrow \\
\text{N-methyl,2-}^{14}\text{Cornithine (1)\textsuperscript{6}} & \rightarrow \text{Nicotine (5; } R=\text{H} \)
\end{align*} \]

* (Number refers to structures found only in this chapter)
However, when the natural precursor $[^2\text{-}^{14}\text{C}]$ornithine (2) is fed to the plants, both the 2' and 5' carbon atoms of the isolated nicotine (5; R=H) are radiolabelled. The latter process is represented by an (X) to (Y) conversion in Scheme A. The authors suggested the plant contained nonselective enzymes which were capable of catalysing the decarboxylation of $\delta$-N-methylornithine (1) to give N-methylputrescine (3) which was utilised by way of the N-methyl-$\delta^1$-pyrrolinium salt (4; R=H) for forming the pyrrolidine ring of the naturally occurring nicotine.

We were interested in extending the general concepts of the above fields of research. Our aim was to investigate the possibilities of an unnatural precursor being converted, by a selected natural plant system, into an unnatural product. That is, to observe an $X'\rightarrow Y'$ conversion, where both the unnatural precursor and product are fairly closely related to their natural counterparts. Thus, in this theoretically possible conversion, the structural relationship between the unnatural product ($Y'$) and the natural product ($Y$) will be exactly the same as that between the unnatural precursor ($X'$) and the natural precursor ($X$).

At the start of our work this type of ($X'$) to ($Y'$) conversion had not been observed in any plant system. However, one example was recently reported by Rapoport, and again was connected with the biosynthesis of nicotine. It had been established previously that 1-methyl-1-pyrrolinium chloride (4; R=H, $\text{Hal}^-=\text{Cl}^-$)→(X), was an efficient precursor for the pyrrolidine ring of nicotine (5; R=H)→(Y). Rapoport showed
that the unnatural precursor $[\text{I}^\text{14C}]$-1,3-dimethyl-l-pyrrolinium chloride (4; R=CH$_3$, Hal$^-_=Cl^-$)-(X'), was incorporated by N. glutinosa tobacco plants into $[\text{I}^\text{14C}]$methylnicotine (5; R=CH$_3$)-(Y'). Two feeding experiments were carried out and the incorporation into 3'-methylnicotine, i.e. the (X')to (Y') conversion value, ranged from 5-11%. Further, the 2' carbon atom of 3'-methylnicotine was assigned an (S) configuration, the same absolute configuration as the 2' carbon atom of the natural nicotine (5; R=H). In this example, only conversion of 1,3-dimethyl-l-pyrrolinium chloride into 3'-methylnicotine (i.e. the X' to Y' conversion) was tested. No direct comparison was made with the natural conversion of 1-methyl-l-pyrrolinium chloride into nicotine (i.e. the X to Y conversion) under the same conditions of feeding.

We considered it important not only to determine if any (X') to (Y') conversion occurred, but if it did, to gain some measure of its efficiency compared with that of the natural (X) to (Y) process. Our approach for this type of study was a little more sophisticated than that adopted by Rapoport, and again is best described in general terms by reference to Scheme A. If the plant system to be studied were fed with a mixture of the unnatural precursor (X') labelled with tritium, and the natural precursor (X) labelled with $^{14}$C, it would then be possible to observe any (X') to (Y') conversion and to compare it directly with the natural (X) to (Y) conversion for a given feeding experiment. The natural precursor therefore would act as an internal standard for a particular experiment.
Similarly it would be possible to measure the extent of the other conversions which might occur, i.e. all those implied by Scheme A.

This then was the reasoning behind our approach which, given that the normal biosynthetic intermediates have been determined, can be applied in principle to the study of the metabolism of a suitable unnatural precursor by any higher plant. We chose to study the application of these ideas to the demethylation of codeine (6; $R_1$=Me, $R_2$=H)-(X) to give morphine (6; $R_1$=H-$R_2$=H)-(Y) in the opium poppy, *P. somniferum*. The reasons for this selection were three-fold. Firstly, the process is fairly simple and believed to involve only one step. The product, morphine is thought to be the final alkaloid (but see below) in the biosynthetic sequence and is not readily metabolised, during the proposed time scale of our experiments, to give other end products. Secondly, the *P. somniferum* plants are fairly easy to grow, and also a great deal is known about the technique of feeding precursors to them. Thirdly, there is some indication that the enzymes involved in the conversion of codeine into morphine are not highly specific and therefore it seemed reasonable that the type of incorporation which we sought might occur. Further, it was hoped by undertaking this study to gain insight into the way in which the enzyme system carries out the demethylation process.

One further point must be made. Some evidence is available to suggest that morphine is metabolised under natural conditions to give other products. Fairbairn and his co-workers found
that $[2-^3H]m$orphine ($6; R_1=R_2=H$) gave other radioactive products when treated with poppy latex over a period of eleven days. The products were phenolic and "morphine like", but no structural determination was carried out. Fairbairn had shown previously\textsuperscript{59} that poppy latex, isolated from the seed capsules of \textit{P.somniferum}, is capable of incorporating precursors into the morphine alkaloids in the same way as the plant itself.

Having outlined the problem we can now discuss in more detail the experimental approach. To define more exactly the mode of action of the demethylating enzyme responsible for the conversion of codeine into morphine in \textit{P.somniferum}, we considered it necessary to investigate all the possible conversions implied by Scheme A. To cover adequately these possibilities it was decided to carry out two different types of feeding experiment. The first type will be described as a double-label feeding experiment although, in the strict sense this is not so. Here, each of the unnatural modified codeine precursors, labelled with tritium, were separately mixed with [N-methyl-$^{14}$C]codeine ($6; R_1=\text{Me}, R_2=H$), which was selected to serve as the internal standard for each feeding. Each of the mixed precursors was then fed separately to the plants. Thus, the conversion of the unnatural precursor ($X'$) into ($Y'$) or into codeine and morphine, was observed by the incorporation of tritium activity. Further, the concurrent metabolism of codeine was followed by incorporation of $^{14}$C activity into morphine, ($X'$), or ($Y'$).

It seemed likely that, if any of the unnatural precursor ($X'$) was converted into codeine and morphine, the incorporation
value would be fairly low compared with the incorporation of [N-methyl-$^{14}$C]codeine into morphine. Thus for a double-label feeding the small amount of tritium activity, representing the conversion of unnatural precursor (X') into codeine and morphine, would be swamped by the $^{14}$C activity present derived from the internal standard [N-methyl-$^{14}$C]codeine. To overcome this possible problem, a second type of feeding experiment was carried out involving the unnatural precursor (X'), labelled with tritium, alone.

The selection of suitable unnatural precursors can now be discussed.

**Selection of Unnatural Precursors for Feeding Experiments:**

The selection of unnatural precursors (X') for feeding experiments was of necessity fairly arbitrary since we had initially no idea which modified codeine compounds (X'), if any, would show incorporation of the type we were seeking, i.e. (X') to (Y'), (X') to (X) to (Y), and (X') to (Y') to (Y). We expected various functional groups in the codeine molecule (6; $R_1=\text{Me, } R_2=$) would be involved in binding with the enzyme during demethylation to morphine. We could only guess which would be important and our selection of unnatural precursors was aimed primarily at defining the possible binding sites associated with the C-ring (Fig.1.). Thus, most of the unnatural precursors involved modifications to the C-ring of codeine although all were fairly closely related to codeine itself. By reference to the type of modification involved the precursors may be placed into three different groups as follows:

(a) Modification to the position of the oxygen atom in the
secondary alcohol group at position C-6 in the C-ring of codeine. The shape of the codeine molecule is illustrated in Fig.1, while a more detailed representation of the configuration of the C-ring is shown in Fig.2. The secondary alcohol function of codeine is on the opposite side of the molecule to the C-15 - C-16 nitrogen bridge, and occupies the 6α-position in Fig.2. The exact position of the oxygen in the codeine molecule may be important during demethylation in the plant. Thus isocodeine (8; R₁=Me, R₂=H) the 6β-epimer of codeine would be a suitable unnatural precursor. Similarly, hydrogenation of the 7,8-octofinic double bond of both codeine and isocodeine will alter the shape of the C-ring from half-boat to chair, and hence the position of the oxygen function. In Fig.2, dihydrocodeine (7; R₁=Me, R₂=H) has the oxygen function at the 6α-position, and dihydroisocodeine (9; R₁=Me, R₂=H) at the 6β-position. The compounds selected as unnatural precursors in this group were :- dihydrocodeine (7; R₁=Me, R₂=H), isocodeine (8; R₁=Me, R₂=H), isocodeine methyl ether (8; R₁=R₂=Me), dihydroisocodeine (9; R₁=Me, R₂=H), and dihydroisocodeine methyl ether (9; R₁=R₂=Me).

b) Modification of the functional group attached to the C-6 position of codeine (6; R₁=Me, R₂=H). The presence of a hydroxyl group at C-6 may be important in binding the molecule (6; R₁=Me, R₂=H) to the enzyme surface during demethylation. Codeine methyl ether (6; R₁=R₂=Me) and dihydrodesoxycodine (10; R = Me) were selected to test this effect. Dihydrodesoxycodine (10; R = Me) possesses a dual advantage as an unnatural precursor.
Diagram of molecular structures labeled (6) to (11).
Not only is there no oxygen containing functional group at position C-6 but also the absence of the 7,8-olefinic double bond ensures that there are no likely centres in the C-ring for co-ordination to the enzyme.

(c) Modification by attachment of a large functional group to the aromatic ring of codeine. The compound selected was 1-bromocodeine (11; R = Me); the reasoning behind this choice will be outlined later during discussion of this feeding experiment.

During work-up of the feeding experiments inactive samples of the four associated alkaloids were used as carriers for dilution analysis. Thus a further consideration in selecting unnatural precursors (X') was that the morphine analogue of the modified codeine precursor should be a known compound and fairly easily prepared on a small scale.

Labelling of Unnatural Precursors.-

All the unnatural precursors were labelled with tritium at position C-2 (6). The starting material was [2-3H]morphine obtained, as described by Kirby and Ogunkoya,\(^6\) by the base-catalysed nuclear exchange of morphine (6; R\(_1\)=R\(_2\)=H) with tritiated water in dimethylformamide. Other workers have shown beyond doubt that the tritiated morphine produced in this way is labelled only at the C-2 position. The stability of the tritium label in [2-3H]morphine, under most of the conditions that it was likely to encounter in our hands, has been established by Battersby.\(^5\)\(^3\)\(^5\) Moreover, it has been demonstrated\(^6\)\(^1\),\(^6\)\(^2\) that monohydric phenols and catechol derivatives do not exchange
ortho and para-tritium under biological conditions similar to those present in P.somniferum. Thus tritium is unlikely to be lost by exchange from the phenolic alkaloids during biosynthesis or isolation.

To establish exactly the required conditions, the exchange was studied again\(^5^3,^5^8\) by n.m.r. spectroscopy using deuterium oxide. From these experiments the best results were obtained by carrying out the exchange in a sealed tube at 100°C for 100 hr. Morphine was isolated as a nearly pure white material only when very rigorous precautions were taken to flush the exchange tube free from oxygen and carbon dioxide prior to sealing.

The exchange was also studied using potassium \(\text{t-butoxide}\) and it was found necessary to use 2 moles of this base to dissolve the morphine in deuterium oxide. Observation using n.m.r. spectroscopy showed the exchange did not produce significant amounts of \([2^-\text{H}]\text{morphine}\). This was in contrast to the results reported by Steinreich,\(^6^3\) but in agreement with the observations of Ingold,\(^6^4\) Raisin, and Wilson. The latter workers had found that the maximum rate of exchange occurred when equal concentrations of phenoxide and undissociated phenol (PhOD) were present.

The labelled morphine was methylated using trimethylanilinium ethoxide\(^6^5\) at 115-120°C to yield \([2^-\text{H}]\text{codeine}\), the starting material for the synthesis of all the unnatural precursors. The trimethylanilinium cation was obtained by the exothermic reaction between methyl toluene-\(p\)-sulphonate and dimethylaniline, to give trimethylanilinium toluene-\(p\)-sulphonate which, on treatment with sodium ethoxide under anhydrous conditions, gave
trimethylanilinium ethoxide, the required methylating reagent. The methylation reaction proceeded well in about 60-70%, and the $[2^{-3}\text{H}]$codeine had essentially the same molar specific activity as the original labelled morphine.

**Synthesis of Unnatural Precursors and their Morphine Analogues.**

In preparing the unnatural precursors we required methods which, firstly gave a high yield of the required product, and secondly would not involve loss of tritium from the codeine molecule. Some of the literature methods were inefficient, and it was therefore necessary, in certain instances, to develop new preparations. Exact conditions required for the preparation of all the unnatural precursors were established using radio-inactive starting materials. In general, the labelled, unnatural precursors were prepared by the same route as their radioinactive counterparts, which were required for radiodilution during feeding work-up. Thus for simplicity only the preparations of the radiolabelled unnatural precursors will be discussed here, except in those cases where a different method was used for the preparation of radioinactive material. Full details of the preparation for both radiolabelled and radioinactive modified codeine compounds appear in the experimental section. The preparation of the morphine analogues, also required for radio dilution, is described in sequence with the corresponding unnatural codeine compound.

$[2^{-3}\text{H}]$Dihydrocodeine (7; $R_1=\text{Me}, R_2=\text{H}$) was prepared $66,67,68$ by hydrogenation of $[2^{-3}\text{H}]$codeine in methanol solution, using palladised charcoal as catalyst.
Preparation of the labelled precursor was carried out on a small scale (ca. 30 mg.) and even after purification via the picrate it was impossible to obtain the material crystalline. However in the preparation of radioactive dihydrocodeine, carried out on a larger scale (ca. 200 mg.), the material was obtained crystalline but only after considerable difficulty. The difficulty encountered in crystallising this compound was most likely due to the presence of a small amount of unreduced codeine which could only be removed by repeated crystallisation of the picrate, followed by regeneration of the free base. The free base was regenerated by pouring a chloroform solution of the picrate salt down a column of neutral grade III alumina.

Similarly, dihydromorphine (7; \(R_1=R_2=\text{H}\)) was prepared by hydrogenation in ethanol solution. The product was readily crystallised, but the reaction consistently produced low yields. It could be that the phenolic alkaloid was adsorbed on the charcoal catalyst. Use of glacial acetic acid as the solvent for hydrogenation failed to produce an increase in the recovery of product.

\(\left[2-^3\text{H}\right]\text{Isocodeine} (8; R_1=\text{Me}, R_2=\text{H}).-\) Originally iso-codeine was prepared by hydrolysis of \(\alpha\)-chlorocodide (12; \(X=\text{Cl}\)) or the \(\beta\)-halogenocodides (14; \(X=\text{Cl, Br, or I}\)). The reaction mixtures resulting from such hydrolysés contained pseudocodeine (14; \(X=\text{OH}\)), allopseudocodeine (15; \(X=\text{OH}\)), in addition to isocodeine which was normally obtained, in very low yield (ca. 6-8%), by fractional crystallisation of suitable derivatives and salts. For the preparation of the labelled precursor it
was necessary to devise a more efficient method. Initially, the method developed involved treatment of \( [2^{-3}\text{H}] \text{codeine toluene-}p\text{-sulphonate} \) \((13; X=\text{TsO})\) with hexadecyltrimethylammonium acetate, prepared from the corresponding bromide and silver acetate, in benzene solution at room temperature. It was expected that, under these non-solvolytic conditions, a clean \( \text{SN}_2 \) attack of acetate at position C-6 would occur, since it is known that codeine toluene-\( p \)-sulphonate reacts with chloride ion to give \( \alpha \)-chlorocodide \((12; X=\text{Cl})\). Indeed, \([2^{-3}\text{H}] \text{isocodeine acetate} \) \((12; X=\text{OAc})\) was isolated, and none of the \( \text{SN}_2\)' product acetyl-allopseudocodeine \((15; X=\text{OAc})\) was detected. The isolated acetate was hydrolysed using aqueous methanolic sodium hydroxide to give \([2^{-3}\text{H}] \text{isocodeine} \) in 40-50\% yield, based on \([2^{-3}\text{H}] \text{codeine} \). It was very important, when preparing \([2^{-3}\text{H}] \text{codeine toluene-}p\text{-sulphonate} \), by treatment of \([2^{-3}\text{H}] \text{codeine} \) with toluene-\( p \)-sulphonyl chloride in pyridine, to carry out the reaction at 0\( ^\circ \)C. The preparation outlined by Karrer and Widmark \(^{72}\) was inadequate and gave predominantly \( \alpha \)-chlorocodide \((12; X=\text{Cl})\). Presumably, \( \text{SN}_2 \) displacement of the tosylate by chloride ion occurs at temperatures higher than ca. 5\( ^\circ \)C.

We hoped originally, by choosing hexadecyltrimethylammonium acetate as the source of acetate, to promote \( \text{SN}_2 \) displacement, and that the hexadecyltrimethylammonium cation would be precipitated from the benzene solution as the tosylate salt, thereby facilitating the work-up procedure. However, isolation of the required product from the reaction mixture was made extremely tedious by the detergent properties of the
hexadecyltrimethylammonium cation. A more satisfactory method for the isolation of isocodeine, especially from larger scale reaction mixtures, was developed. Here, basic hydrolysis of the total reaction mixture obtained from the treatment of codeine toluene-\(p\)-sulphonate with hexadecyltrimethylammonium acetate, generated isocodeine \textit{in situ}. The offending cation was then removed very efficiently by precipitation with Reinecke's salt, \(\text{NH}_4\text{[Cr(NCS)}_4\text{(N\textsubscript{3})}_2\text{]}\). \(\text{H}_2\text{O}\), and pure isocodeine was obtained by chromatography and crystallisation. The yield using this modification was increased to 65-70\%, based on codeine.

Due to the difficulty of recrystallisation, neither the labelled nor the radioinactive codeine toluene-\(p\)-sulphonate was specially purified before use. We were therefore anxious to obtain a quantitative measure of the contamination of isocodeine by codeine, and this was carried out in the following manner. \(\text{[N-methyl-}\text{\textsubscript{14}}\text{C]}\)Codeine was converted into the corresponding toluene-\(p\)-sulphonate and, after treatment with hexadecyltrimethylammonium acetate the total reaction mixture was divided into two portions. One was processed by the modified method already described, to give labelled isocodeine which, after one crystallisation, was mixed with a known amount of radioinactive codeine. The mixture was separated and, after the codeine had been rigorously purified, its activity was determined by scintillation counting. In this way, the crystalline isocodeine was shown to contain 0.035\% codeine, and was considered sufficiently pure for our experiments. This figure could probably have been reduced by further crystallisation of the isocodeine. The other portion of the reaction mixture was
mixed directly with inactive codeine which, after isolation, was purified and counted for $^{14}C$ activity. The reaction mixture was found to contain 1.33% of the original codeine. The contaminating codeine could arise during hydrolysis of the reaction mixture, but is most likely due to contamination of the starting material.

The preparation of isomorphine (8; $R_1=R_2=H$) was carried out according to the method outlined by Makleit and Bognár.\textsuperscript{73} This was not entirely successful and modification of the work-up procedure was required. 3-Q-Acetylmorphine\textsuperscript{74} was treated with a pyridine solution of toluene-p-sulphonyl chloride to yield 3-Q-acetylmorphine toluene-p-sulphonate\textsuperscript{75} (6; $R_1=Ac$, $R_2=Ts$). Hydrolysis with dilute acetic acid should, according to the original authors,\textsuperscript{73} give pure isomorphine which can be crystallised directly. In our experiments hydrolysis produced a complex mixture of phenolic alkaloids, in which isomorphine occurred as the major component. Thus the mixture was acetylated under conditions known to give 3-Q-acetylation, and 3-Q-acetylisomorphine (8; $R_1=Ac$, $R_2=H$) was eventually obtained pure by column chromatography on neutral grade V alumina. Isomorphine was obtained by crystallisation after basic hydrolysis of the 3-Q-acetylisomorphine. The necessity to form the 3-Q-acetyl derivative arose because of the extreme difficulty, encountered throughout the whole of the work, in chromatographing the phenolic alkaloids.

[2-$^3$H]Codeine Methyl Ether (6; $R_1=R_2=Me$).- During the methylation of [2-$^3$H]morphine to give the correspondingly labelled codeine, a small amount of a by-product formed.
The $R_f$ value of the by-product (0.65 alumina/chloroform system) was slightly larger than that of codeine (0.40) and isolation was possible using column chromatography. The n.m.r. spectrum suggested that this by-product was codeine methyl ether. Confirmation came from the m.p., and the material was therefore utilised for feeding experiments. Crystalline codeine methyl ether, prepared by the above method formed in only about 7% yield, and even after extensive investigation of the reaction conditions it was not possible to increase this beyond 12%.

Thus, for preparation of the larger quantities of radioinactive codeine methyl ether, the original method described by Mannich was utilised. This required complete methylation of codeine using dimethyl sulphate and treatment of the reaction mixture with aqueous potassium iodide to give codeine methyl ether methiodide. Iodide ion was exchanged for chloride by treatment with silver chloride, and the derived codeine methyl ether methochloride was dry distilled in vacuo to liberate methyl chloride. Codeine methyl ether was obtained by chromatographing a chloroform solution of the solid material which remained after the distillation.

Preparation of morphine methyl ether ($6; R_1=H, R_2=Me$) also involved the method originally reported by Mannich. Methoxymethylmorphine (16), obtained from the sodium salt of morphine and chlorodimethyl ether, was treated with hydrogen peroxide to yield the suitably protected methoxymethylmorphine $N$-oxide (17). The protected morphine compound (16) was 6-$O$-methylated using dimethyl sulphate, then reduced in aqueous
solution with sulphur dioxide. Morphine methyl ether was obtained pure after extraction and crystallisation.

During the trial preparation of morphine methyl ether, study of the n.m.r. spectra of the various intermediates revealed an interesting effect. The n.m.r. spectrum of the methoxymethylmorphine compound (16) in deuterochloroform, showed the methylene protons H\textsubscript{a} and H\textsubscript{b} (see 16) as an AB system, with chemical shifts $\tau = 4.52$ and 5.00 ($\mathcal{J} = 6.0$ Hz). At first it was thought the methoxymethyl protecting group had been introduced at the chiral centre C-6 (see 16), thus explaining the magnetic non-equivalence of the methylene protons. However, the compound (16) showed no colour reaction with ferric chloride and was insoluble in aqueous sodium hydroxide, showing that the methoxymethyl function was in fact attached only to the aromatic ring and not to C-6. Magnetic non-equivalence and consequent geminal coupling of methylene protons, similar to H\textsubscript{a} and H\textsubscript{b} is not unusual in itself, and a number of other examples have been previously reported. But what is surprising is that the n.m.r. spectrum of methoxymethylmorphine N-oxide (17), which possesses an additional chiral centre, reveals the methylene protons as a sharp singlet, $\tau = 4.80$. Further, we were able to prepare a sample of 3-\textsubscript{Q}-benzylmorphine (6; R\textsubscript{1} = CH\textsubscript{2}Ph, R\textsubscript{2} = H), and its n.m.r. spectrum again showed the methylene protons as a singlet at $\tau = 4.90$. Exactly what causes these methylene protons in one case to display magnetic non-equivalence, and in the other two examples accidental magnetic equivalence, is not fully understood. Time was not available for a fuller.
(16)

(17)

(18)

(19)
investigation, but it might be useful to observe the effects of both solvent and temperature on the n.m.r. spectrum of these compounds.

\( [2-^3\text{H}]\text{Dihydrodesoxycodine} \;(10; R=\text{CH}_3). \quad [2-^3\text{H}]\text{Codeine toluene-\text{p}-sulphonate} \;(13; X=\text{TsO}) \) was again required, and its reduction using lithium aluminium hydride to give \( \Delta^7\)-desoxycodine (18; \( R=\text{CH}_3 \)) proceeded exactly as reported by Rapoport and Bonner. After hydrogenation using Adams catalyst the reaction mixture contained a small amount of impurity, and the labelled dihydrodesoxycodine was chromatographed prior to crystallisation. The impurity is most likely formed by hydrogenolysis, resulting in opening of the oxide bridge and generation of a phenolic compound. Larger scale reaction to produce a quantity of radioinactive material proceeded in the same satisfactory manner.

More difficulty was encountered during the preparation of dihydrodesoxymorphine (10; \( R=\text{H} \)). The use of pyridine hydrochloride for de-\( \text{O} \)-methylation of phenol methyl ethers is well known, and Rapoport has reported that \( \Delta^7 \)-desoxycodine is de-\( \text{O} \)-methylated in this manner at \( 215^\circ \text{C} \) to give \( \Delta^7 \)-desoxymorphine (18; \( R=\text{H} \)). We attempted to repeat Rapoport's preparation, but the reaction mixture became badly charred, and only a trace of \( \Delta^7 \)-desoxymorphine was isolated. However, pyridine hydrochloride de-\( \text{O} \)-methylation of dihydrodesoxycodine gave a fairly clean reaction product which, after chromatography on grade III alumina, gave the required dihydrodesoxymorphine. The phenolic alkaloid, obtained pure by sublimation and crystallisation, was characterised by m.p., optical rotation,
and n.m.r. spectrum. The overall yield from the demethylation was not good (ca. 20%).

The preparation of \textit{L-\textit{bromo-[2-\textit{H}]codeine}} was achieved\textsuperscript{53} by mild bromination of \textit{[2-\textit{H}]codeine acetate} (6; \textit{R_1}=\textit{CH}_3, \textit{R_2}=\textit{Ac}) using only 1 mol. of bromine. Basic hydrolysis of the \textit{\textit{O-acetyl-L-bromo-[2-\textit{H}]codeine}}, followed by extraction and crystallisation gave the required product. \textit{[2-\textit{H}]Codeine acetate} was obtained by acetylation of \textit{[2-\textit{H}]codeine} in pyridine with acetic anhydride.

Once again, preparation of the corresponding morphine analogue proved difficult. An acetic acid/acetic anhydride solution of diacetylmorphine (6; \textit{R_1=R_2}=\textit{Ac}) was brominated by carefully adding a solution of bromine in acetic acid. Evaporation of the solution gave a solid, showing a sharp n.m.r. singlet at \textit{T} 2.88 corresponding to one aromatic proton at C-1 or C-2. The shift down field, when compared with the mid-point of the aromatic quartet \textit{T} 3.38 in diacetylmorphine, suggested that bromination had taken place. The material, which was assumed to be \textit{L-bromodiacetylmorphine}, was hydrolysed with aqueous base. The hydrolysate was acidified to \textit{pH} 3-4 with dilute hydrochloric acid, and then adjusted to \textit{pH} 6 by careful addition of sodium hydrogen carbonate. Rapid, chloroform-propan-2-ol extraction of the aqueous solution and evaporation gave a highly insoluble product, which was chromatographically pure. A white micro-crystalline precipitate was formed after addition of sodium hydrogen carbonate to a hydrochloric acid solution of this insoluble product. Because of the lack of physical data available\textsuperscript{80} on \textit{L-bromomorphine}, a portion of the precipitate was treated with diazomethane. Methylation to give
l-bromocodeine, characterised by m.p. and n.m.r. data, proceeded very easily. A sufficient supply of l-bromomorphine was obtained in this manner.

Isocodeine methyl ether (8; \( R_1=R_2=\text{Me} \)) had previously been prepared \(^{81}\) by methylation of isocodeine using dimethyl sulphate. A suitable preparation of \([2-^3\text{H}]\text{isocodeine methyl ether}\) was achieved by heating \([2-^3\text{H}]\text{codeine toluene-\(p\)-sulphonate in methanol with a solution of sodium methoxide under reflux. Even after rigorous purification, via the picrate, the product would not crystallise. Purification by distillation as reported by Small and Browning \(^{81}\) was of little use. However, proof that the correct compound had been obtained was provided by the n.m.r. spectrum which, with the addition of a signal at \( \delta 6.63 \) (6-OMe), was identical with that obtained for isocodeine.

Both \([2-^3\text{H}]\text{dihydroisocodeine methyl ether}\) (9; \( R_1=R_2=\text{Me} \)) and \([2-^3\text{H}]\text{dihydroisocodeine}\) (9; \( R_1=\text{CH}_3, \ R_2=\text{H} \)), were obtained by catalytic hydrogenation of the corresponding 7,8-unsaturated compound. Both compounds were obtained crystalline, and the n.m.r. spectra were consistent with their structures. The preparation of dihydroisocodeine methyl ether has not been reported previously.

Samples of these labelled precursors were obtained in the manner set out above and administered to \( \text{P.somniferum} \) plants. At the time of these feeding experiments it was thought that preparation of their morphine analogues, necessary for radiodilution, would be relatively simple. However later, in the light of experience gained in preparing the morphine analogues of the modified codeine precursors already described,
it was realised that preparation of isomorphine methyl ether (8; \( R_1=H, R_2=Me \)), dihydroisomorphine methyl ether (9; \( R_1=H, R_2=Me \)), and dihydroisomorphine (9; \( R_1=R_2=H \)) would prove extremely difficult. The time necessary to overcome these difficulties was not available, and therefore analysis of these particular feeding experiments was not possible.

Precursor Feeding Experiments. - Full details of the precursors used in both double- and single-labelled feeding experiments are presented in Table 1. These precursors were administered to *P. somniferum* as follows. Each was dissolved in dilute hydrochloric acid (ca. 1.0 ml.). For the double-labelled feeding experiments, addition of a known small volume of an aqueous solution containing \([N\text{-methyl}^{14}\text{C}]\text{codeine hydrochloride} \) provided the internal reference standard. The acidic solutions of precursors were then adjusted to pH 7 by addition of sodium hydrogen carbonate. Normally, the seed capsules of three of the plants, *P. somniferum* Halle variety, were injected with about half of the aqueous precursor solution using a hypodermic syringe. Most of the remaining precursor solution was injected on the second day, followed on the third day by deionised water (0.5 ml.) to ensure good mixing in the seed capsule. Initial injection of precursor solution took place about five to seven days after petal fall, and the plants were harvested seven to eight days thereafter. Each of the double- and single-label feedings were carried out in the above manner. However, a small quantity of the aqueous precursor solution used in each of the double-labelled feedings was held back.
<table>
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<tr>
<th>Precursor (X')</th>
<th>Weight (mg)</th>
<th>Spec. Activity (mCi/m.mol)</th>
<th>Total Activity (mCi x 10^-2)</th>
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<tr>
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</table>

**Table 1**

(a) double-label feeding in which this sample of unnatural precursor has been mixed with [N-methyl-^14^C]codeine, ca. 0.01 mCi.
(b) single-label feeding
(c) precursor sample could not be crystallised and was used as an oil
(d) feeding experiment carried out in July 1969 season
(e) feeding experiment carried out in July 1970 season
(f) feeding experiment not worked-up
in order to determine the $\text{H}^3/\text{C}^{14}$ ratio. It was then possible to calculate the total activity of $[^{14}\text{C}]$codeine added in each case.

General Procedure for Isolation of Alkaloids.- After harvesting, the plants were removed to the laboratory, and the roots washed with water to remove any soil. In most cases the wet weight of the plants was determined, although in the double-labelled feedings of July 1969 no weighings were made. The three plants associated with each feeding were cut up into small pieces and left to stand for about two weeks in ethanol (ca. 250 ml.). Total alkaloids were extracted from the three plants associated with a particular feeding by, firstly, macerating the plant pieces in the ethanol solution using a Sunbeam Blender. The resulting ethanol and pulp suspension was filtered through celite, and the pulp washed with further quantities of ethanol (total ca. 800 ml.). Evaporation of the ethanol solution gave a green mass which, after dissolving in dilute hydrochloric acid (0.5 N, ca. 150 ml.), was extracted with ether (5 x 50 ml.) to remove non-basic material. The ether extract was washed with water (2 x 50 ml.) and the washings combined with the dilute hydrochloric acid solution. The total acid solution, after adjusting to pH 8 by addition of sodium hydrogen carbonate, was extracted with chloroform-propan-2-ol (v/v, 9:1, 10 x 50 ml.). Evaporation of the organic extract in a tared flask gave the total weight of crude alkaloids. An estimation of the tritium activity present was attempted, but in general the values obtained were inaccurate because colour quenching occurred in the scintillation counter.
The first stage in analysis of the feeding experiments was to dilute the crude alkaloid extract with the radioinactive alkaloids required to serve as carriers. Thus, the extract from the feeding of the modified codeine precursor \((X')\) was diluted with known weights of the phenolic alkaloids morphine and \((Y')\), and non-phenolic alkaloids codeine and inactive \((X')\). Subsequent stages of the work-up required reisolation and purification of these four alkaloids, followed by their radioactive assay. Fairly typically, the alkaloids were first separated into phenolic and non-phenolic fractions. Traditionally \cite{16,33,53} the phenolic and non-phenolic alkaloids (i.e. codeine and morphine) have been separated by extraction from aqueous solution. Initial extraction using chloroform at pH 14 removes the non-phenolic alkaloids, and morphine is then extracted after the pH has been adjusted to 7. This method proved inadequate when investigating the \([2-{}^3\text{H}, \text{N-methyl-}{}^{14}\text{C}]\)-codeine feeding since both the isolated codeine and morphine fractions were still contaminated with the green/brown plant material. Removing this contamination from morphine using normal chromatographic methods proved impossible due to the phenolic properties of the alkaloid. We anticipated that greater difficulties would be encountered with the other feedings which demanded purification of two phenolic alkaloids. Therefore a more suitable method, which would give very clean separation of phenolic and non-phenolic alkaloids and also remove the chemical contamination, was required.

\cite{82} McIntosh and co-workers have reported the use of Permutit ion exchange resin for separating codeine and morphine.
We reinvestigated this method of separation using a fine mesh anion exchange resin, and made suitable adaptations to give our previously defined requirements.

The anion exchange resin was prepared in the manner outlined in the experimental section (Chapter IV). The resin was obtained finally in the hydroxide form, and after washing with 50% aqueous methanol, was slurried into a glass column. A trial run using codeine and morphine to test the separation of non-phenolic and phenolic alkaloids proved successful. The codeine and morphine were dissolved in dilute hydrochloric acid and, after basification with potassium hydroxide, the solution was poured onto the column of resin. Elution with 50% aqueous methanol gave 96% recovery of the non-phenolic alkaloid codeine. The phenolic alkaloid morphine, held by the column because of the ionic properties of the phenolic group, was eluted using 0.1 N hydrochloric acid, the recovery being 94%. Both alkaloids were chemically pure by t.l.c. However, we required to know if there was any radiochemical contamination of the alkaloid, and also if any tritium was exchanged from the [2-3H]morphine under the acidic conditions of the separation.

The following procedure proved suitable to demonstrate that this method of separation was completely suitable for our experiments. A known weight of [2-3H]morphine with specific activity 6.03 x 10^5 dis/min/mg. was mixed with a known weight of radioinactive codeine, and separation carried out using the ion exchange method. The isolated codeine (99% recovery) showed a slight trace of tritium activity (total 259.9 x 10^2 dis/min.) which represented a 0.142% contamination by 64.
labelled morphine. A 92% recovery of pure [2-³H]morphine was achieved, and its specific activity was found to be 6.49 x 10⁵ dis/min/mg. Thus, labelled morphine did not exchange tritium under the separation conditions, and it is reasonable to assume that this situation will apply to all the other phenolic alkaloids discussed in connection with these feeding experiments. In a similar way, [2-³H]codeine with total activity 3.37 x 10⁷ dis/min. was mixed with a known weight of radioinactive morphine. After separation, the morphine (91% recovery) showed a small amount of tritium activity (total 3.79 x 10³ dis/min.), which represented 1.13 x 10⁻²% contamination by the radiolabelled codeine.

Separation of the phenolic and non-phenolic fractions of the crude alkaloid extract, isolated from the [2-³H, N-methyl-¹⁴C]codeine feeding, using the above method was very successful. The coloured plant material was held by the ion exchange medium, and both the isolated fractions were substantially pure. No modification of the method was required for the other feeding experiments, and in all cases the coloured plant material was removed. The observed contamination of the phenolic alkaloid fraction by the non-phenolic (1.13 x 10⁻²%) and vice versa (0.142%) was considered insignificant, especially in view of the rigorous purification that each alkaloid was subjected to even before crystallisation to constant specific activity.

The only common feature in the work-up of the feeding experiments was the dilution with radioinactive alkaloids, and the separation into non-phenolic and phenolic alkaloid fractions. Otherwise, each of these experiments (Table 1).
required a completely different work-up procedure. The double-labelled codeine feeding has already been mentioned, and this will be described first in the following discussion of the analysis of the feeding experiments.

Separation and Purification of Alkaloids.

Double-Labelled Codeine Feeding. - [2-$^3$H]Codeine was mixed with [N-methyl-$^{14}$C]codeine, to give effectively double-labelled [2-$^3$H, N-methyl-$^{14}$C]codeine which was fed to the plants in the way already described. This feeding was carried out so that we could establish independently a typical value for the incorporation of codeine into morphine and confirm that significant de-N-methylation did not occur. The conditions used for feeding this natural precursor were employed later for the unnatural precursor experiments. Moreover, it was possible during the work-up of this feeding to anticipate and overcome problems of separation that were likely to occur with the other feeding experiments.

The traditional 16,33,53 extraction method was used to obtain the non-phenolic alkaloid fraction for this particular feeding experiment. The fraction consisted mainly of codeine which was obtained pure by chromatography on neutral grade III alumina. Initial attempts to crystallise the codeine to constant specific activity resulted in a progressive drop in the $^3$H/$^{14}$C ratio, the value being larger than that calculated for the precursor. The small amount of solution held back from the feeding was used to determine the $^3$H/$^{14}$C ratio (5.32) for the precursor. The labelled codeine isolated from the plant was rechromatographed,
and crystallised to constant specific activity after dilution with more radioinactive codeine. At constant specific activity the $^3\text{H}/^{14}\text{C}$ ratio was 5.69, which was fairly close to the value obtained for the precursor. Radiochemical purity of the codeine was established by treatment with picric acid to form the picrate. $[2-^3\text{H}, \text{N-methyl}^{14}\text{C}]$Codeine picrate was recrystallised and the free base regenerated by decomposition of the alkaloid salt on a column of neutral grade III alumina. The recovered codeine had essentially the same specific activity as that before picrate formation. The recovery of unmetabolised codeine, based on the $^3\text{H}$ activity, was 2.88%. The corresponding value based on the $^{14}\text{C}$ activity was 2.69%.

The morphine isolated from the original extraction procedure was purified to remove contamination by plant material using ion exchange chromatography. Essentially pure morphine was obtained which, after further dilution with radioinactive material, was recrystallised from methanol to constant specific activity. Again the $^3\text{H}/^{14}\text{C}$ ratio decreased progressively finally reaching a value of 5.56. At which point the $^3\text{H}$ molar specific activity of the morphine was $46.0 \times 10^5$ dis/min/mmol. and the $^{14}\text{C}$ molar specific activity was $82.8 \times 10^4$ dis/min/mmol.

Radiochemical purity of the isolated morphine was shown by formation of the diacetyl derivative. A trial experiment using a sample of $[2-^3\text{H}]$morphine indicated that there was no loss of tritium on formation of this derivative. The diacetyl derivative of the biosynthetic morphine showed, after chromatography and crystallisation, a $^3\text{H}$ activity of $47.0 \times 10^5$
dis/min/mmol. and a $^{14}$C activity of $82.4 \times 10^4$ dis/min/mmol. These figures represented a codeine into morphine incorporation of 1.04% based on $^3$H specific activity, very similar to the value of 0.99% based on $^{14}$C specific activity. Surprisingly the $^3$H/$^{14}$C ratio for the isolated codeine (5.69) and morphine (5.57) were not exactly the same, and differed slightly from the ratio determined for the precursor (5.32). Statistical analysis of these ratios (see Appendix) demonstrated that the differences were significant. However, we considered that this did not seriously affect the use of [N-methyl-$^{14}$C]codeine as an internal standard. The results of the natural precursor feeding were therefore largely as expected, and the details are summarised in Table 3.

$[2-^3$H]Dihydrocodeine ($7; R_1=CH_3, R_2=H$) Feeding.

The crude alkaloids from both the double- and single-labelled feedings were separated by ion exchange, and recovery of their respective phenolic and non-phenolic fractions was high (above 95%) with the exception of the phenolic fraction from the double-labelled feeding (recovery 56%). Separation of dihydrocodeine from codeine, and dihydromorphone ($7; R_1=R_2=H$) from morphine was very difficult since the respective pairs of alkaloids ran almost together in most t.l.c. systems. Basic silica p.l.c. plates, made using sodium hydroxide solution instead of water, proved most suitable for separating these pairs of alkaloids. Initially, several separations of the codeine/dihydrocodeine fraction, isolated from the single-labelled feeding, were achieved using alumina p.l.c. plates.
impregnated with silver nitrate.

Dihydrocodeine, obtained from the initial separation of the non-phenolic alkaloids, showed some $^{14}$C as well as $^3$H activity. It was important to know if this $^{14}$C activity arose from a genuine incorporation or because of inadequate separation from codeine; the following procedure was used for purification. The dihydrocodeine ($X'$) was mixed with an equal weight of radio-inactive codeine ($X$). Plate chromatography, as described above, allowed reisolation of the radioactive dihydrocodeine ($X'$). After radiochemical assay, the dihydrocodeine was again diluted with radioinactive codeine and the separation repeated. The whole purification procedure was normally carried out about five times, for each of the four alkaloids associated with the feeding, until we were satisfied that they were free from major radiochemical contamination. This technique was used in the work-up of all the feeding experiments, resulting in rigorous purification of the isolated alkaloids.

The single-labelled feeding experiment was looked at first, and the best conditions for work-up were established. This feeding showed an incorporation of dihydrocodeine into dihydro-morphine of 0.51% (see Table 2). The double-labelled feeding showed similar incorporation of dihydrocodeine into dihydro-morphine (0.59%). The simultaneous codeine into morphine incorporation was 1.26%, making the unnatural conversion 46.7% as efficient as the natural one (Table 3). Although the results from both the double- and single-labelled feedings were substantially in agreement, the latter showed conversion of dihydrocodeine into codeine (0.1%) and into morphine (0.02%)
while the former showed no conversion of this type. The possibility that conversion into codeine and morphine had occurred in the double labelled experiment, but had not been observed due to the relatively high $^{14}C$ activity of both the codeine and morphine, was considered. Further, the codeine and morphine isolated from the single-labelled experiment had a very low tritium count rate i.e. 100-200 cpm/mg. (see Chapter IV). However, it will be recalled that the [2-$^3$H]-dihydrocodeine precursor was obtained as an oil, and was possibly contaminated by [2-$^3$H]codeine. Therefore, a sample of the precursor was mixed with radioinactive codeine, which was reisolated, and purified by chromatography and crystallisation. In this way it was shown that the original precursor material was contaminated by [2-$^3$H]codeine, the maximum value being 0.735%.

The presence of 0.735% [2-$^3$H]codeine in the [2-$^3$H]dihydrocodeine precursor used for the single labelled experiment would mean that the apparent incorporation (0.1%) of dihydrocodeine into codeine is really a 15.4% recovery of unmetabolised codeine. This figure is in agreement with the recovery (N-methyl-$^{14}$C]codeine (19.4%) for the double-labelled experiment. Similarly the apparent incorporation (0.02%) of dihydrocodeine into morphine, corresponds to a conversion of the contaminating codeine into morphine of 2.46%.

The recovery of unmetabolised dihydrocodeine in both the double- and single-label experiments was rather high (31.8% and 39% respectively), as was the recovery of unmetabolised codeine (19.4%). These high values may be due to the precursor's being allowed too short a time for metabolism. It does
demonstrate, however, the importance of the double labelled-feeding experiment which utilises an internal standard.

The labelled dihydromorphine isolated was shown to be radiochemically pure by forming the diacetyl derivative. The derivative was heated with aqueous methanolic potassium carbonate in a sealed tube and the dihydromorphine isolated showed that 96% of the radioactive label had been lost by exchange. Thus the tritium label in the $[2-^3\text{H}]$dihydrocodeine had not been scrambled during its conversion into dihydromorphine.

$[2-^3\text{H}]$Isocodeine ($8; R_1=\text{CH}_3, R_2=\text{H}$) Feeding.- The initial separation and purification using the ion exchange procedure worked well. The non-phenolic alkaloids, codeine and isocodeine, were separated and purified prior to crystallisation by chromatography on neutral grade III alumina. The phenolic alkaloids isolated from the plants were purified by chromatography of their 3-\text{O}-acetyl derivatives on neutral grade V alumina (see above).

In the single-labelled feeding experiment isocodeine showed a 0.27% incorporation into isomorphine, and negligible incorporation into codeine ($< 8.1 \times 10^{-3}$\%) and morphine ($< 0.02$\%)-(Table 2). The double-labelled feeding showed high incorporation of isocodeine into isomorphine (1.38\%), but the $[\text{N-methyl}^{-14}\text{C}]$ codeine incorporation into labelled morphine was even higher (9.1\%). Comparison of the unnatural conversion with the natural one gives an efficiency of only 15.2\% (Table 3). Exchange of the tritium label from the isomorphine, isolated from the double-labelled experiment, showed again that the
labelling pattern of the precursor had been preserved during its biological demethylation.

\[ \text{[2-}^{3}\text{H]Codeine Methyl Ether (6; } R_1=\text{H, } R_2=\text{Me}) \text{ Feeding.} \]

Again the separation of the phenolic and non-phenolic alkaloids isolated from both the double- and single-labelled experiments was fairly good (79-100%). Use of alumina p.l.c. plates gave good separation of the non-phenolic alkaloids, codeine and codeine methyl ether. The purification technique, whereby the isolated radioactive alkaloid \( X' \) is mixed with radio-inactive \( X \) and then reisolated, was employed as in the work-up of all the other feeding experiments. Much more difficulty was again encountered during the separation of the phenolic alkaloids, morphine and morphine methyl ether (6; \( R_1=\text{H, } R_2=\text{Me} \)). Although inadequate, it was possible to obtain some separation of these two alkaloids using p.l.c. alumina plates developed in an n-butanol/di-n-butyl ether/ammonium hydroxide solvent system. Later, however, better separation was obtained using n-butanol/di-n-butyl ether/acidic acid \( \text{as the solvent.} \)

The single-labelled feeding experiment showed incorporation (Table 2) of codeine methyl ether into codeine (0.88%) and morphine (0.05%), but its major conversion was into morphine methyl ether (1.02%). These results were in reasonable agreement with those obtained from the double-labelled experiment (Table 3). Here, codeine methyl ether was incorporated into codeine (0.49%), morphine (0.08%), and into morphine methyl ether (0.62%). The conversion of the \([N\text{-methyl-}^{14}\text{C}]\text{codeine into morphine was 0.90%, giving the unnatural 3,2-dimethylation}\)
process a 69.4% efficiency compared with the natural one. Surprisingly the [N-methyl-\( ^{14}C \)] codeine showed a 0.18% incorporation of \( ^{14}C \) activity into codeine methyl ether. This \( 6.0 \)-methylation was 20.1% as efficient as the natural \( 3.0 \)-demethylation of codeine to give morphine (i.e. the \( X \rightarrow X' \) efficiency value).

A sample of the labelled precursor material was mixed with radioactive codeine which, after reisolation and crystallisation showed that the \([2-^{3}H]\) codeine methyl ether contained 0.006% of codeine. This amount of contamination by codeine was insignificant and therefore the observed incorporation of codeine methyl ether into codeine and morphine was genuine, and not due to the presence of \([2-^{3}H]\) codeine.

\[ [2-^{3}H] \text{Dihydrodesoxycodeine (10; } R = \text{CH}_3) \text{ Feeding.} \]

Although both double- and single-labelled feeding experiments were performed, only the double labelled one was worked-up. Initial separation of the phenolic alkaloids, morphine and dihydridesoxymorphin 7 (10; \( R = \text{H} \), using basic silica p.l.c. plates developed in methanol was not too successful. The dihydridesoxymorphine fraction contained some morphine while the morphine fraction was contaminated by dihydridesoxymorphine. Earlier however, during preparation of dihydridesoxymorphine, it was found that the latter alkaloid was readily soluble in chloroform and easily chromatographed on neutral grade III alumina. Therefore it was not surprising that good separation of the phenolic fractions was achieved using neutral grade V alumina. Grade V alumina allowed the morphine to be eluted, by a chloroform-methanol solvent mixture, after removal of the dihydridesoxymorphine with chloroform. Fractions isolated in this way from the column
were analysed using t.l.c. chromatography and scintillation counting.

The dihydrodesoxycodeine showed very high incorporation into dihydrodesoxymorphine (9.27%), higher indeed than the parallel conversion of [N-methyl-14C]codeine into labelled morphine (8.55%). No incorporation into any of the other alkaloids was observed, and 30.6% of the unnatural precursor was recovered unmetabolised. Therefore this unnatural precursor was demethylated by the plant to give the corresponding unnatural product with greater efficiency than that for demethylation of the natural precursor, codeine.

\[
[2^{-3}H]-1\text{-Bromocodeine (II; }R = \text{CH}_3\text{ ) Feeding.}\]

All the feeding experiments described so far have involved unnatural precursors modified by changes to the C ring of the codeine molecule. Incorporation of all these precursors into unnatural products has been observed. To extend our study we were anxious to investigate the incorporation of other unnatural codeine precursors which had been modified at a different 'site' in the codeine molecule. We decided to investigate the effect of modification to the aromatic ring of the codeine molecule and, in particular, the metabolism of 1-bromocodeine by \textit{P.somniferum}. Originally 1-chlorocodeine was selected as the unnatural precursor, but investigation of the literature indicated that the morphine analogue, 1-chloromorphine, was not a crystalline compound.

Again, both double- and single-labelled feedings of \([2^{-3}H]-1\text{-bromocodeine}\) were carried out, but only the double-labelled experiment was analysed. The phenolic alkaloids isolated from the ion exchange separation were treated with pyridine and
acetic anhydride and their O-diacetyl derivatives obtained. Separation of these two diacetyl derivatives was effected by p.l.c. on alumina plates. But subsequent purification of the phenolic alkaloids, after basic hydrolysis of their diacetyl derivatives, was by p.l.c. on basic silica plates. The non-phenolic alkaloids could not be separated, as previously, by chromatography on alumina. However, their separation was also effected using p.l.c. on basic silica plates. The main problem associated with this feeding experiment was that of the insolubility of 1-bromomorphine (11; R = H). Again, as during its preparation, the alkaloid was obtained as a micro-crystalline material by precipitation from neutral solution.

The \([2-3^\text{H}]\)-1-bromocodeine precursor showed a 0.15% incorporation into labelled 1-bromomorphine. Not only was this the lowest \((X')\) into \((Y')\) conversion we had observed, but also the least efficient (5.9%) relative to the concurrent codeine into morphine conversion (2.57%). A fairly high recovery of unmetabolised 1-bromocodeine (35.2%) and codeine (61%) was observed. Although the isolated 1-bromomorphine was 'crystallised' to constant specific activity and we were able to provide proof of its radiochemical purity by derivative formation, no attempt was made to remove the tritium label from the molecule by exchange. The reason was simply the insolubility of 1-bromomorphine in all suitable solvents. The other three alkaloids were shown to be radiochemically pure in the normal manner.

**Discussion of Results.** The advantage of carrying out both double- and single-labelled experiments has been demonstrated, and there is fairly good agreement between both
sets of results. However, the most important results are those obtained from the double-labelled feeding experiments which utilise the internal reference standard. These results are presented in Table 3, and a graphical representation of the efficiencies of the unnatural conversions compared with the natural one is given in Fig. 4.

It can be readily seen that both the unnatural precursors dihydrocodeine and isocodeine were incorporated into their corresponding unnatural products. Of the two, isocodeine is the least efficiently 3-0-demethylated relative to the natural conversion of codeine into morphine. That dihydrocodeine, which contains no 7,8-olefinic double bond, is incorporated into dihydromorphine suggests that the unsaturation present in the C ring of codeine is not involved in coordinating the molecule to the surface of the demethylating enzyme. It seems likely that the oxygen of the C-6 group in both dihydrocodeine and isocodeine in some way hinders the co-ordination of the particular precursor to the enzyme surface. A better idea of the differing environments occupied by the C-6 hydroxyl group in codeine, dihydrocodeine, and isocodeine can be gained from the following. The C ring of the unsaturated codeine molecule (Fig. 5) has a half boat conformation, giving the hydroxyl group at C-6 a quasi-equatorial position below the plane of ring C and on the opposite side of the molecule to the nitrogen bridge. Thus in isocodeine (Fig. 7) the hydroxyl function occupies a quasi-axial position above the plane of ring C and on the same side of the molecule as the nitrogen bridge. When the C ring is saturated, as in dihydrocodeine (Fig. 6), it has
Results of Single Labelled Feeding Experiments Involving Modified Codeine Precursors

<table>
<thead>
<tr>
<th>Modified codeine precursor</th>
<th>$[2-^3H]$X'</th>
<th>$%\text{recovery}$</th>
<th>X-Codeine</th>
<th>$%\text{incorporation}$</th>
<th>Y'</th>
<th>Y-Morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[2-^3H]$dihydrocodeine</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td>0.51</td>
<td>-</td>
</tr>
<tr>
<td>$[2-^3H]$iso codeine</td>
<td>9.4</td>
<td>$&lt;8.1\times10^{-3}$</td>
<td>0.27</td>
<td></td>
<td>0.02</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>$[2-^3H]$codeine methyl ether</td>
<td>16.4</td>
<td>0.88</td>
<td>1.02</td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.
## Result of Double Labelled Feeding Experiments

Involving Modified Codeine Precursors

<table>
<thead>
<tr>
<th>Modified precursors</th>
<th>$X'$</th>
<th>$X$ Codeine recovery/incorporation</th>
<th>$Y'$</th>
<th>$Y$ Morphine</th>
<th>Efficiency of Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2-\textsuperscript{3}H]codeine</td>
<td>-</td>
<td>2.88</td>
<td>-</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>[N-\textsuperscript{14}CH\textsubscript{2}]codeine</td>
<td>-</td>
<td>2.69</td>
<td>-</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>[2-\textsuperscript{3}H]dihydrocodeine</td>
<td>31.8</td>
<td>zero</td>
<td>0.59</td>
<td>zero</td>
<td>46.7</td>
</tr>
<tr>
<td>[N-\textsuperscript{14}CH\textsubscript{2}]codeine</td>
<td>zero</td>
<td>19.4</td>
<td>zero</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>[2-\textsuperscript{3}H]isocodeine</td>
<td>32.0</td>
<td>&lt;0.01</td>
<td>1.38</td>
<td>&lt;0.01</td>
<td>15.2</td>
</tr>
<tr>
<td>[N-\textsuperscript{14}CH\textsubscript{2}]codeine</td>
<td>&lt;3.7x10\textsuperscript{-3}</td>
<td>16.2</td>
<td>&lt;0.05</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>[2-\textsuperscript{3}H]codeine methyl ether</td>
<td>(1) 18.4</td>
<td>0.49</td>
<td>0.62</td>
<td>0.08</td>
<td>69.4, (b) -54.5, (c) -8.9, (d) -20.1,</td>
</tr>
<tr>
<td>[N-\textsuperscript{14}CH\textsubscript{2}]codeine (2)</td>
<td>0.18</td>
<td>14.6</td>
<td>&lt;116x10\textsuperscript{-4}</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>[2-\textsuperscript{3}H]dihydro-desoxycodeine</td>
<td>30.6</td>
<td>zero</td>
<td>9.27</td>
<td>zero</td>
<td>109</td>
</tr>
<tr>
<td>[N-\textsuperscript{14}CH\textsubscript{2}]codeine</td>
<td>&lt;0.03</td>
<td>38.4</td>
<td>&lt;0.07</td>
<td>8.55</td>
<td></td>
</tr>
<tr>
<td>[2-\textsuperscript{3}H]-1-bromo-codeine</td>
<td>35.2</td>
<td>&lt;2.20x10\textsuperscript{-2}</td>
<td>0.15</td>
<td>&lt;2.2x10\textsuperscript{-2}</td>
<td>5.9</td>
</tr>
<tr>
<td>[N-\textsuperscript{14}CH\textsubscript{2}]codeine</td>
<td>&lt;0.03</td>
<td>61.0</td>
<td>&lt;0.04</td>
<td>2.57</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3

(a).- The efficiency of the unnatural conversion ($X'$) into ($Y'$)

expressed as a % of the natural conversion ($X$) into ($Y$) i.e.

$$X' \rightarrow Y' \text{ value}$$

$$\frac{X'}{X} \rightarrow Y'$$

(b).- $X' \rightarrow X$ efficiency. (c).- $X' \rightarrow Y$ efficiency. (d).- $X \rightarrow X'$ efficiency

$$\frac{X'}{X} \rightarrow Y$$

(1) Total % incorporations of [2-\textsuperscript{3}H]codeine methyl ether = 0.49 + 0.62 + 0.08 = \textbf{1.15%}

(2) Total % incorporations of [N-methyl-\textsuperscript{14}C]codeine = 0.18 + 0.90 = \textbf{1.08%}

78.
Efficiency of $X'\rightarrow Y'$ Conversions for Feeding Experiments.

\[
\frac{X'\rightarrow Y'}{X\rightarrow Y} \%
\]

- Dihydrocodeine: 69.4%
- Isocodeine: 54.1%
- Codeine methyl ether: 20.1%
- 1-bromo-codeine: 5.9%
- Dihydrodesoxycodine: 109%

Fig. 4.
a slightly deformed chair conformation. Thus the hydroxyl group at C-6 is located under the plane of the ring in an axial position and, on the opposite side of the molecule to the nitrogen bridge. Dreiding models of codeine (Fig.5), dihydrocodeine (Fig.6), and isocodeine (Fig.7) were constructed, and a more quantitative illustration of the way in which the position of the oxygen function is changing was obtained.

Measurement of the distance from the C-6 oxygen to, the nitrogen (a), to the oxygen of the aromatic methoxyl (ArOCH₃) (b), and to the oxygen of the oxide bridge (c), was made for each of the three models. The distances measured (a), (b), and (c) are illustrated in Figs. 5, 6, and 7; their values are given in Table 4.

\[
\begin{array}{ccc}
(a) & (b) & (c) \\
\hline
\text{Codeine (Fig.5)} & 6.5 & 4.5 & 2.6 \\
\text{Dihydrocodeine (Fig.6)} & 6.0 & 3.8 & 2.5 \\
\text{Isocodeine (Fig.7)} & 5.5 & 6.2 & 3.5 \\
\end{array}
\]

Table 4

Originally, from the results of the dihydrocodeine and isocodeine feedings, it was thought that the precursors might be co-ordinated to the enzyme via the lone-pair electrons of the C-6 oxygen or via hydrogen bonding involving the hydroxyl group. Therefore the differing positions of the C-6 oxygen were making co-ordination difficult (compared with codeine) and hence reducing the efficiency of 3-0-demethylation. If this was the case dihydrodesoxycodine, which possesses no C-6 oxygen, should show very inefficient incorporation, by \textit{P. somniferum}, into
Fig. 5

Fig. 6

Fig. 7
its corresponding unnatural product. However, the incorporation of dihydrodesoxycocdine into dihydrodesoxymorphine was more efficient than the codeine demethylation process. Therefore the presence of the C-6 hydroxyl group is unnecessary for 3-0-demethylation. However, when the C-6 hydroxyl group is present its exact position is important the most efficient demethylation occurring for codeine rather than for dihydrocodeine or isocodeine.

The above measurements, made for codeine (Table 4), are the same as those for codeine methyl ether, which showed very good incorporation into morphine methyl ether (0.62%). Further, we also observed incorporation into codeine (0.49%) and into morphine (0.08%), a result which is in agreement with the findings of Brockmann-Hanssen and also Rapoport. Comparison of the total percentage incorporations of codeine methyl ether (1.15%), and codeine (1.08%), into both natural and unnatural products suggests that the former is at least as good and possibly a better metabolised precursor than codeine (Table 3). The unnatural incorporation of codeine methyl ether into morphine methyl ether was 69.4% as efficient as the natural one, and results from both double- and single-labelled experiments were in good agreement. Surprisingly, we also observed the incorporation of codeine into codeine methyl ether (0.18%). This suggests that codeine methyl ether occurs naturally in P.somniferum and that some sort of equilibrium exists between codeine and its methyl ether. If this is correct, then there might be two possible routes for the conversion of thebaine into codeine, one proceeding via

82.
Evidence provided by both Rapoport and Battersby favours the route which involves codeinone as an intermediate. Further work is therefore necessary to establish the exact nature of the intermediate involved during the natural conversion of thebaine into codeine. Probably the most suitable experiments would involve feeding radiolabelled thebaine, and investigating its incorporation into all the alkaloids that can theoretically serve as the intermediates. It would also be interesting to investigate if codeinone is incorporated into morphinone under conditions similar to those in which codeine methyl ether is incorporated into morphine methyl ether.

We turn now to the feeding experiments with the unnatural precursor 1-bromocodeine. Introduction of a bromine atom at C-1 in codeine is likely to exercise both an electronic and a steric effect on the 3-methoxyl group. Therefore it was not surprising to observe the very low efficiency for the conversion of 1-bromocodeine into 1-bromomorphine. The low incorporation value is most likely due to the steric influence of the bromine atom which hinders the close approach of the enzyme to the methoxyl group and thereby inhibits demethylation. Even though the incorporation value is low it is surprising that the enzyme system still possesses the ability to accept this unnatural precursor.

We were able to show in all our other feeding experiments, by exchange of the tritium label, that all the unnatural phenolic alkaloids were biosynthesised from the unnatural precursors without scrambling of the label. As indicated previously the tritium label, assumed to be at C-2, of the isolated radioactive
l-bromomorphine was not removed by exchange due to solubility problems. However, it was considered unlikely, in view of our previous experiences, that the unnatural precursor l-bromocodeine was broken down into smaller components before being incorporated, by the plant, into l-bromomorphine.

In conclusion, we have observed that a particular demethylating enzyme or system of enzymes, in P.somniferum, is capable of accommodating unnatural precursors and converting them with surprising efficiency into their corresponding unnatural products. We have been able, through these studies, to gain some insight into the way in which this enzyme system works.

In extending the work which has been described here, it would be very useful to study the parts played by both the nitrogen associated with the C-15-C-16 bridge and the oxygen of the oxide bridge during demethylation of suitable unnatural precursors. It may be that the lone-pair electrons of the nitrogen play an important part during the demethylation of codeine, and feeding of a codeine metho-salt would provide evidence for this. Similarly the oxygen of the oxide bridge may be important, and feeding the unnatural codeine type precursor (19), to P.somniferum, would be of interest. This compound (19) is modified in both the C ring and the aromatic ring close to the site for demethylation; there is also one less hetero atom available for the donation of lone-pair electrons.

Studies, similar to our own, could be usefully applied to other plant systems. It may be, that work of this type will help to increase our understanding of the mechanisms by which alkaloids are biosynthesised in their natural systems.
and certainly of the substrate specificity of the enzymes involved. A great deal of interest is now shown in the mechanisms and enzymology of alkaloid formation, and it is this area which will provide the most challenging topics for future research.
Chapter III

Oxonium Ion Intermediates in the Rearrangement of Thebaine
The importance of the L-benzylisoquinoline, reticuline, and its phenol oxidative coupling for the biosynthesis of morphine has been described in Chapter I. Study of the biosynthesis of the Erythrina alkaloids, erythratine (4) and erythraline (5) in Erythrina cristagalli, has demonstrated the importance of the L-benzylisoquinoline (1) as a precursor. After the initial para-para phenol oxidative coupling of \( \alpha \)-norprotosinomenine (1) to give (2), it is thought that rearrangement, involving the nitrogen lone-pair electrons, occurs to give ultimately erysodienone (3) (see Scheme 1) which subsequently gives erythratine (4) and erythraline (5) as indicated. A similar type of rearrangement is involved in the suggested biosynthetic pathway to protostephanine (11; \( R_1=R_2=\text{Me} \)) in Stephania japonica Hiers. The postulated pathway involves initially para-para phenol oxidative coupling of the L-benzylisoquinoline (6) to give the dienone (7; \( R=H \)). The dienol (8), formed by \( \text{O} \)-methylation and reduction of (7; \( R=H \)), may then undergo the rearrangement (8) \( \rightarrow \) (9) \( \rightarrow \) (10), followed finally by reduction of (10) to give protostephanine (11; \( R_1=R_2=\text{Me} \)). Some evidence for this pathway was provided by Hackett who observed that the morphinandiennone (7; \( R=H \)) was incorporated (2.9%) by \( S. \) japonica into protostephanine. Also, Battersby et al. have been able to synthesise protostephanine by a route which is related to the above biosynthetic pathway (Scheme 2.). These workers oxidised the di phenol (6), using ferricyanide, to give the dienone (7; \( R=H \)) which was
Scheme 2.

(6) → (7) → (8) → (9) → (10) → (11)
Q-methylated to give protostephanone (7; R=Me). Reduction of (7; R=Me) gave a quantitative yield of the epimeric dienols (8) which, rearranged to give (12) on catalytic treatment with sulphuric acid. When the dienone (12) was heated with magnesium iodide and the products reduced with lithium aluminium hydride the two phenols (11; \(R_1=H, R_2=\text{Me}\)) and (11, \(R_1=R_2=H\)) were isolated. Q-Methylation of these two phenols, using diazomethane, gave protostephanine (11; \(R_1=R_2=\text{Me}\)). A similar dienone (13) was obtained in vitro, from a mixture of the \(N\)-acetylnorsinoactinols (14) kept at room temperature in \(\text{IN} \) hydrochloric acid; the dienols (14) were derived from norsinoccutine (15). This result was at the time a little surprising to the authors in view of the accepted rearrangement of the salutaridinols (described in Chapter I) to give thebaine.

Another morphinandienone alkaloid found to undergo a similar acid catalysed rearrangement, involving the nitrogen lone pair electrons, is amurine (16). It was observed that (16), when heated with \(3\text{N}\) hydrochloric acid gave (21). The mechanism suggested for this rearrangement is outlined, \((16)\rightarrow (21)\), and is analogous to that proposed by Stork for the transformation of thebaaine (22) into thebenine. It was thought that when thebaaine (22) is dissolved in concentrated hydrochloric acid, as in the initial stages of the latter transformation, that the resultant red solution contains the protonated form of the dienone (23; \(R=H\)). Attempts to isolate the dienone from the red solution have always given ill defined products,
however, reduction of the solution gives metathebainone\(^91,92\) (24). We were interested not only to identify the major constituents of this red solution, but also, because of the general biosynthetic significance, to study in detail the mechanism of the rearrangement. We investigated spectrophotometrically the reactions of both thebaine and its metho-salts in concentrated hydrochloric acid and in trifluoroacetic acid. The metho-salts of thebaine were used in our study since, in these compounds, the nitrogen lone pair electrons are not available for aromatisation of the C-ring. Fleischhacker et al.\(^93\) later reported the preparation of 7,8-dehydrometa-thebainone methoperchlorate (25; R=Me) by treating thebaine methoperchlorate with aqueous perchloric acid.

The rearrangement, in concentrated hydrochloric acid, of thebaine and its methiiodide and methochloride was studied using u.v. spectrometry. The final solutions had identical u.v. spectra and corresponded well with that recorded for thebaine in hydrochloric acid. However preparative scale work was unsatisfactory due to the low solubility of the thebaine metho-salts in concentrated hydrochloric acid. Use of trifluoroacetic acid did not help, but it was found that the u.v. spectra of the metho-salts in this acid were identical with those obtained in hydrochloric acid. The work described above was carried out by Mr R. T. Channon in these laboratories.

We anticipated that the problem of solubility could be overcome by using thebaine methotrifluoroacetate

93.
and trifluoroacetic acid. The u.v. spectral changes for the rearrangement of thebaine methotrifluoroacetate in concentrated hydrochloric acid were studied. The reaction proceeded at qualitatively the same rate as for the other metho-salts ($t_{1/2}$ ca. 2 min at $34^\circ$C) and the u.v. spectra of the final solutions were identical to those observed by Channon. The shape and intensity of the absorption curve at ca. 400 nm., for all the metho-salts, was sensitive to acid concentration; addition of concentrated sulphuric acid caused enhancement while addition of water reduced absorption in this region. It appeared that quaternisation of thebaine did not significantly alter the course of the acid-catalysed rearrangement.

Thus, thebaine methotrifluoroacetate, prepared from the corresponding methiodide by treatment with silver trifluoroacetate, was selected for the detailed investigation of the rearrangement. This salt dissolved readily in trifluoroacetic acid to give a solution which rapidly developed an intense absorption band at 420 nm. (Fig. 1. curve A). This band diminished in intensity with time at ambient temperature, and was eventually replaced by a broad band (Fig. 1. curve B), $\lambda_{\text{max.}}$ 322 nm. ($\varepsilon$ 7,130). The high solubility of thebaine methotrifluoroacetate in trifluoroacetic acid enabled us to observe the rearrangement using n.m.r. spectroscopy. A quantity of the metho-salt (ca. 60-70 mg.) was dissolved in trifluoroacetic acid and immediately transferred to an n.m.r. tube. The spectrum of the freshly
generated red solution showed the methoxonium ion (25; \( R = R' = \text{Me} \)) to be present as the major species (see Table 5). The presence of the ion (25; \( R = R' = \text{Me} \)) was indicated primarily by the position of the 6-methoxy-group which resonated at lower field (\( \tau \approx 5.34 \)) than the aromatic methoxy-group (\( \tau \approx 5.94 \)). The olefinic proton signals showed the expected coupling constants (\( J_{5,7} = 2.2 \) and \( J_{7,8} = 10.1 \) Hz). The methoxy-singlet, occurring at \( \tau \approx 5.34 \), diminished with time and was replaced by a signal at \( \tau \approx 5.98 \) which is presumably due to methyl trifluoroacetate. After 1h. the solution, then yellow, gave an n.m.r. spectrum fully consistent with that expected for the quaternary dienone (23; \( R = \text{Me} \)).

The rearrangement was carried out on a preparative scale, and thebaine methotrifluoroacetate was allowed to react to completion (the solution was yellow) in trifluoroacetic acid at room temperature. The solvent was removed in vacuo and the trifluoroacetate anion was replaced by chloride, using an ion exchange resin, to give 7,8-dehydromethaetinebainone methochloride (23; \( R = \text{Me} \)) as an amorphous yellow powder; \( \nu_{\text{max}} \approx 1660 \text{ cm}^{-1} \), \( \lambda_{\text{max}} \approx 304 \text{ nm} \) (EtOH), \( \epsilon = 8650 \). The same salt was obtained more directly by treating thebaine methotrifluoroacetate with concentrated hydrochloric acid.

The u.v. spectrum of the prepared dienone (23; \( R = \text{Me} \)) was observed. This showed a wavelength shift (\( \lambda_{\text{max}} \approx 304 \rightarrow 322 \) and \( 440 \text{ nm} \)) upon addition of alkali.
Fig. 1.

Electronic absorption spectra of dehydrometathebainone derivatives in CF$_3$CO$_2$H; (A) the methoxonium ion (25; R' = R'' = Me), (B) the dienone (23; R = Me); the wavelength scale expands two-fold below 350 nm.

N.m.r. spectra of dehydrometathebainone derivatives

<table>
<thead>
<tr>
<th>Solvent</th>
<th>H-5</th>
<th>H-7</th>
<th>H-8</th>
<th>MeO</th>
<th>MeN</th>
<th>J values (J in Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(23; R = Me)</td>
<td>3.58</td>
<td>3.61</td>
<td>2.50</td>
<td>6.17</td>
<td>6.59</td>
<td>D$_2$O</td>
</tr>
<tr>
<td>(2.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.14</td>
</tr>
<tr>
<td>2.78</td>
<td>3.26</td>
<td>2.30</td>
<td>5.96</td>
<td>6.46</td>
<td>CF$_3$CO$_2$H</td>
<td></td>
</tr>
<tr>
<td>(25; R' = Me)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.89</td>
</tr>
<tr>
<td>(2.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(10.0)</td>
</tr>
<tr>
<td>1.96</td>
<td>*</td>
<td>1.40</td>
<td>5.34</td>
<td>6.32</td>
<td>CF$_3$CO$_2$H</td>
<td></td>
</tr>
<tr>
<td>(25; R' = Me)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.82</td>
</tr>
<tr>
<td>(25; R'' = H)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.99</td>
<td>H$_2$SO$_4$</td>
</tr>
<tr>
<td>R'' = Me)</td>
<td>2.09</td>
<td>*</td>
<td>1.46</td>
<td>5.90</td>
<td>6.32</td>
<td>CF$_3$COH</td>
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<td>(25; R = H)</td>
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<td></td>
<td>6.82</td>
</tr>
<tr>
<td>R'' = Me)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&amp; H$_2$SO$_4$</td>
</tr>
</tbody>
</table>

* Signals for H-7 overlap aromatic quartet, ca. 2.9

Table 1.
resembling that observed for metathebainone (λ<sub>max</sub> 296–317 and 425 nm) and is consistent with the presence of an enone chromophore conjugated with a phenyl residue which carries an ortho-hydroxyl-group. The dienone (23; R=Me) was hydrogenated to give metathebainone methochloride. Chloride ion was exchanged for iodide and the observed u.v. and i.r. spectra were identical with those obtained for the product isolated after treating an authentic sample of metathebainone with methyl iodide. The dienone (23; R=Me) was characterised further as its crystalline Reineckate salt. Addition of concentrated sulphuric acid (ca. 2% v/v) to a solution of the dienone (23; R=Me) in trifluoroacetic acid generated the hydroxonium ion (25; R'=H, R''=Me); λ<sub>max</sub> 407 nm. (ε 11,800). The n.m.r. spectrum of this ionic species (see Table 1) closely resembled that of the initially observed 6-+Q-methyl derivative (25; R'=R''=Me).

The spontaneous demethylation of the methoxonium ion (25; R'=R''=Me) to give the dienone (23; R=Me), in trifluoroacetic acid, was studied further. The transformation was not significantly accelerated by an increased concentration of trifluoroacetate anion, produced by addition of sodium acetate to the solution. Also, when thebaine methotrifluoroacetate was dissolved in trifluoroacetic acid containing concentrated sulphuric acid (2% v/v) (hereafter abbreviated to CF<sub>3</sub>CO<sub>2</sub>H·H<sub>2</sub>SO<sub>4</sub>) a solution of the red methoxonium ion (25; R=R''=Me), λ<sub>max</sub> 420 nm. (ε 14,800), was produced which was stable.
for several h. Therefore it appeared that neither the trifluoroacetate anion nor the resonance stabilised hydrogen sulphate anion were sufficiently good nucleophiles to promote loss of the methyl group. However, addition of water (ca. 2% v/v) to the red trifluoroacetic acid solution produced an increase in the rate of demethylation, while the use of freshly dried trifluoroacetic acid as solvent had the reverse effect. Thus the demethylation of the oxonium ion must involve attack of water at C-6 as the initial step.

Thebaine itself dissolved in trifluoroacetic acid to give a transient red compound, $\lambda_{\text{max}}$ 420 nm, presumably the methoxonium ion (25; $R'$=Me, $R''$=H). This species decomposed too rapidly at 35°C to be conveniently studied by n.m.r. spectroscopy. In CF$_3$CO$_2$H-H$_2$SO$_4$, however, thebaine formed a stable red solution, $\lambda_{\text{max}}$ 420 nm ($\epsilon$ 15,400). The n.m.r. spectrum of this solution was observed and indicated, particularly by occurrence of a singlet at $\tau$ 5.30, that the ion (25; $R'$=Me, $R''$=H) was present. The solution of thebaine in trifluoroacetic acid was kept at room temperature until disappearance of the 420 nm absorption band was complete. At this point the solution showed no significant u.v. absorption above 300 nm, and was yellow in colour. It seemed likely, in view of other evidence, that decomposition of the ion (25; $R'$=Me, $R''$=H) in the absence of mineral acid involved loss of the proton from nitrogen and collapse of the dienone.
system to give the iminium salt (26). An indication of this came from the n.m.r. of the bleached solution which showed a one-proton singlet at $\tau$ 1.90 corresponding to the vinyl proton of the iminium group in (26). We hoped to demonstrate the presence of (26) by reducing the bleached solution to give neodihydrothebaine (27). Hydrogenation gave a product not unlike an authentic sample of (27) which was provided by Dr. K. W. Bentley. Final proof was provided by Mr. Channon. He reduced an ethanol solution of the gummy residue, obtained by evaporation of the acid solvent, using sodium borohydride to give neodihydrothebaine, identical with the authentic sample. Previous preparations of neodihydrothebaine involved$^{95,96}$ successive treatment of thebaine with magnesium iodide and a hydridic reducing agent.

Thus the red solution obtained by dissolving thebaine in concentrated hydrochloric acid appears, from the u.v.--visible spectrum, to contain the protonated diene (23; $R=H$) and the hydroxonium ion (25; $R'=R''=H$), these being in the ratio of ca. 3:1. However the amount of hydroxonium ion present depends upon the age and hence concentration of the 'concentrated' hydrochloric acid. Basification of the solution, as a preliminary step to isolation of the products, may induce aromatisation of the dienone ring by removal of the proton attached to nitrogen.

In view of our findings it is possible$^{97}$, in the case of the rearrangement of $N$-acetyl norsinoacetinols (14), observed by Stuart et al.$^{88}$, that the expected dihydrofuran ring closure was achieved first and it was
this product which then underwent further rearrangement to
give the dienone (13).
Chapter IV

Experimental Section
General Methods

Solvents.- All solvents were distilled at least once. Benzene and ether were dried over sodium wire after distillation. Tetrahydrofuran was initially dried over sodium wire, then with lithium aluminium hydride from which it was distilled just before use. In general, chloroform was only purified by distillation before use. However when dry chloroform was required, the solvent was shaken with magnesium sulphate and calcium chloride (after distillation) and decanted as required.

Physical Methods.- Melting points were determined on a Kofler block apparatus, and are uncorrected.

$^1$H Nuclear magnetic resonance spectra were determined at 60 M.Hz. on a Perkin-Elmer R10 spectrometer. In general spectra were determined in deuterochloroform and tetramethylsilane was used as the internal standard. Some spectra were determined in D$_2$O and also DCl and t-butanol was used as the internal standard. The spectrometer operated at a temperature of 34°C.

Infra-red spectra were determined in chloroform solution (solution cell 0.1 mm.) or in Nujol mull using Perkin-Elmer 237 or 257 spectrophotometers. All spectra were calibrated using polystyrene film at 1601, and 909.8 cm.$^{-1}$

Ultra-violet spectra were determined in ethanolic solutions (see part B of this Chapter) using a Pye-Unicam SP-800 spectrophotometer.

Optical rotation measurements were made at ca. 20°C in chloroform on ethanol solution.

Hydrogenation.- All hydrogenations were carried out at atmospheric pressure and ambient temperature, ca. 20°C.
Chromatography.- (a) Unless otherwise stated all column chromatography was carried out using neutral grade III alumina with chloroform as solvent. In general, the weight of alumina used was fifty times that of the material to be separated.

(b) Analytical thin layer chromatography on alumina was carried out using Merck GF$_{254}$ Alumina. The mixture, consisting of water (68 ml.) and alumina (45 g.), was spread in a 0.25 mm. layer on twenty (20 cm. x 5 cm.) plates. The plates were activated at 140°C for 2 hr.

(c) Preparative thin layer (p.l.c.) chromatography was carried out using Merck PF$_{254}$ Alumina. The mixture, consisting of water (120 ml.) and alumina (90 g.), was spread in a 0.5 mm. layer on five (20 cm. x 20 cm.) or one (1 m.) plates. Water was allowed to evaporate from the plates for about 12 hr.; then they were activated at 120°C for 2 hr.

(d) Thin layer chromatography on silver nitrate/alumina plates was exactly as for (b), and (c) except that the water used in each case contained, respectively, 4.5 and 9.0 g. of silver nitrate.

(e) Separations on basic silica plates utilised Merck GF$_{254}$ silica for analytical and PF$_{254}$ silica for preparative work. The silica (50 g.) was slurried with 0.1N sodium hydroxide (90 ml.) and spread in a 0.5 mm. layer onto five (20 cm. x 20 cm.) plates (or one x 1 m plate). Analytical plates used half quantities with a 0.25 mm. layer. The plates were activated at 140°C for 2 hr. before use.

In general, for preparative work, ca. 100 mg. of material was spread on a 1 m. plate, while ca. 20-30 mg. of material was used for a 20 cm. x 20 cm. plate.
Analytical plates were examined using the Dragendorff Reagent, and also iodine.

**Counting Methods.** - Both $\text{H}^3$ and $\text{C}^{14}$ were determined by liquid scintillation counting using a Beckman CPM-100 Liquid Scintillation System. The liquid scintillator consisted of 2,4-diphenyloxazole (PPO) (1.9 g), and 1,4-Bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl POPOP) (0.1 g.) dissolved in Analar, sulphur-free toluene (500 ml.). The liquid scintillator was stored in a dark bottle, under nitrogen, at ca. $0^\circ$C. After use, the scintillator liquid was flushed with dry, oxygen and carbon dioxide free nitrogen.

The instrument was used to count in two channels, Channel I counting mainly $\text{H}^3$ activity, and Channel II counting mainly $\text{C}^{14}$ activity. The 'voltage window' was set to give optimum efficiency for counting in both Channels I and II, however, a certain amount of cross-over was observed i.e. $\text{C}^{14}$ activity contributed to the Channel I count. The efficiency of counting and the amount of cross-over was determined using the hexadecane standards, $[\text{C}^{14}]n$-hexadecane and $[\text{H}^3_2]n$-hexadecane, of known specific activity ($1.73 \times 10^3$ and $0.44 \times 10^2$ dis/min/mg respectively).

For determining the activity of a sample containing both $\text{C}^{14}$ and $\text{H}^3$ the following simultaneous equations apply.

\[
\begin{align*}
C_1 &= aA + bB \\
C_2 &= xA + yB.
\end{align*}
\]

$C_1$ and $C_2$ are the counts indicated by the instrument for Channel I and Channel II respectively. $A$ and $B$ are the actual activities (dis/min) of, respectively, $\text{H}^3$ and $\text{C}^{14}$ in the sample. The % efficiency of counting $\text{H}^3$ in Channel I is represented by (a),
while that of counting $^{14}$C is represented by (b). The efficiency of counting $^3$H in Channel II is represented by $x$, and of $^{14}$C by $y$. Using the standards, the following typical values were obtained; 

$a = 44\%$, $b = 25\%$, $x = 0.055\%$ (considered negligible), and $y = 63\%$.

The $^3$H and $^{14}$C activity of a crystalline solid was determined by weighing a known amount (ca. 0.5 mg.) into a counting bottle, and adding dimethylformamide (0.1 ml.) and scintillation liquid (5.0 ml.). The sample prepared in this way was then counted by the instrument.

Dimethylformamide was doubly distilled and dried over calcium hydride. It was checked periodically for contamination.

Throughout this Chapter and also in Chapter II Specific and Total Activities are measured in 'disintegrations'/min/mg., and 'disintegrations'/min, and these will be written as dis/min/mg. and dis/min respectively. When the actual counts, recorded by the instrument, are given as counts per min this will be written as cpm. Specific Activity has been abbreviated to s.a.

**Feeding Work-up.** - (a) All chloroform-propan-2-ol extractions use these solvents in a v/v ratio of 9:1.

(b) Volumetric Count - In the early stages of isolation of an alkaloid from a particular feeding it was neither possible nor convenient to crystallise before determining the specific activity in the normal way. However some estimation of the activity present was required. This was carried out by addition of ethanol (5.0 ml.) to an accurately known weight of the alkaloid. An aliquot of the ethanol solution (0.1 ml.) was removed and placed in a counting bottle, then evaporated in a stream of dry nitrogen. 106.
Liquid scintillator (5.0 ml.) and dimethylformamide (0.1 ml.) were added and the count determined.

(c) Washing - This procedure was used for purifying both the non-phenolic and the phenolic alkaloids isolated from the plants. Thus, if, after separation of the alkaloids A₁ and A₂, it was found that A₁ contained activity most likely to have arisen by contamination with A₂, the following purification was carried out. Active alkaloid A₁ (x mg.) was mixed with a radioinactive sample of A₂ (x mg.) and separation repeated. Thus A₁ has been "washed" once with radioinactive A₂ to remove traces of active A₂.

(A) Experimental details associated with Chapter II.

Preparation of Radioinactive Alkaloids.

Dihydrocodeine. 68 - Codeine (0.199 g.) in dry methanol (10 ml.) was hydrogenated (uptake 16.3 ml., H₂; calc. 16.1 ml.) using 10% palladised charcoal as catalyst (25 mg.). The catalyst was removed by filtration through celite, and evaporation of the solvent gave a colourless oil. The oil, in ethanol (2 ml.), was treated with an ethanolic solution (1 ml.) of picric acid (0.145 g.). The dihydrocodeine picrate was crystallised twice from ethanol (m.p. 168°C decomp.), and the free base recovered from the picrate by pouring a chloroform solution through a column of alumina. Dihydrocodeine was obtained as pure white
crystals from ethanol (0.164 g., 83%), m.p. 110-112° C (lit., 112-113° C); \( ^{1}H N M R \) (CDCl) \( 3.34 \) (q, 1,2-H), 5.42 (d, \( J \) 5.0 Hz, 5β-H), ca. 6.0 (broad s, 6-H), 6.15 (s, Ar-OMe), 7.70 (s, N-Me).

**Dihydromorphine.** Morphine (0.214 g.) was hydrogenated (uptake, 19.3 ml H\(_2\); calc. 18.1 ml.) in ethanol (20 ml.) using 10% Pd/C (20 mg.) as catalyst. The solution was evaporated after filtration through celite to remove the catalyst. Crystallisation of the dihydromorphine (97 mg, 45%) was from ethanol; m.p. 153-155° C (lit., 155-157° C); diacetyldihydromorphone derivative \( ^{1}H N M R \) (CDCl) \( 3.32 \) (q, 1,2-H), 4.95 (broad s, 5-H), 5.52 (d, \( J \) 5.5 Hz, 6-H), 7.65 (s, N-Me). Hydrogenation, as above, but in glacial acetic acid, gave dihydromorphine in only 37% yield.

**Isocodeine:**

(i) Hexadecyltrimethylammonium acetate. Hexadecyltrimethylammonium bromide (4.0 g.) in ethanol (20 ml.) was added to a finely powdered suspension of silver acetate (1.8 g.) in ethanol (20 ml.). The mixture was shaken in the dark for 2 hr., then filtered through celite to remove the precipitated silver bromide. Evaporation of the solvent in vacuo gave the required quaternary ammonium acetate as a waxy solid (4.1 g). The purity of this material (typically 93%) was determined by titration, of an aqueous solution, with hydrochloric acid using methyl red indicator.

(ii) Codeine toluene-p-sulphonate. Codeine (1.44 g.) in pyridine (1.6 ml.), cooled to 0° C, was treated with an ice-cold solution of toluene-p-sulphonyl chloride (1.02 g, 1.1 mol.) in pyridine (1.3 ml.). After being kept in ice for 4 hr., the mixture was poured into ice-water with rapid stirring. A pink precipitate formed on addition of concentrated ammonia (ca. 2 ml.), and the supernatant liquid was decanted. The
pink crystalline material was washed with water, and filtered off. Water was removed in vacuo over calcium chloride and phosphorus pentoxide to give crystalline codeine toluene-\(p\)-sulphonate (1.99 g, 99\%), m.p. 119-121°C (lit., 121°C).

(iii) Isocodeine.- The codeine toluene-\(p\)-sulphonate (1.99 g) in benzene (20 ml.) was treated with hexadecyltrimethylammonium acetate (2.14 g, 1.2 mol.) and agitated for 12 hr., at room temperature. The benzene was evaporated and the residue dissolved in 50\% aqueous methanol (50 ml.) then treated with 2N sodium hydroxide (10 ml.). After 12 hr. at room temperature the reaction mixture was neutralised with solid carbon dioxide and treated with Reineckes's salt, \(\text{NH}_4[\text{Cr(CNS)}_4(\text{NH}_3)_2]\). \(\text{H}_2\text{O}\) (2.3 g.), in water (80 ml.). The resulting red precipitate was filtered off through celite and washed with portions of chloroform-methanol (1:1) (total (80 ml.). Evaporation of the combined filtrate and washings gave a red oil which was chromatographed on alumina (50 g.) in chloroform. Evaporation of the chloroform gave isocodeine which crystallised from ethyl acetate as prisms (0.98 g, 68\% from codeine), m.p. 173-174°C (lit., 171-172°C), \([\alpha]_D=149^0\) (c 0.48 in CHCl\(_3\)) (lit., 99-150.6° in CHCl\(_3\)); \(\Upsilon\) (CDCl\(_3\))\(^{100}\) 4.00 (m, 7-H), 4.40 (q, \(J\) 10 and 1.7 Hz, 8-H), 5.20 (broad s, 5-H), and 5.78 (d, \(J\) 5.7 Hz, 6-H).

Radiodilution Analysis of Isocodeine.- [N-methyl\(^{14}\)C]Codeine (307 mg, s.a., 2.58 x 10\(^5\) dis/min/mg; total activity 7.92 x 10\(^7\) dis/min) was converted into its toluene-\(p\)-sulphonate (373 mg.) which was treated, without prior crystallisation, with hexadecyltrimethylammonium acetate (405 mg.) (as above). After evaporation of the benzene, the total reaction mixture was
dissolved in methanol (25 ml.) and divided into two equal portions. Isocodeine (104 mg, s.a. 2.73 x 10^5 dis/min/mg; total 3.82 x 10^7 dis/min), m.p. 170-171°C (lit., 171-172°C) was isolated from one portion in the described manner. After the one crystallisation from ethyl acetate this material was mixed with radioactive codeine (74.4 mg.). The mixture was separated chromatographically on alumina and the codeine rigorously purified, then crystallised from benzene to constant specific activity, m.p. 155-156°C (lit., 156-157°C). Radiochemical purity was demonstrated by picrate formation. The specific activity of the purified codeine (133 dis/min/mg.) corresponded to the presence of 0.035% codeine in the isocodeine sample. The other portion of the reaction mixture was mixed directly with radioactive codeine (63.1 mg.). The alkaloids were separated after hydrolysis of the mixture and treatment with Reinecke's salt. The specific activity of the codeine, m.p. 155°C (lit., 156-157°C), (s.a. 8419 dis/min/mg.) showed that 1.33% of the original codeine was present in the total reaction mixture. The isocodeine isolated (106 mg, 69%), m.p. 172-173°C (lit., 171-172°C), had a ^14C specific activity of 2.80 x 10^5 dis/min/mg.

Isomorphine.—Morphine (9.66 g.) was stirred in water (966 ml.) with sodium hydrogen carbonate (97 g.) and acetic anhydride (49 ml.) added dropwise. After all the morphine had dissolved, the aqueous solution was extracted with chloroform (6 x 50 ml.) and evaporation in vacuo gave 3-O-acetylmorphine as a colourless oil (10.95 g., 98%); ν max. (CHCl₃) 2920, 1758 (AcOAr,C=O), 1493, 1450, 995 cm⁻¹; δ (CDCl₃) 3.35.
(q, 1,2-H), 4.28 (q, J 10 Hz and J 2 Hz, 7-H), 4.78 (q, J 10 and J 2 Hz, 8-H), 5.10 (d, J 8 Hz, 5-H), 5.82 (broad m, 6-H), 7.60 (s, N-Me), 7.75 (s, AcOAr). The 3-O-acetylmorphine (10.95 g.) in pyridine (44 ml.) was treated at 0°C with an ice-cold pyridine (43 ml.) solution of toluene-p-sulphonyl chloride (7.03 g, 1.1 mol.). After 6 hr. at 0°C, the pyridine solution was poured, with rapid stirring, into water (1 l.) saturated with sodium hydrogen carbonate. The aqueous mixture was extracted using chloroform (6 x 50 ml.) which, after drying, was evaporated in vacuo to yield 3-O-acetylmorphine toluene-p-sulphonate \(^75\) (12 g, 90% from morphine) as a red gum; \(\delta^1(\text{CDCl}_3)\) 2.15, 2.62 (q, TsOAr-H), 3.35 (q, 1,2-H), 7.55 (s, N-CH\(_3\)), 7.73 (s, Ar-CH\(_3\)).

3-O-Acetylmorphine toluene-p-sulphonate (12 g.) was heated under reflux \(^73\) for 4 hr. with a mixture of glacial acetic acid (58 ml.) and water (500 ml.). The reaction mixture was made alkaline using conc. ammonium hydroxide then extracted with ethyl acetate (4 x 50 ml.). The solvent was evaporated in vacuo to give a mixture (7.2 g.) which was treated, in water (700 ml.) and sodium hydrogen carbonate (70 g.), with acetic anhydride (35 ml.) to give the 3-O-acetyl derivatives. \(^74\) The slow removing fraction (\(R_f\) 0.15 on alumina plates developed in chloroform) was isolated by chromatographing on neutral grade V alumina (230 g.) in chloroform. The isolated 3-O-acetyl derivative (2.46 g.), \(\nu\) \(_{\text{max.}}\) 1758 cm\(^{-1}\), was hydrolysed by treating an aqueous-methanol (1:1) (20 ml.) solution with 2N sodium hydroxide (8 ml.). Solid carbon dioxide was added to adjust to pH-7;
extraction with chloroform-propan-2-ol (9:1) (6 x 20 ml.) and evaporation gave a colourless oil (1.98 g.). Crystallisation from ethyl acetate-methanol gave isomorphine as prisms (0.75 g., 8% from morphine), m.p. 249°C (lit., 251-252°C, [α]_D-164º (c 0.39 in MeOH) (lit. 73-167º in MeOH); T (DC1) 3.23 (s, 1,2-H), 3.98 (m, 7-H), 4.33 (q, J 10 Hz and 2 Hz, 8-H), 5.10 (broad s, 5-H), 5.78 (d, J 5.7 Hz, 6-H), 7.02 (s, N-Me). It was found necessary to dry the compound at 120°C for 24 hr. to remove all traces of methanol after crystallisation.

**Codeine methyl ether.**- The preparation was essentially that described by Mannich with slight modification. Codeine (3.0 g.) in 1N sodium hydroxide solution (15 ml.) at 0°C was treated with dimethyl sulphate (1.98 ml.) and stirred vigorously in ice until all traces of the methylating reagent disappeared. Further 10 N sodium hydroxide (0.99 ml.) and dimethyl sulphate (0.99 ml.) were added and the mixture again stirred. The process was repeated twice more, followed, after 4 hr., by drop-wise addition of a solution of potassium iodide (20 g.) in water (20 ml.). An oil separated which crystallised on cooling and gave after filtration and washing with cold water codeine methyl ether methiodide (4.58 g, 98%), m.p. 238-240°C (lit., 241°C). Iodide ion was exchanged for chloride by shaking with freshly prepared silver chloride (2.01 g.) in water (10 ml.) for 4 hr. The precipitated silver iodide was filtered off through celite, and evaporation of the filtrate gave codeine methyl ether methochloride (3.64 g, 98%), 203-207°C (lit., 208°C). Dry distillation/sublimation (0.5 mm Hg, at 140°C for 2 hr.) of codeine methyl ether methochloride
liberated methyl chloride. The residue and sublimed material were warmed with 2 N hydrochloric acid, then filtered to remove undissolved solid. Basification to pH-10 and extraction with chloroform followed by evaporation in vacuo gave a light brown gum (2.23 g.). The major fraction (Rf 0.65) was isolated as a colourless oil by column chromatography on alumina in chloroform. Crystallisation was from ethanol to give codeine methyl ether (1.74 g, 56%), m.p. 140-141°C (lit., 76; 140-141°C); \[^{1}H\]NMR (CDCl\(_3\)) \[3.45 (q, 1,2-H), 4.25 (m, 7-H), 4.65 (q, J 10 Hz and 2 Hz, 8-H), 5.0 (q, J 6.5 Hz and 1.5 Hz, 5-H), 6.20 (s, ArOCH\(_3\)), 6.50 (s, -OCH\(_3\)), 7.56 (s, N-CH\(_3\)).

Morphine methyl ether. 76 - Sodium ethoxide solution (12.0 ml.), prepared from sodium (4.6 g.) and ethanol (110 ml.) followed by addition of water (57 ml.), was added to morphine (2.5 g.) in ethanol (8.0 ml.). Ether (ca. 20 ml.) was added to the resulting pale yellow solution, and the derived sodium salt of morphine (obtained as a white precipitate) (2.76 g.) was filtered off and dried in vacuo with phosphoric oxide. A stirred ice-cooled suspension of the morphine sodium salt in dry ethanol free, chloroform (15 ml.) was treated drop-wise with a solution of chlorodimethyl ether (1.2 ml.) in chloroform (1.6 ml.). After 1 hr., the solution was washed with 5% sodium hydroxide (10 ml.), dried over sodium sulphate and evaporation of the chloroform in vacuo gave a yellow gum (Rf 0.55 on alumina plates in chloroform) which showed only slight contamination by t.l.c. Chromatography on a column of alumina (60 g.) in chloroform gave pure 3-O-methoxymethylmorphine (0.86 g.) as a colourless
The methoxymethylmorphine was treated with hydrogen peroxide (2 ml.) in a platinum basin, and slightly warmed. A yellow solution resulted after 12 hr. at room temperature, and the excess of hydrogen peroxide and water was removed in vacuo over phosphoric oxide. The oil formed was dissolved in methanol, then evaporated down to dryness to yield a brown micro-crystallised powder - methoxymethylmorphine $\text{H}_2\text{O}$ (1.03 g); $\Gamma$ (DC1) 3.25 and 3.47 (ABq, $\delta 9$ Hz, 1,2-H), 4.27 (m, 7-H), 4.62 (m, 8-H), 4.80 (s, -O-C-O), 4.92 (d, $\delta 6$ Hz, 5-H), 6.52 and 6.57 (s, N-CH$_3$). The methoxymethylmorphine $\text{H}_2\text{O}$ (1.03 g) in 2N sodium hydroxide solution (5 ml.) was vigorously stirred at 0°C with dropwise addition of dimethyl sulphate (0.7 ml.). Another portion of dimethyl sulphate (0.4 ml.) and 10 N sodium hydroxide was added after 2 hr., and the process repeated after a further 2 hr. The aqueous solution was acidified by addition of 2N sulphuric acid and the hydroiodide salt of the 6-0-methylmorphine $\text{H}_2\text{O}$ was formed by addition of potassium iodide (1.7 g.) in water (5 ml.). The resulting white precipitate was not filtered off as described by Mannich. Instead, the aqueous solution of the hydroiodide was saturated with sulphur dioxide gas, then kept with stirring at 40°C for 2 days. The solution was neutralised with sodium hydrogen carbonate and extracted with chloroform-propan-2-ol ($10 \times 20$ ml.). The extract was dried (MgSO$_4$) and evaporated in vacuo to yield a colourless oil (363 mg.). Crystallisation
of the oil from ethanol gave morphine methyl ether (288 mg, 11%), m.p. 239-240°C (lit., 76°C); \( \gamma \) (DCl) 3.25 (q, 1,2-H), 4.72 (d, J 6 Hz, 5-H), 5.82 (m, 6-H), 6.50 (s, 6-OCH\textsubscript{3}), (s, N-CH\textsubscript{3}); \( \gamma \) (D\textsubscript{2}O/K-\textsuperscript{t}-Butoxide) 3.40 (s, 1,2-H), 4.92 (d, J 6 Hz, 5-H), 6.45 (s, 6-OCH\textsubscript{3}), 7.62 (s, N-CH\textsubscript{3}).

3-0-Benzylmorphine\textsuperscript{103} - Morphine (171 mg.) in ethanol (5 ml.) was heated under reflux for 3 hr. with 2N sodium ethoxide (1 ml.) and benzyl chloride (0.5 ml.). Water (20 ml.) and 5% sodium hydroxide (2 ml.) were added after cooling, and the solution extracted with chloroform (4 x 10 ml.). After drying over magnesium sulphate the solution was evaporated in vacuo to give an oil which was essentially pure (major fraction \( R_f \) 0.18) by t.l.c. on alumina plates developed in chloroform. Purification by chromatography on a column of alumina in chloroform followed by crystallisation from ethyl acetate gave 3-0-benzylmorphine (64 mg, 29%), m.p. 132-134°C (lit.,\textsuperscript{103} 132°C); \( \gamma \) (CDCl\textsubscript{3}) 3.30 and 3.47 (ABq, J 9 Hz, 1,2-H), 4.32 (m, 7-H), 4.78 (m, 8-H), 4.90 (s, Ar-CH\textsubscript{2}-O-), 5.17 (d, J 6 Hz, 5-H), 5.82 (broad m, 6-H), 7.62 (s, N-CH\textsubscript{3}).

Dihydrodesoxycodeine\textsuperscript{71} - Codeine (3.78 g.) was converted into codeine toluene-\( \beta \)-sulphonate which was dried in vacuo at 60°C for 12 hr. The crystalline derivative (4.74 g.) in dry tetrahydrofuran (15 ml.) at 0°C was treated dropwise with a slurry of lithium aluminium hydride (0.5 g.) in tetrahydrofuran (15 ml.) under a dry nitrogen atmosphere. The pink tosylate solution turned yellow during reduction and, after the last traces of lithium aluminium hydride had been washed into the reaction mixture, the whole was heated under gentle reflux for 3 hr. After cooling to room
temperature ether (50 ml.) was added followed cautiously by water to destroy excess lithium aluminium hydride. Celite was added and the mixture filtered, the residue being washed with ether (20 ml.). The filtrate was extracted with 3N hydrochloric acid (8 x 25 ml.) and after basification to pH-10 with aqueous potassium hydroxide, this extract was extracted with chloroform (6 x 25 ml.). The organic extract was dried over magnesium sulphate then evaporated in vacuo to yield a colourless oil (3.42 g.) which contained two components (major fraction R_f 0.80) by t.l.c. (alumina-chloroform).

Chromatography of the major fraction on a column of alumina in chloroform followed by crystallisation from ethanol gave

\[ \Delta^7\text{-desoxycodine} \] (3.31 g., 93%), m.p. 83-84°C (lit., T_l 82-83°C), \([\alpha]_D^0 = 64^\circ (c 0.62 \text{ in } \text{CH}_2\text{OH}) \text{ (lit., } T_l -68^\circ \text{ in } \text{CH}_2\text{OH}); \gamma(\text{CDCl}_3)^{98} 3.45 \text{ (q, } 1,2-\text{H}), 4.30 \text{ (m, 7-H), } 4.67 \text{ (q, } J 8 \text{ Hz and } 2 \text{ Hz, } 8-\text{H),} \\
5.15 \text{ (t, } J 5 \text{ Hz, } 5-\text{H), } 5.75 \text{ (broad s, } 6-\text{H), } 6.20 \text{ (s, } \text{ArOCH}_3), \\
7.58 \text{ (s, } \text{N-CH}_3). \]

The \[ \Delta^7\text{-desoxycodine} \] (989 mg.) in methanol (20 ml.) was hydrogenated (uptake 88.2 ml. \( \text{H}_2 \); calc. 86.1 ml.) using platinum oxide (20 mg.) as catalyst. The mixture was filtered through celite, and evaporation of the solvent gave an oil (1.014 g.) consisting of two components (major fraction R_f 0.70) by t.l.c. (alumina-chloroform). Column chromatography and crystallisation from methanol, of the resulting oil, gave dihydrodesoxycodine (849 mg., 86%), m.p. 103-105°C (lit., T_l 102-105°C); \gamma(\text{CDCl}_3) 3.40 \text{ (q, } 1,2-\text{H), } 5.45 \text{ (t, } J 8 \text{ Hz, } 5-\text{H),} \\
6.20 \text{ (s, } \text{ArOCH}_3), 7.60 \text{ (s, } \text{N-CH}_3). 

\textbf{Dihydrodesoxymorphine.}- Dihydrodesoxycodine (1.08 g.), prepared as described above, was mixed with pyridine hydrochloride (6.0 g.), obtained by passing hydrogen chloride through an ether
solution of pyridine. The mixture was heated at 212-215°C under a nitrogen atmosphere of 15 min., after which the reaction mixture was immediately cooled by addition of water (25 ml.). Non-phenolic material was removed by ether extraction (3 x 10 ml.) after the solution had been made alkaline with 2N sodium hydroxide. The aqueous basic solution was adjusted to pH-6 by addition of 2N hydrochloric acid then extracted with chloroform-propan-2-ol (9:1) (6 x 10 ml.) which was dried over magnesium sulphate. Evaporation of the organic solvent gave a brown gum, a chloroform solution of which was poured through a column of alumina. The colourless oil obtained after evaporation of the solvent was sublimed (0.2 mm of Hg, 170-180°C for 4 hr.) and the derived solid material was dissolved in chloroform. Evaporation of the chloroform in vacuo gave a foam which crystallised with ethyl acetate to give dihydrodesoxymorphine (310 mg, 30%), m.p. 189°C (lit., 188-189°C), [α]_D-68° (c 1.26 in CH₃OH) (lit., [α]_D-67.2 g, 1.31 in ethanol); ν (CDCl₃) 3.25 and 3.40 (ABq, J 9 Hz, 1,2-H), 5.42 (broad m, 5-H), 7.52 (s, N-CH₃).

1-Bromocodeine. 53 - Codeine (1.015 g.) was set aside for 20 hr. in dry pyridine (45 ml.) and acetic anhydride (22.5 ml.); the solution was then evaporated in vacuo. The residue in water (50 ml.) was treated with sodium hydrogen carbonate and the 6-0-acetylcoditne extracted with chloroform (6 x 20 ml.); ν (CDCl₃) 3.32 and 3.47 (ABq, J 9 Hz, 1,2-H), 4.90 (broad s, 5-H), 6.15 (s, ArOCH₃), 7.57 (s, N-CH₃), 7.85 (s, 6-0 Ac). The acetylcodine (1.11 g.) in acetic acid (46 ml.) and acetic anhydride (2.3 ml.) was
treated drop-wise with a solution of bromine (260 mg.) in acetic acid (370 ml.) then kept in the dark at room temperature for 48 hr. Evaporation of the solvents in vacuo followed by basification with aqueous sodium hydrogen carbonate (50 ml.) allowed 6-O-acetyl-l-bromocodeine to be obtained as a gum after extraction with chloroform (5 x 20 ml.); \(\text{^1H}(\text{CDCl}_3)\) 3.27 (s, 2-H), 5.00 (broad s, 5-H), 6.30 (s, ArOCH\(_3\)), 7.70 (s, N-CH\(_2\)), 8.00 (s, 6-0 Ac). The acetyl derivative in aqueous-methanol (1:1) (30 ml.) was treated with 2N sodium hydroxide (5 ml.) and the solution set aside for 5 hr. Neutralisation with solid carbon dioxide was followed by chloroform (5 x 20 ml.) extraction. The solvent was dried over magnesium sulphate and evaporated to give a colourless gum, which crystallised from ethanol to give 1-bromocodeine as needles (946 mg, 73%) m.p. 162-163°C (lit., 104° 161-162°C); \(\text{^1H}(\text{CDCl}_3)\) 3.20 (s, 2-H), 4.30 (q, J 10 Hz and 2 Hz, 7-H), 4.75 (q, J 10 Hz and 2 Hz, 8-H), 5.15 (q, J 6 Hz and 1.5 Hz, 5-H), 5.80 (broad m, 6-H), 6.22 (s, ArOCH\(_3\)), 7.60 (s, N-CH\(_2\)).

1-Bromomorphine.- Morphine (1.02 g.) in pyridine (10 ml.) and acetic anhydride (5 ml.) was heated for 2 hr. on a water bath to yield diacetylmorphine (1.28 g.). The crystalline derivative in acetic acid (48 ml.) and acetic anhydride (3 ml.) was treated drop-wise with a solution of bromine (285 mg.) in acetic acid (380 ml.) then kept in the dark at room temperature for 45 hr. The solvents were evaporated off in vacuo and water (60 ml.) was added to the residue which was made alkaline with sodium hydrogen carbonate and extracted with chloroform-propan-2-on (5 x 20 ml.). The colourless gum.
obtained after evaporation was hydrolysed using aqueous-methanol (1:1) (30 ml.) and 2N sodium hydroxide (5 ml.). After 4 hr. the solution was acidified with 2N hydrochloric acid, then solid sodium hydrogen carbonate carefully added to give pH-6, followed by rapid extraction with chloroform-propan-2-ol (5:1) (5 x 25 ml.). The solvent was dried over magnesium sulphate and evaporated in vacuo to give an off-white solid. This solid was dissolved with warming in 2N hydrochloric acid (ca. 4 ml.), sodium hydrogen carbonate added to pH-7, and after standing at room temperature for 4 hr. the micro-crystalline precipitate filtered off. After drying in vacuo at 100°C the isolated product (380 mg., 28% from morphine), m.p. > 250°C was shown to be 1-bromomorphine in the following way. The compound (81 mg.) was suspended in methanol (5 ml.) and treated at 0°C with ethereal diazomethane solution (ca. 3 ml.), then stirred at room temperature for 12 hr. The product, isolated by chromatography on a column of alumina and crystallised from ethanol, was 1-bromocodeine (78 mg.), m.p. 160-131°C (lit., 161-162°C); \( \tau \) (CDCl\(_3\)) 3.15 (s, 2-H), 4.30 (q, \( j \) 10 Hz and 2 Hz, 7-H), 4.75 (q, \( j \) 10 Hz and 2 Hz, 8-H), 5.15 (d, \( j \) 6 Hz, 5-H), 5.80 (broad m, 6-H), 6.20 (s, ArOCH\(_3\)), 7.57 (s, N-CH\(_3\)).

Preparation of Radiolabelled Precursors.

\( [2-^{3}H] \)Morphine.- This material was prepared on numerous occasions and the following is typical of the method used. A solution of anhydrous morphine (0.48 g.) in dry dimethylformamide (2 ml.) was treated, in the exchange tube, with tritiated water (3.6 mCi/mmol.) (0.5 ml., 100 mCi). The tube
was flushed with dry, carbon dioxide and oxygen free, nitrogen for 1 hr., then sealed. The morphine was dissolved by heating the tube on a hot plate, then the solution was heated in boiling water for 100 hr., cooled and poured into water (10 ml.). The crystallised morphine was collected (410 mg.), washed with cold water, and dried in vacuo at 100°C. The specific activity of [2-\(^3\)H]morphine, obtained in this way, was typically 1.4 mCi/mmol.

\[\text{[2-}^{3}\text{H]}\text{Codeine:}\]

The methylation of [2-\(^3\)H]morphine was also repeated several times; the following method was typical.

(i) Trimethylanilinium Ethoxide.- Methyl toluene-p-sulphonate (9.3 g.) and dimethyl aniline (6.05 g.) were heated at 120°C to yield the trimethylanilinium toluene-p-sulphonate salt as an ice blue solid. The salt (5.0 g.) was shaken with 1N sodium ethoxide solution (16.3 ml.), prepared from sodium (1.15 g.) and dry ethanol (50 ml.), for 12 hr. under anhydrous conditions. The methylating solution (trimethylanilinium ethoxide) was obtained by filtration through celite under strictly anhydrous conditions. The concentration of the methylating reagent was determined by titration in water against 0.1N hydrochloric acid using methyl orange as indicator (normally 1 ml. contained 1.15 \times 10^{-3} \text{ mol. of methylating reagent}).

(ii) Methylation of [2-\(^3\)H]Morphine.- A suspension of [2-\(^3\)H]morphine (0.4 g. s.a. 1.43 mCi/mmol.) in toluene, after azeotroping to remove water, was cooled to ca. 14°C and treated with twice the required volume of methylating reagent.
reagent (2.4 ml., 2 mol.), then stirred for 15 min. After the morphine had dissolved the solution was heated until ethanol distilled over (ca. 75°C) under reflux (ca. 111°C) for 10 mins. The solution was cooled and filtered through celite then extracted with warm (ca. 60°C) 2N sulphuric acid (5 x 25 ml.). The acid extract was basified to pH-10 by addition of aqueous potassium hydroxide then steam distilled to remove traces of dimethyl aniline. The aqueous basic solution was extracted with chloroform (5 x 25 ml.), which was washed with water, then dried over sodium sulphate. The solvent was evaporated in vacuo to give a colourless oil which contained codeine (Rf 0.40 alumina-chloroform) as the major fraction by t.l.c.; a by-product was also present (Rf 0.65). Chromatography on a column of alumina in chloroform followed by crystallisation from benzene gave [2-3H]codeine (0.253 g., 61%, s.a. 1.43 mCi/mmol.), m.p. 155°C (lit., 156-157°C); (CDCl3)98,100 3.40 (q, 1.2-H), 4.30 (q, J 10 Hz and 2 Hz, 7-H), 4.75 (q, J 10 Hz and 2 Hz, 8-H), 5.15 (d, J 6 Hz, 5-H), 5.85 (broad m, 6-H), 6.15 (s, ArOCH3), 7.56 (s, N-CH3).

[2-3H]Dihydrocodeine.- [2-3H]Codeine (44 mg. s.a. ca. 1.3 mCi/mmol.) in methanol (10 ml.) was hydrogenated (uptake 4.6 ml. H2; calc. 3.5 ml.) using 10% palladised charcoal (6 mg.) as catalyst. The catalyst was removed by filtration through celite, and evaporation of the solvent gave a colourless oil (31 mg., 70%, s.a. 1.209 mCi/mmol). The oil was purified via the picrate salt, but could not be induced to crystallise. However, the n.m.r. spectrum and t.l.c. of the product were identical with those of the radioinactive dihydrocodeine.

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[2-3H]Isocodeine.—[2-3H]Codeine (102 mg, s.a. 1.45 mCi/mmol.) was converted into the correspondingly labelled codeine toluene-$p$-sulphonate. The derivative (149 mg, s.a. ca. 1.4 mCi/mmol.) in benzene (5 ml.) was treated with a solution of hexadecyltrimethylammonium acetate (164 mg.) in benzene (5 ml.) then stirred for 12 hr. at room temperature. The solvent was evaporated, water (20 ml.) added, and the mixture carefully (swirling rather than shaking to avoid formation of an emulsion) extracted with benzene (4 x 5 ml.). Separation of the aqueous and organic layers was achieved by centrifuging. The benzene solvent was evaporated off and the product purified by p.l.c. on alumina plates (two 20 cm x 20 cm) developed in chloroform. In this way 6-O-acetyl-[2-3H]isocodeine was obtained as a colourless oil (62 mg, 55%); $\delta$ (CDCl$_3$) 3.40 (q, 1,2-H), 4.23 (m, 7-H), 4.82 (d, $\tilde{J}$ 6 Hz, 8-H), 5.25 (broad s, 5-H), 6.18 (s, ArOCH$_3$), 7.58 (s, N-$CH_3$), 8.00 (s, 6-0Ac). The [2-3H]isocodeine acetate (50 mg.) in aqueous-ethanol (1:1) (7 ml.) was treated with 2N sodium hydroxide (2 ml.) and set aside for 12 hr. The solution was neutralised with solid carbon dioxide then extracted with chloroform (4 x 5 ml.). The solvent was dried over magnesium sulphate and evaporated in vacuo to yield an oil which after chromatography on a column of alumina in chloroform was crystallised from ethyl acetate to give [2-3H]isocodeine (33.8 mg, 34% from codeine, s.a. 1.212 mCi/mmol.) m.p. 170-172°C (lit., 99 172-173°C); the t.l.c. of the product was identical with that of radioinactive material.

[2-3H]Codeine methyl ether.—All the fast moving fractions (R$_f$ 0.65) obtained from the [2-3H]morphine methylations were
combined. The total by-product was chromatographed on a column of alumina in chloroform. Crystallisation from methanol gave [2-\textsuperscript{3}H]codeine methyl ether (46 mg, s.a. 1.294 mCi/mmol.), m.p. 139-140°C (lit., 76 140-141°C); \((\text{CDCl}_3) 3.45 (q, 1,2-H), 4.25 (m, 7-H), 4.65 (q, J 10 Hz and 2 Hz, 8-H), 5.0 (q, J 6 Hz and 2 Hz, 5-H), 6.20 (s, ArOCH\textsubscript{3}), 6.50 (s, OCH\textsubscript{3}), 7.56 (s, N-CH\textsubscript{3}). From each methylation of [2-\textsuperscript{3}H]morphine typically 26 mg of labelled codeine methyl ether (ca. 7%) was obtained.

[2-\textsuperscript{3}H]Dihydrodesoxycodeine.- [2-\textsuperscript{3}H]Codeine toluene-\(\beta\)-sulphonate (181 mg.), obtained from labelled codeine (258 mg, s.a. 1.24 mCi/mmol.), in dry tetrahydrofuran (5 ml.) was treated at 0°C under a dry nitrogen atmosphere with a slurry of lithium aluminium hydride (30 mg.) in tetrahydrofuran (5 ml.). After the initial reaction, the solution was heated under reflux for 4 hr. with stirring. The reaction mixture was cooled then ether (10 ml.) added followed by careful addition of water, to destroy excess of lithium aluminium hydride. The mixture was filtered through celite, and the filtrate extracted with 2N hydrochloric acid (6 x 5 ml.). The aqueous acid solution was adjusted to pH-10 by addition of aqueous potassium hydroxide, then extracted with chloroform (4 x 10 ml.) which, after drying over magnesium sulphate, was evaporated in vacuo to yield a colourless gum. Chromatography on a column of alumina in chloroform gave [2-\textsuperscript{3}H]-\(\Delta^7\)-desoxycodeine (108 mg.) which was hydrogenated (uptake 12.6 ml. of H\textsubscript{2}; calc. 9.6 ml.) in methanol using platinum oxide (20 mg.) as catalyst. Catalyst was removed by filtration through celite and the product chromatographed on a column of alumina in chloroform. Crystallisation from methanol gave [2-\textsuperscript{3}H]dihydrodesoxycodeine (99 mg, 42% from codeine, 123.
s.a. 0.818 mCi/μmol.), m.p. 103°C (lit., 102-105°C); again the n.m.r. spectrum and t.l.c. of the product were identical with those of the radioinactive sample.

l-Bromo-[2-3H]codeine.-[2-3H]Codeine acetate, prepared from [2-3H]codeine (145 mg, s.a. 1.28 mCi/mmol.), in acetic acid (6 ml.) and acetic anhydride (0.6 ml.) was treated drop-wise with a solution of bromine (40 mg.) in acetic acid (25 ml.) then kept in the dark at room temperature for 48 hr. The solvents were evaporated off and the residue in water (30 ml.) was made alkaline with sodium hydrogen carbonate and extracted with chloroform (4 x 15 ml.). After drying, the chloroform was evaporated in vacuo to give an oil (174 mg.) which was hydrolysed in aqueous-methanol (1:1) (10 ml.) by treatment with 2N sodium hydroxide. After neutralisation with solid carbon dioxide and evaporation the residue in water (20 ml.) was extracted with chloroform (4 x 15 ml.). The oil, obtained after evaporation, was purified by chromatographing on a column of alumina in chloroform. Crystallisation from ethanol gave l-bromo-[2-3H]codeine (132 mg, 72% from codeine, s.a. 0.929 mCi/mmol.), m.p. 162-163°C (lit., 162-163°C); both the n.m.r. spectrum and the t.l.c. of the product were identical with those of the radioinactive sample.

[2-3H]Isocodeine methyl ether.- [2-3H]Codeine toluene-p-sulphonate (188 mg, s.a. ca. 1.3 mCi/mmol.) in dry methanol (8 ml.) was treated with 0.43N sodium methoxide (3 ml.), then heated under reflux for 10 hr. The solvent was evaporated off and the residue, in water (10 ml.), extracted with chloroform (4 x 10 ml.). Evaporation of the solvent in vacuo gave a gum which, even after chromatography and purification via the picrate, could not be
induced to crystallise. Distillation in vacuo and also sublimation as suggested by Small and Browning did not produce crystalline material. Thus, $[\text{2-}^3\text{H}]\text{Isocodeine methyl ether (118 mg, 80}\%\ 1.091\ \text{mCi/mmol.})$ was obtained as an oil; $\tau(CDCl_3)$ 3.40 (q, 1,2-H), 4.15 (m, 7-H), 4.38 (q, $J\ 9\ \text{Hz}$ and $2\ \text{Hz}$, 8-H), 5.20 (s, 5-H), 6.17 (s, ArOCH$_3$), 6.63 (s, 6-OCH$_3$), 7.59 (s, N-CH$_3$).

$[\text{2-}^3\text{H}]\text{Dihydroisocodeine methyl ether.}$ $[\text{2-}^3\text{H}]\text{Isocodeine methyl ether (48 mg, s.a. 1.2 mCi/mmol.)}$ was hydrogenated (uptake 5.2 ml. $H_2$; calc. 3.8 ml.) in methanol (20 ml.) using 10% palladised charcoal (10 mg.). The catalyst was removed by filtration and the solvent evaporated to yield a colourless gum which, in methanol (2 ml.), was treated with a methanolic (3 ml.) solution of picric acid (40 mg.). The derived picrate was recrystallised from ethanol (m.p. 174°C decomp.) and pure $[\text{2-}^3\text{H}]\text{dihydroisocodeine methyl ether recovered from the salt by pouring a chloroform solution through a column of alumina.}$

Crystallisation from ethanol gave $[\text{2-}^3\text{H}]\text{dihydroisocodeine methyl ether (29 mg, 59}\%\ 1.138\ \text{mCi/mmol.}),$ m.p. 116-117°C; $\tau(CDCl_3)$ 3.32 (q, 1,2-H), 5.60 (d, $J\ 8\ \text{Hz}$, 5-H), 6.12 (s, ArOCH$_3$), 6.54 (s, 6-OCH$_3$), 7.61 (s, N-CH$_3$).

$[\text{2-}^3\text{H}]\text{Dihydroisocodeine.}$ $[\text{2-}^3\text{H}]\text{Isocodeine (62 mg, s.a. ca. 1.2 mCi/mmol.)}$ in methanol (10 ml.) was hydrogenated (uptake 6.3 ml. of $H_2$; calc. 5.3 ml.) using 5% palladised charcoal (20 mg.). Filtration and evaporation of the solvent gave a colourless gum which was purified via the picrate. Crystallisation from ethanol gave $[\text{2-}^3\text{H}]\text{dihydroisocodeine (43 mg, 70}\%\ 1.015\ \text{mCi/mmol.}),$ m.p. 197-198°C (lit.,$^{105}$ 199-200°C); $\tau(CDCl_3)^{98}$ 3.32 (t, 1,2-H) 5.65 (d, $J\ 8\ \text{Hz}$, 5-H), 6.15.

125.
(s, ArOCH$_3$), 7.60 (s, N-CH$_3$).

$^{14}$C-Codeine.- This material was obtained as the hydrochloride from the Radio-Chemical Centre, Amersham, with s.a. $^{14}$C $48$ mCi/mmol.

Cultivation of Plants.

The seeds of *P. somniferum*, Halle variety, were sown during March in good soil. The plants used during the 1970 season were cultivated in a greenhouse which was maintained at ca. 18°C. The plants were watered (using rain water) each day, and thinned out about every two weeks. Those plants used during the 1969 season were grown in a sheltered garden (kindly supplied by G.W.K.). The plants normally flowered about the middle of July and the precursor feedings, full details of which have been given in Chapter II, were carried out later in the month.

Ion Exchange Separation of Phenolic and Non-Phenolic Alkaloids.

(i) Preparation of Resin.- The anion exchange resin (50 g.), De-Acidite FF (Permutit Co.Ltd., SRA 71, 100-200 mesh), in the chloride form was stirred with 0.5N sodium hydroxide solution (100 ml.) then, after ca. 10 min., the mixture was filtered. The resin, after continuous washing with deionised water until the filtrate was neutral to pH paper, was stirred with a saturated solution of sodium chloride (100 ml.). The resin suspension was again filtered and washed with deionised water until the washings were neutral. This whole cycle of exchange and washing was repeated three more times and finally, after the resin had been obtained in the hydroxide form, was stirred

126.
with aqueous-methanol (1:1 (100 ml.). After filtering off the
methanol solution the resin (ca. 30 g.) was slurried into a
glass column (2 x 20 cm.) using aqueous-methanol.

(ii) Separation of Radioinactive Codeine and Morphine.-
Codeine (33 mg.) was mixed with morphine (29 mg.) then dissolved
in 2N hydrochloric acid (5 ml.) followed by basification to pH 12
with aqueous potassium hydroxide. The basic solution was poured
onto the ion exchange column which was eluted with aqueous-methanol
(1:1) (150 ml.) to give the non-phenolic alkaloid. The column of
resin was then eluted with 0.5N hydrochloric acid (250 ml.) to
remove the phenolic alkaloid. The acid solution was neutralised
by addition of sodium hydrogen carbonate and the solvents
evaporated in vacuo. Water (20 ml.) was added to the residue then
extraction with chloroform-propan-2-ol (9:1) (6 x 30 ml.) and
drying over magnesium sulphate gave, after evaporation in vacuo,
morphine (27 mg., 94% recovery). The aqueous methanol solution
was evaporated to dryness, water (20 ml.) added and the solution
extracted with chloroform-propan-2-ol (1:1) (9:1) to give codeine
(31 mg., 96% recovery). The above method was repeated in detail for
separating the material isolated from the plants into phenolic
and non-phenolic fractions.

(iii) Separation of [2-3H]Morphine and Radioinactive Codeine.-
Morphine (27.186 mg) was mixed with [2-3H]morphine (3.128 mg,
s.a. 1.32 mCi/mmol.) then crystallised from methanol to give
[2-3H]morphine, s.a. 6.03 x 10^5 dis/min/mg and total activity
183 x 10^5 dis/min. Radioinactive codeine (52.551 mg.) was
added and the two alkaloids separated using the ion exchange method.
The isolated codeine (52.3 mg, 99% recovery) had s.a. 497 dis/min/mg

127.
and total activity $0.2599 \times 10^5$ dis/min representing 0.142% contamination by morphine. Morphine (27.9 mg, 92% recovery) had s.a. $6.49 \times 10^5$ dis/min/mg.

(iv) Separation of $[2-^3H]$Codeine and Radioinactive Morphine.-

$[2-^3H]$Codeine (5.162 mg, s.a. 1.03 mCi/mmol) was diluted with radioinactive codeine (42.461 mg.) and the total crystallised from benzene to give $[2-^3H]$codeine (38.438 mg.) of s.a. $8.76 \times 10^5$ dis/min/mg and total activity $33.7 \times 10^6$ dis/min. Radioinactive morphine (42.741 mg) was added and the ion exchange separation carried out. Morphine (38.659 mg, 91% recovery) was isolated with s.a. 98 dis/min/mg and total activity $37.9 \times 10^2$ dis/min, representing $1.13 \times 10^{-2}$% contamination by codeine.

Work-Up of Precursor Feeding Experiments.

The method of isolating the crude alkaloids from the plants was described in Chapter II.

$[2-^3H, N\text{-methyl}^{14}C]$Codeine Feeding:-

The total alkaloids extracted (62 mg.) from the plant contained codeine together with a trace of thebaine by t.l.c. on alumina and morphine by t.l.c. on basic silica. A volumetric count suggested a recovery of at least 23% of the radioinactivity fed, based on $^3H$. Radioinactive codeine (29.39 mg.) and morphine (24.9 mg.) were added to the material extracted from the plant and the mixture dissolved in 0.5N hydrochloric acid (20 ml.) and then basified to pH-12 by addition of 1N sodium hydroxide. The basic solution was extracted with ether (4 x 10 ml.) to remove the non-phenolic alkaloids, mainly codeine and some thebaine. Solid carbon dioxide was added to adjust the pH to 7 - 8 and the aqueous solution extracted with chloroform-propan-2-ol.

128.
(10 x 10 ml.) to remove the phenolic alkaloid morphine.

Non-Phenolic Alkaloids.—The ether solution was dried and evaporated in vacuo to give a residue which, by t.l.c. on alumina, contained mainly codeine and some thebaine. The residue was chromatographed on a column of alumina in chloroform to give codeine (22.3 mg.) which was diluted with radioinactive codeine (16.4 mg; total 38.7 mg.) then crystallised four times from benzene. After each crystallisation the specific activity of the codeine was determined and showed that the $^{3}\text{H}/^{14}\text{C}$ ratio decreased and differed from the value calculated for the precursor (see Appendix -Table 1). The $^{3}\text{H}/^{14}\text{C}$ ratio was determined using two methods as follows. The first involved addition of ethanol (ca. 2 ml.) to the small amount of precursor solution, remaining from the feeding, then a small volume was removed and a volumetric count determined (gave ratio as 5.42:1). The more accurate method involved addition of radioinactive codeine (ca. 60 mg.) to the ethanol solution of the precursor followed, after evaporation of the ethanol, by crystallisation of the codeine from benzene, m.p. 155-156$^\circ$C (lit., 156-157$^\circ$C) and specific activity determination (gave ratio as 5.32:1). After the four crystallisations the $^{3}\text{H}/^{14}\text{C}$ ratio for the codeine recovered from the plants did not compare well with that of the original precursor, and even after the mother liquors and crystalline codeine were recombined and chromatographed on a column of alumina the crystallised codeine had a ratio of 10.92:1. Thus the total pure codeine (38.7 mg.) was further diluted with a larger amount of radioinactive codeine (146.6 mg;
total 185.3 mg.). Crystallisation from benzene to constant specific activity (Table 1. - Appen.) gave codeine, m.p. 155-157°C (lit. 156-157°C) with $^3$H s.a. $150 \times 10^2$ dis/min/mg. and $^{14}$C s.a. $26.4 \times 10^2$ dis/min/mg. i.e. $^3$H/$^{14}$C ratio 5.69.

Calculation of Codeine Recovery:

(i) Recovery of [2-$^3$H]codeine.

Total $^3$H activity as codeine = $\frac{150 \times 10^2 \times 185.3 \times 29.39}{22.3}$ dis/min.

Total $^3$H activity fed = $0.0578 \times 2.2 \times 10^9$ dis/min. — (Table 1 Chapter II)

% $^3$H activity recovered as codeine = $\frac{150 \times 185.3 \times 29.39 \times 10^4}{22.3 \times 0.578 \times 2.2 \times 10^9}$

= 2.88%

(ii) Recovery of [N-methyl-$^{14}$C]codeine.

Total $^{14}$C activity isolated as codeine = $\frac{26.4 \times 10^2 \times 185.3 \times 29.39}{22.3}$ dis/min.

Total $^{14}$C activity fed = $0.0578 \times 2.2 \times 10^9$ dis/min.

% $^{14}$C activity recovered as codeine = $\frac{26.4 \times 185.3 \times 29.39 \times 5.32 \times 10^4}{22.3 \times 0.0578 \times 2.2 \times 10^9}$

= 2.69%

The radiochemical purity of the isolated codeine was demonstrated via the picrate salt which was recrystallised from ethanol, m.p. 196-197°C (lit., 196-197°C). Pure codeine was obtained, in the normal way, by pouring a chloroform solution of the salt through a column of alumina.

Phenolic Alkaloids.— The green coloured phenolic extract (26 mg.) was dissolved in 2N hydrochloric acid (5 ml.) then, after basification to pH12 with aqueous potassium hydroxide was poured down the ion exchange column, as described previously, and completely colourless morphine (16.1 mg, 65% recovery) was isolated.
Radioinactive morphine (36.5 mg; total 52.6) was added and crystallisation to constant specific activity from methanol gave morphine, m.p. 245-247°C (lit., 247-248°C) with \(^3\text{H}\) s.a. 162 \(\times 10^2\) dis/min/mg i.e. 46.2 \(\times 10^5\) dis/min/mmol, and \(^{14}\text{C}\) s.a. 29.0 \(\times 10^2\) dis/min/mg i.e. 82.7 \(\times 10^4\) dis/min/mmol. The diacetyl derivative of the morphine was prepared and after crystallisation from ethyl acetate, m.p. 171-173°C (lit., 173°C) gave the molar specific activity as: \(^3\text{H}\) = 47.0 \(\times 10^5\) dis/min/mmol, \(^{14}\text{C}\) = 82.4 \(\times 10^4\) dis/min/mmol.

Calculation of Codeine into Morphine Incorporation:

(i) Incorporation based on \(^3\text{H}\) activity. -
Total \(^3\text{H}\) activity isolated as morphine = \(\frac{162 \times 10^2 \times 52.6 \times 24.9}{\text{dis/min}}\) 16.1%

\(\%\) incorporation based on \(^3\text{H}\) activity = \(\frac{162 \times 52.6 \times 24.9 \times 10^4}{16.1 \times 0.0578 \times 2.2 \times 10^9}\)

= 1.04%

(ii) Incorporation based on \(^{14}\text{C}\) activity.-
Total \(^{14}\text{C}\) activity isolated as morphine = \(\frac{29.0 \times 10^2 \times 52.6 \times 24.9}{\text{dis/min}}\) 16.1%

\(\%\) incorporation based on \(^{14}\text{C}\) activity = \(\frac{29.0 \times 52.6 \times 24.9 \times 3.2 \times 10^4}{16.1 \times 0.0578 \times 2.2 \times 10^9}\)

= 0.99%

The fact that \([2-^3\text{H}]\)morphine did not exchange \(^3\text{H}\) during its diacetylation was shown in the following way. \([2-^3\text{H}]\)Morphine (ca. 20 mg, molar s.a. 40.3 \(\times 10^7\) dis/min/mmol.) in dry distilled pyridine (4 ml.) was treated with acetic anhydride (2 ml.) then heated on a water bath for 3 hr. The solvents were evaporated off, and the solid chromatographed on a column of alumina in chloroform. Crystallisation from ethyl acetate
gave diacetylmorphine (22 mg.), m.p. 171-172°C (lit., 173°C) with water s.a. 42.4 x 10^7 dis/min/mmol).

Single Labelled \([2-^3H]\)Dihydrocodeine Feeding:-

The plants (wet weight 109 g.) gave a total alkaloid extract (38.2 mg.) which, by volumetric count, suggested a 44% recovery of \(^3\)H activity. Radioinactive codeine (28.2 mg.), dihydrocodeine (32.03 mg.) [total non-phenolic alkaloids 60.23 mg.], morphine (33.5 mg.), and dihydromorphine (40.2 mg.) [total phenolic alkaloids 73.7] were added for dilution and separation into phenolic and non-phenolic alkaloid fractions achieved using the ion exchange procedure.

Non-Phenolic Alkaloids.- The non-phenolic alkaloids were initially separated to give dihydrocodeine and codeine using p.l.c. on alumina plates (20 x 20 cm.) impregnated with silver nitrate and developed in methanol (respective \(R_f\) values 0.2 and 0.3). Subsequent separations were using basic silica plates developed in methanol (respective \(R_f\) values 0.5 and 0.4).

Dihydrocodeine Fraction.- Initial separation gave dihydrocodeine (29.6 mg.) which was pure by t.l.c. on basic silica; volumetric count showed that the total activity present was 326 x 10^5 dis/min. Attempts to crystallise the dihydrocodeine (27.7 mg.) failed even after addition of further radioinactive dihydrocodeine (37.9 mg; total 67.5 mg.). A chloroform solution of the alkaloid was poured through a column of alumina to remove traces of silver nitrate originating from the initial separation. The gum, obtained after evaporation of solvent, was sublimed (2mm. of Hg, ca. 140°C) and the resulting oil eventually crystallised from ether to give dihydrocodeine of constant \(^3\)H specific activity 4.84 x 10^5 dis/min/mg. 132.
Recovery of \([2-^3\text{H}]\)dihydrocodeine.

Total \(^3\text{H}\) activity isolated as dihydrocodeine = \(\frac{4.84 \times 10^5 \times 67.5 \times 32.03}{27.7} \) dis/min

Total \(^3\text{H}\) activity fed (Table I. Chapter II) = 0.044 \times 2.2 \times 10^9 dis/min

\% recovery of dihydrocodeine = \(\frac{4.84 \times 67.5 \times 32.03 \times 10^7}{27.7 \times 0.044 \times 2.2 \times 10^9} \)

= 39%

Codeine Fraction.- Codeine (28.1 mg.) isolated initially was shown, by t.l.c. on basic silica, to be contaminated with dihydrocodeine and volumetric count showed the total activity present was 287 \(\times 10^4\) dis/min. More radioinactive codeine (39.9 mg; total 68.0 mg.) was added, and after rechromatographing on basic silica plates the recovered codeine (65.6 mg.) showed a total activity of \(\text{ca.} \ 714 \times 10^3\) dis/min by volumetric count and the presence of some dihydrocodeine by t.l.c. Further chromatography on basic silica plates gave codeine (45.9 mg.) with total activity \(807 \times 10^2\) dis/min. Silicic acid was removed from the codeine by pouring a chloroform solution through a column of alumina, and the recovered material (23.3 mg.) was diluted with radioinactive codeine (17.7 mg; total 41.0 mg.) and mixed with dihydrocodeine (30.4 mg.). Separation on basic silica plates gave codeine (34.0 mg.) with total \(^3\text{H}\) activity \(353 \times 10^2\) dis/min. A second wash with radioinactive dihydrocodeine (29.4 mg.) produced codeine (32.0 mg.) with an estimated total activity of \(215 \times 10^2\) dis/min by volumetric count. Treatment on a column of alumina removed silicic acid and crystallisation from benzene to constant specific activity gave codeine, m.p. 156-157 (lit., \(101\) 156-157) with \(^3\text{H}\) s.a. 920 dis/min/mg. (actual determinations gave; 148 cp.m. for 0.367 mg., and 131 cp.m. for 0.322 mg. of the codeine).
The [2-3H]dihydrocodeine precursor was shown (see later) to contain 0.735% [2-3H]codeine. Thus total activity of [2-3H]codeine present in precursor (see Table 1. Chapter II) = 0.735 x 4.4 x 2.2 x 10^5 dis/min.

Total 3H activity isolated as codeine = \(\frac{920 \times 41.0 \times 67.9}{23.3}\)

% recovery of contaminating [2-3H]codeine = \(\frac{920 \times 41.0 \times 67.9 \times 10^{-3}}{0.735 \times 4.4 \times 2.2 \times 23.3}\)

= 15.4%

The codeine was shown to be radiochemically pure via the picrate which was recrystallised from ethanol, m.p. 194-196°C (lit., 196-197°C) and the regenerated codeine showed, after crystallisation, 3H specific activity of 925 dis/min/mg.

**Phenolic Alkaloids.** The alkaloids, dihydromorphine and morphine, from ion exchange separation (61.8 mg, 100% recovery) were separated by pl.c. on basic silica plates developed in methanol (respective R_f values were 0.5 and 0.4).

**Dihydromorphine Fraction.** The dihydromorphine (36.6 mg.) isolated showed, by t.l.c. on basic silica, that some morphine was present. A wash with radioinactive morphine (30.0 mg.) gave dihydromorphine (29.9 mg.) which was treated with water (10 ml.), then extracted with chloroform-propan-2-ol (4 x 5 ml.) to give dihydromorphine (24.4 mg.) which was freed from silicic acid. Dilution with radioinactive material (16.9 mg.; total 41.3 mg.) followed by crystallisation from ethanol to constant specific activity gave the dihydromorphine, m.p. 155-157°C (lit., 155-157°C) with 3H s.a. 72.2 x 10^2 dis/min/mg, i.e. 20.7 x 10^5 dis/min/mmol. The diacetyl derivative was formed (method as for morphine) which crystallised from ethyl acetate, m.p.
164-166°C (lit. 165-167°C) molar specific activity 20.8 x 10^5 dis/min/mmol.

Calculation of dihydrocodeine into dihydromorphine incorporation:

\[
\text{Total } ^3\text{H activity isolated as dihydromorphine} = \frac{72.2 \times 10^2 \times 41.3 \times 40.2 \times 10^4}{24.4 \times 0.044 \times 2.2 \times 10^9}
\]

% incorporation = \[
\frac{72.2 \times 41.3 \times 40.2 \times 10^4}{24.4 \times 0.044 \times 2.2 \times 10^9}
\]

= 0.51%

Morphine Fraction.- Morphine (32.1 mg.) obtained from the initial separation was pure by t.l.c., and the total activity present estimated from the volumetric count was 443 x 10^2 dis/min. The separation gave material (31.1 mg.) with total activity 155 x 10^2 dis/min. The alkaloid was extracted from aqueous solution to remove silicic acid and crystallised from ethanol to constant specific activity to give morphine, m.p. 247-248°C (lit., 247-248°C) with ^3H s.a. 525 dis/min/mg. i.e. 15.0 x 10^4 dis/min/mmol. (actual determinations after correction for background gave; 102 cpm for 0.437 mg, and 208 cpm from 0.909 mg. of the morphine). The diacetyl derivative was formed and crystallised from ethyl acetate, m.p. 171-172°C (lit., 173°C), molar specific activity 14.6 x 10^4 dis/min/mmol. Thus,

\[
\text{Total } ^3\text{H activity isolated as morphine} = 525 \times 33.5 \text{ dis/min.}
\]

% incorporation of contaminating [2-^3H]codeine into morphine

\[
\frac{525 \times 33.5}{0.735 \times 4.4 \times 2.2 \times 10^3}
\]

= 2.46%

Dilution Analysis on [2-^3H]Dihydrocodeine Precursor.- The precursor material, [2-^3H]dihydrocodeine (28.1 mg, s.a. 1.209 mCi/mmol; total 2.48 x 10^8 dis/min), was mixed with radioinactive
codeine (31.9 mg.) and the codeine reisolated by p.l.c. on basic silica plates. The codeine (30.5 mg.) isolated was mixed with radioinactive dihydrocodeine (29.2 mg.) and the separation, in which a narrow band associated with codeine was cut from the plate, was repeated. Crystallisation from benzene to constant specific activity gave codeine (24.9 mg.), m.p. 156°C (lit., 156-157°C), with specific activity $56.9 \times 10^3$ dis/min/mg, total $182 \times 10^4$ dis/min representing 0.735% contamination. Radiochemical purity of the codeine was demonstrated via the picrate (gave s.a. $56.7 \times 10^3$ dis/min/mg.).

**Double-Labelled [2-^3H]Dihydrocodeine Feeding:**

The total alkaloids (43.4 mg.) extracted from the plants were diluted with codeine (37.3 mg.), dihydrocodeine (41.4 mg.) [total non-phenolic 78.7 mg.], morphine (32.1 mg.), and dihydromorphine (35.3 mg.) [total phenolic 67.4 mg.]. Separation by ion exchange gave non-phenolic alkaloids (92.6 mg.) and phenolic alkaloids (37.3 mg, 56% recovery). The $^3$H/$^{14}$C ratio for the precursor was determined by volumetric count and found to be 3.62:1, i.e. 0.0119 mCi of $^{14}$C activity was fed.

**Non-Phenolic Alkaloids.** - The alkaloids were separated by p.l.c. on basic silica and gave codeine (38.2 mg.) and dihydrocodeine (43.0 mg.) which by t.l.c. (basic silica-methanol) showed a trace of contamination by each other.

**Codeine Fraction.** - The small amount of contaminating dihydrocodeine was removed by p.l.c. on basic silica, however, the recovered codeine (24 mg.) still showed slight $^3$H activity. Radioinactive dihydrocodeine (22.2 mg.) was added and chromatography gave codeine (18.7 mg.) which showed only $^{14}$C activity. Dilution
with radioinactive codeine (18.5 mg.; total 37.2 mg.) followed by crystallisation to constant specific activity from benzene gave codeine, m.p. 154-155°C (lit., 156-157°C), with only $^{14}$C present, s.a. $68.6 \times 10^3$ dis/min/mg. Formation of the picrate and regeneration of the free base gave codeine with $^{14}$C s.a. $68.0 \times 10^3$ dis/min/mg.

Recovery of $[\text{N-methyl-}^{14}\text{C}]$codeine.-
Total $^{14}$C activity isolated as codeine=$68.6\times10^3\times37.2\times37.3$ dis/min.

\[
\text{% recovery} = \frac{68.6\times10^3\times37.2\times37.3 \times 10^5}{18.7 \times 0.0119 \times 2.2 \times 10^9}
\]

\[
= 19.4\%
\]

Dihydrocodeine Fraction.- After one further separation on basic silica plates the recovered dihydrocodeine (40.0 mg.) was mixed with radioinactive codeine (42.0 mg.) and then rechromatographed. The recovered dihydrocodeine (25.8 mg.) was pure and showed only $^3$H activity and was diluted with radioinactive material (65.0 mg., total 90.8 mg.). Many attempts were made at direct crystallisation but all were without success; eventually the material was sublimed (2.4 mm of Hg, 153°C) and the resultant oil seeded with radioinactive dihydrocodeine to give a crystalline product. The process was repeated twice and the derived dihydrocodeine showed a $^3$H s.a. $21.0 \times 10^4$ dis/min/mg.

Recovery of $[2-^3\text{H}]$dihydrocodeine.-
Total $^3$H activity isolated as dihydrocodeine=$21.0\times10^4 \times 90.8 \times 41.1$ dis/min.

\[
\text{% recovery} = \frac{21.0\times90.8\times41.1 \times 10^6}{25.8 \times 0.0434 \times 2.2 \times 10^9}
\]

\[
= 31.8\%
\]
Phenolic Alkaloids.- Ion exchange separation gave low recovery of the total phenolic alkaloids (37.3 mg.), thus radioinactive morphine (21.4 mg.; total 40.0 mg.) and dihydromorphine (30.1 mg.; total 48.7 mg.) were added. It was assumed that an equal amount of each of the phenolic alkaloids was present in the total isolated i.e. 18.6 mg. The alkaloids were again separated by p.l.c. on basic silica plates developed in methanol.

Dihydromorphine Fraction.- After the initial separation the dihydromorphine contained traces of morphine and volumetric count showed mainly $^{3}$H but a small amount of $^{14}$C activity. Radioinactive morphine (30 mg.) was added, and the recovered dihydromorphine (31.6 mg.) was pure by t.l.c. showing only $^{3}$H activity by volumetric count. Extraction gave dihydromorphine (29.1 mg.), free from silicic acid, and crystallisation from ethanol to constant specific activity gave the alkaloid, m.p. 153-156°C (lit., 67 155-157), with $^{3}$H s.a. $60.3 \times 10^{2}$ dis/min/mg. i.e. $17.3 \times 10^{5}$ dis/min/mmol. The diacetyl derivative crystallised from ethyl acetate, m.p. 166-168°C (lit., 108 165-167°C) and had a molar specific activity of $17.2 \times 10^{5}$ dis/min/mmol.

Incorporation of dihydrocodeine into dihydromorphine.-

Total $^{3}$H activity isolated as dihydromorphine=$60.3x10^2x48.7x35.3$ dis/18.6 min

% incorporation

$$\frac{60.3x48.7x35.3x10^4}{18.6x0.0434x2.2x10^9} = 0.59\%$$

Morphine Fraction.- After initial separation, the morphine was found to be contaminated with a small amount of dihydromorphine. A second separation gave morphine (35.8 mg.), pure by t.l.c., but still containing some $^{3}$H activity and this was removed by washing.
with radioinactive dihydromorphine (38.4 mg.). Silicic acid was removed to give morphine (21.6 mg.) which was then diluted with radioinactive material (11.7 mg.; total 33.3 mg.). Crystallisation from methanol to constant specific activity gave morphine, m.p. 243-246°C (lit., 102 247-248), with $^{14}$C s.a. $31.3 \times 10^2$ dis/min/mg. i.e. $8.92 \times 10^5$ dis/min/mmol.

The diacetyl derivatives of the morphine crystallised from ethyl acetate and gave m.p. 173°C (lit., 107 173°C) molar specific activity $9.12 \times 10^5$ dis/min/mmol.

Incorporation of $[^{14}$C$]$codeine into morphine. -

Total $^{14}$C activity isolated as morphine = $31.3 \times 10^2 \times 33.3 \times 40.0 \times 32.1$ dis/min/21.6 x 18.6 = $31.3 \times 33.3 \times 40.0 \times 32.1 \times 10^4$

% incorporation = $\frac{31.3 \times 33.3 \times 40.0 \times 32.1 \times 10^4}{21.6 \times 18.6 \times 0.0119 \times 2.2 \times 10^9}$ = 1.26%.

Exchange of $^3$H label in $[2-^3$H]Diacetyldihydromorphine from Double-Label Feeding.- The $[2-^3$H]diacetyldihydromorphine (15.7 mg., s.a. $46.0 \times 10^2$ dis/min/mg.; total $72.3 \times 10^3$ dis/min.) obtained from the double-labelled feeding was diluted with radioinactive material (51.6 mg., s.a. $10.7 \times 10^2$ dis/min/mg.) then treated, in an exchange tube, with an aqueous (2 ml.) methanol (0.5 ml.) solution of potassium carbonate (51.2 mg., 3 mol.). After flushing, with oxygen and carbon dioxide free nitrogen, the tube was sealed and heated at ca. 100°C for 119 hr. After cooling, the tube was opened and the contents poured into water. Extraction with chloroform-propan-2-ol, followed by crystallisation from ethanol to constant specific activity gave dihydromorphine (34.1 mg.); the $^3$H specific activity was

139.
$54\text{ dis/min/mg.}$ representing $96.1\%$ loss of $^3\text{H}$ activity.

**Single-Labelled $[2-^3\text{H}]$Isocodeine Feeding:**

The plants (wet weight $84\text{ g.}$) gave a total alkaloid extract ($122\text{ mg.}$) which was diluted with radioinactive samples of codeine ($88.9\text{ mg.}$), isocodeine ($63.6\text{ mg.}$) [total non-phenolic $152.5\text{ mg.}$], morphine ($49.4\text{ mg.}$), and isomorphine ($84.4\text{ mg.}$) [total phenolic $133.8\text{ mg.}$]. The ion exchange separation was utilised.

**Non-Phenolic Alkaloids.** The total phenolic fraction isolated ($192.0\text{ mg.}$) was found by t.l.c. to contain both codeine and isocodeine. The fraction was coloured green presumably by contaminating plant material which explained the additional material recovered above the weight of dilution compounds ($152.5\text{ mg.}$). Separation of the two non-phenolic alkaloids was by chromatography on a column of alumina in chloroform. This system gave good separation since the $R_f$ of isocodeine is 0.1 and of codeine 0.4.

**Codeine Fraction.** The codeine ($77.4\text{ mg.}$) isolated from the column chromatography was pure by t.l.c. and showed negligible $^3\text{H}$ activity by volumetric count. Crystallisation from benzene to constant specific activity gave codeine, m.p. $154-156\degree\text{C}$ (lit., $101\degree-102\degree\text{C}$), with $^3\text{H}$ s.a. 76 dis/min/mg. (actual determinations gave; 46 cpm. for 1.396 mg., 34 cpm. for 1.007 mg. of the codeine). Because of the very small amount of $^3\text{H}$ activity present no proof of radiochemical purity was sought.
Estimate of \([2-^{3}H]\)isocodeine incorporation into (or: contamination of) codeine.-

Total \(^{3}\text{H}\) activity isolated as codeine = 76 \times 88.9 \text{ dec/min}.

Total \(^{3}\text{H}\) activity fed = \(0.0374 \times 2.2 \times 10^{9}\) \text{ dis/min}

Estimated max \% incorporation

\[
= \frac{76 \times 88.9 \times 10^{2}}{0.0374 \times 2.2 \times 10^{9}}
\]

\[
= 8.1 \times 10^{-3}\% 
\]

\text{i.e. } (8.1 \times 10^{-3}\%)

Isocodeine Fraction.- Isocodeine (56.0 mg.) isolated by column chromatography was, from t.l.c., pure and free from codeine. Direct crystallisation to constant specific activity from ethyl acetate gave isocodeine, m.p. 170-171\(^{\circ}\text{C}\) (lit., 171-172\(^{\circ}\text{C}\)), with \(^{3}\text{H}\) s.a. \(121 \times 10^{3}\) dis/min/mg. No suitable derivative is available for proving the radiochemical purity of the isocodeine but the value of specific activity given above remained constant over three crystallisations.

Recovery of \([2-^{3}H]\)isocodeine.-

Total \(^{3}\text{H}\) activity isolated as isocodeine = \(121 \times 10^{3} \times 63.6\) \text{ dec/min}.

\% recovery

\[
= \frac{121 \times 63.6 \times 10^{5}}{0.0374 \times 2.2 \times 10^{9}}
\]

\[
= 9.4\% 
\]

Phenolic Alkaloids.- The ion exchange separation gave the phenolic alkaloid fraction which contained about equal amounts of morphine and isomorphine by t.l.c. on basic silica (respective \(R_{f}\) values 0.4 and 0.2). The alkaloids were converted into their \(3-O\text{-acetyl}\) derivatives\(^{74}\) for separation by treating the phenolic fraction (92.4 mg.) in aqueous (9.3 ml.) sodium bicarbonate (920 mg.) with acetic anhydride (0.47 ml.). Chloroform (4 x 10 ml.) extraction and solvent evaporation gave 141.
the two 3-O-acetyl-derivative (113 mg.) which were separated by chromatographing on a column of neutral grade V alumina.

**Isomorphine Fraction:** The 3-O-acetylisomorphine (48.1 mg.) isolated after separation showed a $^3$H specific activity of $3.89 \times 10^3$ dis/min/mg., and total activity $18.7 \times 10^4$ dis/min by volumetric count. The t.l.c. showed the 3-O-acetylisomorphine to be pure, however, radioinactive 3-O-acetylmorphine (44.5 mg.) was added and the mixture rechromatographed. The recovered 3-O-acetylisomorphine (28.2 mg.) was pure from t.l.c. and the estimated total activity from the volumetric count gave $14.18 \times 10^4$ dis/min. The compound was further purified by p.l.c. on alumina plates, then hydrolysed in aqueous-methanol (1:1)(10 ml.) by treatment with 2N sodium hydroxide (2 ml.) to give isomorphine (26.5 mg.) which was diluted with radioinactive material (36.6 mg.; total 59.1 mg.). Crystallisation to constant specific activity from methanol-ethyl acetate gave isomorphine, m.p. 253-225°C (lit., 102 251-252°C), with $^3$H s.a. $11.6 \times 10^2$ dis/min/mg. i.e. $331 \times 10^3$ dis/min/mmol. The isomorphine was treated with diazomethane to yield isocodeine which was crystallised from ethyl acetate, m.p. 171-172°C (lit., 99 171-172°C), and molar specific activity $329 \times 10^3$ dis/min/mmol.

Incorporation of [2-$^3$H]isocodeine into isomorphine.-

Total $^3$H activity isolated as isomorphine = $11.6 \times 10^2 \times 59.1 \times 84.4 \times 10^4$ dis/min

\[
\% \text{ incorporation} = \frac{11.6 \times 59.1 \times 84.4 \times 10^4}{26.5 \times 0.0374 \times 2.2 \times 10^9}
\]

\[
= 0.27\%
\]
From other experiments the average value for conversion of codeine into morphine is 1.06% which gives for the above an efficiency (i.e. $\frac{X'}{X} \rightarrow \frac{Y'}{Y}$ value) of 25.1% C.f. value from double-labelled feeding was 15.2%.

Morphine Fraction.—After initial separation the isolated 3-0-acetylmorphine (11.1 mg.) appeared pure by t.l.c., a volumetric count estimated the $^3$H specific activity as $13.7 \times 10^2$ dis/min/mg. and total activity $15.2 \times 10^3$ dis/min. The 3-0-acetylmorphine was diluted with radioinactive material (29.5 mg.; total 40.6 mg.) and "washed" using radioinactive 3-0-acetylisomorphine (32.3 mg.). The recovered alkaloid (24.2 mg.) was hydrolysed with aqueous methanolic sodium hydroxide to give morphine (23.3 mg.) which was further diluted with radioinactive morphine (10.5 mg.; total 33.8 mg.). Crystallisation from methanol to constant specific activity gave the morphine, m.p. 244-246°C (lit., 247-248°C), which was essentially radioinactive, i.e. $^3$H s.a. 68 dis/min/mg. (actual determinations gave; 17 cpm for 0.631 mg, and 20 cpm for 0.721 mg. of the morphine).

Estimate of [2-3H]isocodeine incorporation into morphine.—
(or [2-3H]isomorphine contamination of morphine)

Total $^3$H activity isolated as morphine=$68 \times 33.8 \times 40.6 \times 49.4 \times 10^2$ dis/min

Estimated max % incorporation

$$\frac{68 \times 33.8 \times 40.6 \times 49.4 \times 10^2}{23.2 \times 11.1 \times 0.0374 \times 2.2 \times 10^9}$$

= 0.021%

i.e. < 0.02%
Double-Labelled \([2-{^3}H]\)Isocodeine Feeding:

The alkaloid extract (37.7 mg.) was diluted with radio-inactive samples of codeine (64.9 mg.), isocodeine (50.1 mg.) [total non-phenolic 115.0 mg.], morphine (78.9 mg.), and isomorphine (46.5 mg.) [total phenolic 125.4 mg.]. The non-phenolic (135.7 mg.) and phenolic (105.8 mg., 85%) fractions were obtained by ion exchange chromatography.

Non-Phenolic Alkaloids.- These alkaloids were separated to give codeine and isocodeine by chromatographing on a column of alumina in chloroform. The \( ^3H/^{14}C \) ratio was determined for the precursor (described previously) and found to be 3:13:1 i.e. 0.0126 mCi of \(^{14}C\) activity were fed.

Codeine Fraction.- The codeine recovered after separation (61.9 mg.) was found, by volumetric count, to contain mainly \(^{14}C\) activity and also a total \(^3H\) activity of \(38.1 \times 10^6\) dis/min. Thus the codeine was mixed with radioinactive isocodeine (55.6 mg.) and the alkaloids reseparated. The codeine (42.9 mg.) recovered showed negligible \(^3H\) activity to be present. Crystallisation to constant specific activity from benzene gave codeine, m.p. 152-154°C (lit., 101 156-157°C), with \(^{14}C\) s.a. \(69.0 \times 10^3\) dis/min/mg. and \(^3H\) s.a. 144 dis/min/mg. The codeine obtained after picrate formation had \(^{14}C\) s.a. \(69.0 \times 10^3\) dis/min/mg.

(i) Recovery of \([N\text{-methyl}-^{14}C]\)codeine.-
Total \(^{14}C\) activity isolated as codeine = \(69.0 \times 10^3 \times 64.9\) dis/min.

\[
\text{% recovery} = \frac{69.0 \times 64.9 \times 10^5}{0.0126 \times 2.2 \times 10^9} = 16.2%.
\]
(ii) Estimate of [2-\textsuperscript{3}H]isocodeine incorporation into (or contamination by) codeine.-

Total \textsuperscript{3}H activity isolated as codeine= 144x64.9 dis/min.

Total \textsuperscript{3}H activity fed = 0.0396x2.2x10\textsuperscript{9} dis/min.

Estimated max. \% incorporation = \frac{144x64.9x10^2}{0.0396x2.2x10^9} = 1.006x10^2 \%

i.e. <0.01%.

Isocodeine Fraction.- Separation gave isocodeine (59.1 mg.) which was pure by t.l.c. and volumetric count showed mainly \textsuperscript{3}H activity but also a total \textsuperscript{14}C activity of 16.5 x 10\textsuperscript{4} dis/min.

Radioininactive codeine (52.6 mg.) was added and separation gave isocodeine (42.4 mg.), pure by t.l.c., showing a total \textsuperscript{14}C activity of 1032 dis/min. Crystallisation from ethyl acetate to constant specific activity gave isocodeine, m.p. 170-172\textdegree C (lit., 171-172\textdegree C), having \textsuperscript{3}H s.a. 55.9 x 10\textsuperscript{4} dis/min/mg. and \textsuperscript{14}C s.a. 36 dis/min/mg. The \textsuperscript{3}H specific activity remained constant over three successive crystallisations.

(i) Recovery of [2-\textsuperscript{3}H]isocodeine.-

Total \textsuperscript{3}H activity isolated as isocodeine= 55.9x10\textsuperscript{4}x50.1 dis/min.

\% recovery = \frac{55.9x50.1x10^6}{0.0396x2.2x10^9} = 32%.

(ii) Estimate of [N-methyl-\textsuperscript{14}C]codeine incorporation (contamination) into isocodeine.-

Total \textsuperscript{14}C activity isolated as isocodeine = 36x50.1 dis/min.

Estimated max. \% incorporation = \frac{36x50.1x10^2}{0.0126x2.2x10^9} = 3.73x10^{-3}%

i.e. <0.003%
Phenolic Alkaloids.- The total phenolic alkaloids (105.8 mg.) were converted into their 3-O-acetyl-derivatives and separated by chromatographing on a column of neutral grade V alumina, to give 3-O-acetylisomorphine and 3-O-acetylmorphine.

Morphine Fraction.- The 3-O-acetylmorphine was obtained pure after the column chromatography, and showed no \(^{3}\text{H}\) activity to be present. Hydrolysis using aqueous methanolic sodium hydroxide followed by extraction gave morphine (41.1 mg.). Crystallisation to constant specific activity was from methanol and gave morphine, m.p. 247-248°C (lit., 102 247-248°C), having \(^{14}\text{C}\) s.a. 32.0 x 10\(^3\) dis/min/mg., i.e. 9.12 x 10\(^6\) dis/min/mmol. and no \(^{3}\text{H}\) activity. The diacetyl derivative was formed and crystallised from ethyl acetate to give material, m.p. 174-175°C (lit., 107 173°C), with molar specific activity 8.98 x 10\(^6\) dis/min/mmol.

Incorporation of \([N\text{-methyl-}\text{\textsuperscript{14}}\text{C}]\text{codeine into morphine.}\) Total \(^{14}\text{C}\) activity isolated as morphine =32.0x10\(^3\)x78.9 dis/min.

\[
\text{% incorporation} = \frac{32.0 \times 78.9 \times 10^5}{0.0126 \times 2.2 \times 10^9} = 9.1\%
\]

Isomorphine Fraction. - A volumetric count on the 3-O-acetylisomorphine (31.0 mg.), obtained after the initial separation, gave the total \(^{3}\text{H}\) activity as 10.9 x 10\(^5\) dis/min and total \(^{14}\text{C}\) activity as 5.77 x 10\(^4\) dis/min. The fraction was diluted with radioinactive 3-O-acetylisomorphine (24.6 mg.; total 55.6 mg.), then rechromatographed on neutral grade V alumina. The recovered 3-O-acetylisomorphine (37.5 mg.) still showed \(^{14}\text{C}\) activity and was mixed with radioinactive
3,2-acetylmorphine (32.4 mg.) then reseparated. The isolated 3,2-acetylisomorphine (10.5 mg.) was free from $^{14}$C activity and after further dilution with radioinert material (55.6 mg; total 66.1 mg.) was hydrolysed in aqueous methanolic sodium hydroxide to give isomorphine (63.3 mg.). Crystallisation to constant specific activity was from ethyl acetate-methanol and gave isomorphine, m.p. 247-250 (lit., 251-252°C), with $^3$H s.a. $2.2 \times 10^2$ dis/min/mg. and $^{14}$C s.a. 25 dis/min/mg.

(i) Incorporation of [2-$^3$H]isocodeine into isomorphine.-

Total $^3$H activity isolated as isomorphine = 22.1 × $10^2$ × 66.1 × 55.6 × 46.5 dis/10.5 × 31.0 min

% incorporation = $2.2 \times 66.1 \times 55.6 \times 46.5 \times 10^4$

10.5 × 31.0 × 0.02962.2 × 10^9

= 1.38%.

(ii) Estimate of [N-methyl-$^{14}$C]codeine incorporation into isomorphine.- (or $^{14}$C contamination of isomorphine)

Total $^{14}$C activity isolated as isomorphine = 25 × 66.1 × 55.6 × 46.5 dis/10.5 × 31.0 min

Estimated max.% incorporation = $2 \times 66.1 \times 55.6 \times 46.5 \times 10^2$

10.5 × 31.0 × 0.0126 × 2.2 × 10^9

= 0.047%

i.e. < 0.05%

The isomorphine could not be shown to be radiochemically pure since no suitable derivative is available. Methylation, as in the single labelled experiment, to give isocodeine, was discounted since it would then not be possible to exchange the $^3$H label at C-2. Removal of the $^3$H label was necessary to demonstrate that the precursor had been incorporated intact.
The specific activity of the isomorphine isolated from this latter experiment was constant over three successive crystallisations, and the incorporation was 15.2% efficient (i.e. \( x' \rightarrow y' \)) which is in reasonable agreement with the value estimated for the single-labelled feeding (see earlier).

Exchange of \(^3\text{H}\) label in \([2-^{3}\text{H}]\text{Isomorphine from Double-Labelled Feeding.}\) The isolated \([2-^{3}\text{H}]\text{isomorphine (24.3 mg.;} \text{s.a. 22.1 x 10}^2 \text{dis/min/mg.}) was diluted with radioactive alkaloid (24.7 mg.; giving 49.0 mg. with} \text{s.a. 11 x 10}^2 \text{dis/min/mg.}). After treating with aqueous (2 ml.) methanolic (0.5 ml.) potassium carbonate (28 mg.) in an exchange tube the resultant solution was heated to 100°C for 106 hr. The recovered isomorphine (32.4 mg.) had a \(^3\text{H}\) specific activity of 61 dis/min/mg., representing a 94.9% loss of the original activity present.

Single-Labelled \([2-^{3}\text{H}]\text{Codeine Methyl Ether Feeding.-}\) After extraction from the plants (wet weight 83 g.) the total alkaloids (47.0 mg.) were diluted with radioactive samples of codeine (40.0 mg.), codeine methyl ether (47.8 mg.) [total non-phenolic 87.8 mg.], morphine (40.2 mg.) and morphine methyl ether (42.3 mg.) [total phenolic 82.5 mg.]. The fractions were separated by ion exchange chromatography.

Non-Phenolic Alkaloids.- This fraction (93.1 mg.) was found, by t.l.c., to contain both codeine and codeine methyl ether. Separation and purification of these alkaloids was achieved by p.l.c. on alumina plates developed in chloroform, giving initially codeine (39.8 mg.) and codeine methyl ether (50.0 mg.)

148.
Codeine Fraction. - The isolated codeine (39.8 mg; estimated
\( ^{3} \)H s.a. 4.20 x \( 10^{4} \) dis/min/mg, total 16.7 x \( 10^{5} \) dis/min.) was
mixed with radioactive codeine methyl ether (38.2 mg.) and
reisolated. The codeine (30.4 mg; estimated \( ^{3} \)H s.a. 3.30
x \( 10^{4} \) dis/min/mg, total 10.0 x \( 10^{5} \) dis/min.) recovered still
showed \( ^{3} \)H activity by volumetric count, and was therefore washed
a second time with radioactive codeine methyl ether (70.5 mg.).
The recovered codeine (26.8 mg; estimated \( ^{3} \)H s.a. 0.94 x \( 10^{4} \)
dis/min/mg, total 2.52 x \( 10^{5} \) dis/min) was chromatographed on
a column of alumina, and the isolated material (22.8 mg.) was
diluted with radioactive codeine (24.7 mg; total 47.5 mg.).
Crystallisation to constant specific activity from benzene gave
codeine, m.p. 155°C (lit., 101 156-157°C), with \( ^{3} \)H s.a. 108 x
\( 10^{2} \) dis/min/mg. The picrate was formed, m.p. 196-197°C (lit., 106
196-197°C), and the resulting codeine had \( ^{3} \)H s.a. 108 x \( 10^{2} \)
dis/min/mg.

Incorporation of [2-\( ^{3} \)H]codeine methyl ether into codeine.-
Total \( ^{3} \)H activity isolated as codeine=\( 108 x 10^{2} x 47.5 x 40.0 \)
dis/min
\( \frac{22.8}{22.8} \)
Total \( ^{3} \)H activity fed
=0.0466x2.2x10^{9} \text{ dis/min.}
\( =108x47.5x40.0x10^{4} \)
\( 22.8x0.0466x2.2x10^{9} \)
= 0.88%.

Codeine Methyl Ether Fraction. - The codeine methyl ether
(50.0 mg.) isolated from the initial separation was 'washed' with
radioactive codeine (51.0 mg.), and the recovered alkaloid
(36.4 mg.) was diluted with radioactive codeine methyl ether
(29.9 mg.; total 66.3 mg.). Crystallisation to constant
specific activity from ethanol gave codeine methyl ether, m.p.

149.
139-141°C (lit., 76 140-141°C), with $^3$H s.a. $19.4 \times 10^4$ dis/min/mg.

The picrate was formed, m.p. 182-184, and regeneration of the free base on a column of alumina gave codeine methyl ether with $^3$H s.a. $19.3 \times 10^4$ dis/min/mg.


Total $^3$H activity recovered as codeine MeO $=19.4 \times 10^4 \times 66.3 \times 47.8 \times 10^6$ dis/min.

\[
\% \text{ incorporation} = \frac{19.4 \times 66.3 \times 47.8 \times 10^6}{36.4 \times 0.0466 \times 2.2 \times 10^9} = 16.4\%
\]

Phenolic Alkaloids. - The total phenolic alkaloids (64.0 mg., 79%) obtained were separated to give morphine and morphine methyl ether by p.l.c. on alumina plates developed in n-butanol/di-n-butyl ether/ammonium hydroxide (v:v:25:70:5). The separation was not very good although the alkaloids obtained were fairly pure. All the subsequent separations were carried out using the solvent system n-butanol/di-n-butyl ether/acetic acid (v:v:40:50:10).

Morphine Fraction. - The morphine isolated initially (38.9 mg.) was mixed with radioinactive morphine methyl ether (40.0 mg.) and the separation effected. The morphine (41.8 mg.) recovered was contaminated (from t.l.c.) by morphine methyl ether and was rechromatographed on alumina. It was not possible to crystallise directly the recovered morphine (29.5 mg.) and radioinactiv material (19.0 mg.; total 48.5 mg.) was added. Even then crystallisation was very difficult and eventually effected to constant specific activity using methanol, and gave morphine, m.p. 246-247°C (lit., 247-248°C), with $^3$H s.a. 748 dis/min/mg.
i.e. \(2.15 \times 10^5\) dis/min/mm. The diacetyl derivative was formed and crystallised from ethyl acetate, m.p. 171-173°C (lit., 107-173°C), with molar specific activity \(2.07 \times 10^5\) dis/min/mm.

Incorporation of \([2-^3\text{H}]\)codeine methyl ether into morphine.

Total \(^3\text{H}\) activity isolated as morphine = \(748 \times 48.5 \times 10^2\) dis/min

\[
\% \text{ incorporation} = \frac{748 \times 48.5 \times 10^2}{29.5 \times 0.0466 \times 2.2 \times 10^3} = 0.048%.
\]

Morphine Methyl Ether Fraction.

Morphine methyl ether isolated from the initial separation (40.1 mg.) was mixed with radioinactive morphine (40.0 mg.) and rechromatographed. The recovered morphine methyl ether (36.0 mg.) was pure except for some contamination by acetic acid. Therefore the alkaloid was dissolved in 0.5N hydrochloric acid (10 ml.), filtered, and after basification to pH 8 with sodium hydrogen carbonate morphine methyl ether (23.3 mg.) was extracted with chloroform-propan-2-ol; dilution with radioinactive material (23.8 mg.; total 47.1 mg.) was carried out. Crystallisation to constant specific activity from ethanol gave morphine methyl ether, m.p. 243°C (lit., 76 242°C), with \(^3\text{H}\) s.a. \(12.6 \times 10^3\) dis/min/mg. Radiochemical purity of the morphine methyl ether was demonstrated via the picrate which was crystallised from ethanol, m.p. 264°C (decomp.). Morphine methyl ether, obtained from the picrate by pouring a chloroform-propan-2-ol (1:1) solution through a column of alumina, had a \(^3\text{H}\) specific activity of \(12.2 \times 10^3\) dis/min/mg.

Incorporation of \([2-^3\text{H}]\)codeine methyl ether into morphine.

151.
methyl ether.

Total $^3$H activity isolated as morphine methyl ether = $12.2 \times 10^{-3} \times 47.1 \times 42.5$ dis/min

\[
\% \text{ incorporation} = \frac{12.2 \times 47.1 \times 2.3 \times 10^5}{23.3 \times 0.0466 \times 2.2 \times 10^9}
\]

= 1.02%

Double-Labelled [2-$^3$H]Codeine Methyl Ether Feeding:

The alkaloid extract (26.1 mg.) from the plants was diluted with radioactive codeine (71.7 mg.), codeine methyl ether (59.3 mg.) [total non-phenolic 121.0 mg.], morphine (36.2 mg.), and morphine methyl ether (37.2 mg.) [total phenolic 73.4 mg.].

Phenolic and non-phenolic fractions were obtained by ion exchange chromatography. The $^3$H/$^{14}$C ratio for the precursor was determined by volumetric count and found to be 2.87:1, i.e. 0.0107 mCi of $^{14}$C activity were fed.

Non-Phenolic Alkaloids.- The non-phenolic alkaloid fraction (137.3 mg.), recovered from the ion exchange separation, contained both codeine and codeine methyl ether. As with the single-labelled experiment these alkaloids were separated by p.l.c. on alumina plates and gave codeine (56.2 mg.) and codeine methyl ether (55.5 mg.).

Codeine Fraction.- Volumetric count of the codeine (56.2 mg.), recovered from the initial separation, showed that both $^3$H and $^{14}$C activity were present. Thus a first wash with radioactive codeine methyl ether (54.2 mg.) was carried out and gave codeine (44.5 mg.) which again showed $^3$H and $^{14}$C activity using volumetric count methods. A second wash with radioactive codeine methyl ether (43.0 mg.) was carried out and the recovered codeine still contained $^3$H and $^{14}$C activity. The alkaloid was further purified by chromatographing on a column of alumina (37.0 mg., codeine
recovered) and then diluted with radioinactive codeine (28.8 mg.; total 65.8 mg.). Crystallisation to constant specific activity from benzene gave codeine, m.p. 155-157°C (lit., 156-157°C), with $^{14}C$ s.a. $26.3 \times 10^3$ dis/min/mg, and $^3H$ s.a. $23.8 \times 10^2$ dis/min/mg. Formation of the picrate, m.p. 197°C, (lit., 196-197°C), and treatment on a column of alumina gave codeine with $^{14}C$ s.a. $26.9 \times 10^3$ dis/min/mg, and $^3H$ s.a. $25.8 \times 10^2$ dis/min/mg.

(1) Recovery of [N-methyl-$^{14}C]$codeine.

Total $^{14}C$ activity isolated as codeine $= \frac{26.9 \times 10^3 \times 65.8 \times 71.7}{37.0}$ dis/min.  

% recovery $= \frac{26.9 \times 65.8 \times 71.7 \times 10^5}{37.0 \times 0.0107 \times 2.2 \times 10^9}$  

= 14.6%

(ii) Incorporation of [2-$^3H$]codeine methyl ether into codeine.

Total $^3H$ activity isolated as codeine $= \frac{25.8 \times 10^2 \times 65.8 \times 71.7}{37.0}$ dis/min.  

Total $^3H$ activity fed $= 0.0309 \times 2.2 \times 10^9$ dis/min.  

% incorporation $= \frac{25.8 \times 65.8 \times 71.7 \times 10^4}{37.0 \times 0.0309 \times 2.2 \times 10^9}$  

= 0.49%

Codeine methyl ether Fraction.- The volumetric count of the codeine methyl ether (55.5 mg.), isolated after the initial separation, gave the $^3H$ s.a. $22.3 \times 10^4$ dis/min/mg. and $^{14}C$ s.a. $11.4 \times 10^2$ dis/min/mg. i.e. $^3H/^{14}C$ ratio was 196 (allowance was made for the contribution of the $^3H$ activity to the Channel II count, i.e. 0.055%). Thus the alkaloid was mixed with radioinactive codeine (67.8 mg.) and volumetric count of the recovered codeine methyl ether (32.1 mg.) estimated its $^3H$ s.a. $27.0 \times 10^4$ dis/min/mg. and
$^{14}$C s.a. $10.1 \times 10^2$ dis/min/mg. i.e. $^3$H/$^{14}$C ratio is 268.

After a second wash with radioactive codeine (37.1 mg.) volumetric count of the recovered codeine methyl ether (14.2 mg.) gave $^3$H s.a. $34.2 \times 10^4$ dis/min/mg. and $^{14}$C s.a. $14.7 \times 10^2$ dis/min/mg. i.e. $^3$H/$^{14}$C ratio of 233. The alkaloid was rechromatographed on a column of alumina (the recovery being 9.6 mg.) then diluted with radioactive codeine methyl ether (41.3 mg.; total 50.9 mg.). Crystallisation to constant specific activity from ethanol gave codeine methyl ether, m.p. $142^\circ$ C (lit., 76 $140-141^\circ$ C, with $^3$H s.a. $41.3 \times 10^3$ dis/min/mg, and $^{14}$C s.a. 148 dis/min/mg. (actual corrected determinations, in Channel II gave; 84 cpm for 0.725 mg., and 81 cpm for 0.660 mg. of the codeine methyl ether) i.e. $^3$H/$^{14}$C ratio was 279 which is in reasonable agreement with that obtained throughout the separation stages. Again proof that the codeine methyl ether was radiochemically pure was via the picrate, m.p. $186^\circ$ C decomp., the regenerated alkaloid having $^3$H s.a. $39.1 \times 10^3$ dis/min/mg, $^{14}$C s.a. 136 dis/min/mg. i.e. $^3$H/$^{14}$C ratio was 287.


Total $^3$H activity recovered as codeine MeO=$39.1x10^3x50.9x59.3$ dis/min

\[
\% \text{ recovery} = \frac{39.1x50.9x59.3x10^5}{9.6} \\
= 18.4\%
\]

(ii) Incorporation of $[N$-methyl-$^{14}$C]codeine into codeine methyl ether.

Total $^{14}$C activity isolated as codeine MeO=$136x50.9x59.3$ dis/min.

\[
\% \text{ incorporation} = \frac{136x50.9x59.3x10^2}{9.6x0.0107x2.2x10^9} \\
= 0.182\% 
\]
Phenolic Alkaloids.- The total phenolic alkaloids isolated (75.6 mg.) were separated to give morphine and morphine methyl ether using p.l.o. on alumina plates developed in the n-butanol/di-n-butyl ether/ammonium hydroxide solvent system. However, as indicated for the single-labelled experiment, the recovery of alkaloids was not good, and all subsequent chromatography utilised the acetic acid solvent system.

Morphine Fraction.- The morphine (31.2 mg.) isolated contained a trace of morphine methyl ether, and was therefore chromatographed on alumina plates using the acetic acid solvent system. A volumetric count for the recovered morphine (11.0 mg.) gave the specific activities, \( ^3H \times 207 \times 10^2 \text{ dis/min/mg.} \), and \( ^1C \times 167 \times 10^2 \text{ dis/min/mg.} \). The morphine (11.0 mg.) was diluted with radiolabeled material (16.4 mg.; total 27.4 mg.) and washed with radiolabeled morphine methyl ether (27.1 mg.). Again volumetric count on the recovered morphine (17.1 mg.) gave the specific activities as, \( ^3H \times 559 \text{ dis/min/mg.} \), and \( ^1C \times 24.4 \times 10^2 \text{ dis/min/mg.} \). C.f. values obtained for crystalline alkaloid. Crystallisation to constant specific activity from methanol gave morphine, m.p. 244-246°C (lit., 102 247-248°C), with \( ^3H \) s.a. 563 dis/min/µg. i.e. 16.1 x 10^4 dis/min/mmol., and \( ^1C \) s.a. 23.4 x 10^2 dis/min/µg. i.e. 66.7 x 10^4 dis/min/mmol. The diacetyl-derivative was formed and crystallised from ethyl acetate, m.p. 171-173°C (lit., 107 175°C), having the molar specific activities, \( ^3H \times 16.2 \times 10^4 \text{ dis/min/mmol.} \), \( ^1C \times 64.8 \times 10^4 \text{ dis/min/mmol.} \).

1. [N-methyl-\( ^1C \)]Codeine incorporation into morphine.-

Total \( ^1C \) activity isolated as morphine = \( 23.4 \times 10^2 \times 27.4 \times 36.2 \text{ dis/min/11.0} \).
\[
\% \text{ incorporation} = \frac{23.4 \times 27.4 \times 36.2 \times 10^4}{11.0 \times 0.0107 \times 2.2 \times 10^9} = 0.90\
\]

(ii) Incorporation of \([2-^3\text{H}]\)codeine methyl ether into morphine.

Total \(^3\text{H}\) activity isolated as morphine = \(563 \times 27.4 \times 36.2 \times 10^2\) dis/min.

\[
\% \text{ incorporation} = \frac{563 \times 27.4 \times 36.2 \times 10^2}{11.0 \times 0.0309 \times 2.2 \times 10^9} = 0.08\
\]

Morphine Methyl Ether Fraction.- The morphine methyl ether (25.9 mg.) isolated initially had specific activities, \(^3\text{H}\) \(14.8 \times 10^3\) dis/min/mg, \(^1\text{C}\) \(12.6 \times 10^2\) dis/min/mg. by volumetric count. A 'wash' with radioinactive morphine (28.0 mg.) was carried out, but the recovered morphine methyl ether (14.2 mg.) still showed slight \(^1\text{C}\) activity. Radioinactive alkaloid (15.5 mg.; total 29.7 mg.) was added for dilution, but even after purification via the hydrochloride and also the picrate the morphine methyl ether would not crystallise from ethanol. The recovered morphine methyl ether (15.3 mg.) was diluted with further radioinactive material (38.6 mg.; total 53.9 mg.) and crystallised to constant specific activity from ethanol, m.p. 242°C (lit., 242°C), giving \(^3\text{H}\) s.a. \(14.9 \times 10^2\) dis/min/mg. The picrate, m.p. 269°C (decomp.), was again, as with the single-labelled experiment, used to prove the radiochemical purity of the morphine methyl ether; \(^3\text{H}\) s.a. \(14.6 \times 10^2\) dis/min/mg. and \(^1\text{C}\) s.a. ca. 10 dis/min/mg.

(1) Incorporation of \([2-^3\text{H}]\)codeine methyl ether into morphine methyl ether.-

Total \(^3\text{H}\) activity isolated as morphine Me0 = \(\frac{14.9 \times 10^2 \times 53.9 \times 29.7 \times 37.2 \text{dis}}{15.3 \times 14.2 \text{min}}\).
% incorporation = \frac{14 \times 53.9 \times 29.7 \times 37.2 \times 10^4}{15.3 \times 1^{14.2} \times 2 \times 0.0309 \times 2.2 \times 10^9} = 0.62%

(ii) Estimate of [N-methyl-14C]codeine incorporation into morphine methyl ether.

Total 14C activity isolated as morphine MeO = \frac{10 \times 53.9 \times 29.7 \times 37.2 \text{ dis/ min.}}{15.3 \times 1^{4.2}}

Estimated max. % incorporation = \frac{10 \times 53.9 \times 29.7 \times 37.2 \times 10^2}{15.3 \times 1^{4.2} \times 2 \times 0.0107 \times 2.2 \times 10^9} = 116 \times 10^{-4}

Exchange of 3H Label in [2-3H]Morphine Methyl Ether from Double-Label Feeding.- The isolated [2-3H]morphine methyl ether (36.0 mg., s.a. 14.6 \times 10^2 \text{ dis/min/mg}; total 52.5 \times 10^3 \text{ dis/min}) was diluted with radioactive alkaloid (26.5 mg.; total 62.5 mg., s.a. 840 dis/min/mg.). After treatment with a solution of methanol (0.5 ml.), water (2 ml.) and potassium carbonate (22 mg.) at \text{ca.} 100^\circ \text{C} for 119 hr. the morphine methyl ether (52.1 mg.) isolated showed a 3H specific activity of 28 dis/min/mg. This represented a loss of 96.7% of the 3H activity originally present.

Dilution Analysis on [2-3H]Codeine Methyl Ether Precursor.- The precursor material, [2-3H]codeine methyl ether (14.2 mg., s.a. 1.294 mCi/mmol; total 12.9 \times 10^7 \text{ dis/min}), was mixed with radioactive codeine (17.4 mg.) then the codeine was reisolated by p.l.c. on alumina plates. The codeine (12.6 mg.) was diluted with radioactive alkaloid (20.0 mg., total 32.6 mg.) and then crystallised from benzene to constant specific activity, m.p. 157^\circ \text{C} (\text{lit.,} 156-157^\circ \text{C}), with 3H s.a. 172 dis/min/mg. i.e. a total activity of 77.5 \times 10^2 \text{ dis/min.} This represents a 0.006% contamination of the precursor by codeine and is insignificant.
Double-Labelled \([2-\text{H}]\text{Dihydrodesoxycodeine Feeding.}\)

The plants (wet weight 140 g.) were extracted in the manner described to give the total alkaloids (164.3 mg.) which was diluted with radioactive codeine (95.4 mg.), dihydrodesoxycodeine (58.7 mg.) [total non-phenolic 154.1 mg.], morphine (65.4 mg.), and dihydrodesoxymorphine (73.9 mg.) [total phenolic 139.3 mg.]. The total mixture was treated on a column of ion exchange resin to give the phenolic (88.3 mg, 64%) and non-phenolic (203.7 mg.) alkaloid fractions. The \(^3\text{H}/\text{\(\text{U}\)}\) ratio for the precursor was found by volumetric count to be 4.45:1 i.e. 0.00919 mCi of \(^{14}\text{C}\) activity were fed.

Non-Phenolic Alkaloids.- The non-phenolic alkaloids, codeine and dihydrodesoxycodeine, were separated by p.l.c. on alumina plates developed in chloroform (respective \(R_f\) values 0.40 and 0.70). Good separation was achieved.

Codeine Fraction.- The codeine (98.2 mg.) recovered after initial separation showed only \(^{14}\text{C}\) activity by volumetric count. However, a wash with radioactive dihydrodesoxycodeine (94.6 mg.) was carried out, and the recovered codeine (87.2 mg.) was chromatographed on a column of alumina to give pure alkaloid (86.4 mg.). Crystallisation to constant specific activity from benzene gave codeine, m.p. 156-157°C (lit., 101 156-157°C), with \(^{14}\text{C}\) s.a. 81.7 x 10³ dis/min/mg, and no \(^3\text{H}\) activity. The codeine derived via the picrate, m.p. 195-197°C (lit., 106 196-197°C), gave \(^{14}\text{C}\) s.a. 80.7 x 10³ dis/min/mg.

Recovery of [N-methyl-\(^{14}\text{C}\)]codeine.-

Total \(^{14}\text{C}\) activity isolated as codeine = 80.7 x 10³ x 95.4 dis/min

\[
\text{% recovery} = \frac{80.7 \times 95.4 \times 10^5}{0.00919 \times 2.2 \times 10^9}
\]

\[
\text{158.} = 38.4\% 
\]
Dihydrodesoxycodine Fraction.- The separated dihydrodesoxycodine (85.4 mg.) contained mainly $^3$H activity and some $^{14}$C activity, thus a wash with radioactive codeine (80.2 mg.) was carried out. The recovered dihydrodesoxycodine (28.4 mg.) contained only a small amount of $^{14}$C activity and, after chromatography on a column of alumina the recovered material (22.7 mg.) was diluted with radioactive dihydrodesoxycodine (41.3 mg; total 64.0 mg.). Crystallisation to constant specific activity from methanol gave dihydrodesoxycodine, m.p. 103-104°C (lit. 102-105°C), having $^3$H s.a. $169 \times 10^3$ dis/min/mg. and a max. estimated $^{14}$C s.a. $10^4$ dis/min/mg. The picrate, m.p. 206-207°C (lit., 71 207°C), was formed to prove radiochemical purity and the regenerated dihydrodesoxycodine had a $^3$H s.a. of $167 \times 10^3$ dis/min/mg.

(ii) Recovery of [2-$^3$H]dihydrodesoxycodine.-
Total $^3$H activity isolated as dihydrodesoxycodine = $167 \times 10^3 \times 2.2 \times 10^9$ dis/min.
Total $^3$H activity fed = $0.0407 \times 2.2 \times 10^9$ dis/min.
% recovery $= \frac{167 \times 64.0 \times 58.7 \times 10^5}{22.7 \times 0.0407 \times 2.2 \times 10^9}$
$= 30.5\%$

(ii) Estimate of codeine incorporation (contamination) into dihydrodesoxycodine.-
Total $^{14}$C activity isolated as dihydrodesoxycodine = $10^4 \times 64.0 \times 58.7$
Estimated max % incorporation $= \frac{10^4 \times 64.0 \times 58.7 \times 10^2}{22.7 \times 0.00919 \times 2.2 \times 10^9}$
$= 0.08$
i.e. $\leq 0.08\%$

Phenolic Alkaloids.- The two phenolic alkaloids were initially separated by p.l.c. on basic silica plates developed in methanol.
The slow moving fraction isolated contained mainly dihydrodesoxy-
morphine and some morphine, while the faster moving fraction
contained mainly morphine together with some dihydrodesoxy-
morphine. Both the separation and recovery of alkaloids was
inefficient, but good separation and purification was obtained
using a column of neutral grade V alumina with chloroform as
solvent.

Morphine Fraction.- The initial p.l.c. separation gave the
morphine (37.2 mg.) fraction which contained, by volumetric count,
\(^{14}\text{C}\) activity and a large amount of \(^{3}\text{H}\) activity. Chromatography
on grade V alumina gave morphine (27.5 mg.) which contained only
\(^{14}\text{C}\) activity and was then diluted with radioactive alkaloid
(62.6 mg. total 90.1 mg.). Crystallisation to constant specific
activity from methanol gave morphine, m.p. 246-247\(^{0}\text{C}\) (lit.,\(^{102}\)
247-248\(^{0}\text{C}\)), having \(^{14}\text{C}\) s.a. \(80.9 \times 10^2\) dis/min/mg. i.e. \(230 \times \)
\(10^4\) dis/min/mmol. The diacetyl derivative was prepared, and
crystallisation from ethyl acetate gave the diacetylmorphine,
m.p. 172-173\(^{0}\text{C}\) (lit.,\(^{107}\) 173\(^{0}\text{C}\)), with molar specific activity
\(224 \times 10^4\) dis/min/mmol.

Incorporation of [N-methyl-\(^{14}\text{C}\)]codeine into morphine.-
Total \(^{14}\text{C}\) activity isolated as morphine=\(80.9 \times 10^2 \times 90.1 \times 65.4\) dis/min
\(27.5\)
\(= 8.55\%\)

Dihydrodesoxymorphine Fraction.- Initial separation by p.l.c.
gave the dihydrodesoxymorphine fraction (29.4 mg.), containing, by
t.l.c., some morphine, and by volumetric count some \(^{14}\text{C}\) activity.
After chromatography on grade V alumina the recovered dihydrodesoxymorphine (26.2 mg.), showing mainly $^3\text{H}$ activity, was 'washed' with radioinactive morphine (30.2 mg.) by chromatography on the column of alumina. Dihydrodesoxymorphine (24.0 mg.) was recovered pure, and was diluted with radioinactive material (78.2 mg.; total 102.2 mg.). After repeated attempts at crystallisation only 65.5 mg. remained and this was diluted with radioinactive alkaloid (28.5 mg.; total 94.0 mg.) which was chromatographed on neutral grade III alumina to give dihydrodesoxy­morphine (87.8 mg.). The material was sublimed (0.5 mm. of Hg, 170°C), and crystallised to constant specific activity from ethyl acetate to give dihydrodesoxymorphine, m.p. 186-188°C (lit., 79 188-189°C), having $^3\text{H}$ s.a. $190 \times 10^2$ dis/min/mg. and an estimated $^{14}\text{C}$ s.a. $31$ dis/min/mg. The dihydrodesoxymorphine which was recovered and crystallised, m.p. 187-188°C, after formation of the picrate m.p. 216°C (decomp.), had a $^3\text{H}$ specific activity of $18.4 \times 10^3$ dis/min/mg.

(ii) Incorporation of $[2-^3\text{H}]$dihydrodesoxycodine into dihydrodesoxymorphine.

Total $^3\text{H}$ activity isolated as dihydrodesoxymorphine =

$$\frac{18.4 \times 10^3 \times 94.0 \times 102.2 \times 73.9 \text{ dis/}}{65.5 \times 24.0 \text{ min}}$$

% incorporation =$$\frac{18.4 \times 94.0 \times 102.2 \times 73.9 \times 10^5}{65.5 \times 24.0 \times 0.047 \times 2.2 \times 10^9}$$

= 9.27%.

(ii) Estimate of codeine incorporation (or $^{14}\text{C}$ contamination)

into dihydrodesoxymorphine.

Total $^{14}\text{C}$ activity isolated as dihydrodesoxymorphine =

$$\frac{31 \times 94.0 \times 102.2 \times 73.9 \text{ dis/min.}}{65.5 \times 24.0}$$

161.
Estimated max % incorporation
\[ \frac{21 \times 94.0 \times 102.2 \times 73.9 \times 10^2}{65.5 \times 24.0 \times 0.009 \times 2.2 \times 10^9} = 0.069\% \]

i.e. \( < 0.07\% \)

Exchange of \( ^3\text{H} \) Label in \([\text{2-}^3\text{H}]\text{Dihydrodesoxymorphine}\) from Double-Label Feeding.- The isolated \([\text{2-}^3\text{H}]\text{dihydrodesoxymorphine}\) (66.7 mg., s.a. 18.4 \( \times 10^3 \) dis/min/mg; total 123 \( \times 10^4 \) dis/min) was treated in methanol (0.5 ml.) with water (2 ml.) and potassium carbonate (32 mg.) then heated at ca. 100°C for 104 hr. The dihydrodesoxymorphine isolated (42.4 mg.) had a \( ^3\text{H} \) specific activity of 74 dis/min/mg., representing a loss of 95.9% of the original \( ^3\text{H} \) activity present.

Double-Labelled \([\text{2-}^3\text{H}]\text{-1-Bromocodeine}\) Feeding.-

The total alkaloids (192 mg.) extracted from the plants (wet weight 145 g.) were diluted with radioactive codeine (72.7 mg.), 1-bromocodeine (74.3 mg.) [total non-phenolic 147.0 mg.], morphine (67.8 mg.) and 1-bromomorphine (77.5 mg.) [total phenolic 145.3 mg.]. The total alkaloid mixture was, by treatment on a column of ion exchange resin, separated into phenolic (164 mg.) and non-phenolic (223 mg.) alkaloid fractions. Again the \( ^3\text{H}/^{14}\text{C} \) ratio was determined by volumetric count and was found to be 7.37, i.e. 0.00456 mCi of \( ^{14}\text{C} \) activity was fed.

Non-Phenolic Alkaloids.- No separation of the alkaloids could be achieved using p.l.c. on alumina plates. Reasonable separation to give 1-bromocodeine and codeine was obtained by p.l.c. on basic silica plates developed in methanol (respective \( R_f \) values being 0.45 and 0.35.). An initial attempt to separate the alkaloids was made using alumina plates but was unsuccessful, thus
the recovered fractions were combined and the separation repeated using the basic silica plates.

Codeine Fraction.—Volumetric count on the recovered codeine (39.6 mg.) gave the total $^{14}$C activity as $302 \times 10^4$ dis/min and $^3$H activity as $247 \times 10^4$ dis/min i.e. $^3$H/$^{14}$C ratio was 0.82. The alkaloid was mixed with radioinactive 1-bromocodeine (48.1 mg.) and reseparation on basic silica plates gave codeine (21.2 mg.) with total $^{14}$C activity $161 \times 10^4$ dis/min and $^3$H activity $34.0 \times 10^2$ dis/min i.e. $^3$H/$^{14}$C ratio was $2.11 \times 10^{-3}$. The alkaloid was diluted with radioinactive codeine (48.4 mg.; total 69.6 mg.) and the total chromatographed on a column of alumina in chloroform. Crystallisation to constant specific activity from benzene gave codeine, m.p. 157-158°C (lit., 156-157°C), having $^{14}$C s.a. $246 \times 10^2$ dis/min/mg, and estimated $^3$H s.a. 68 dis/min/mg.

The picrate was prepared, m.p. 196°C (lit., 196-197°C), and radiochemical purity demonstrated in the normal way i.e. $^{18}$C s.a. $258 \times 10^2$ dis/min/mg.

(i) Recovery of [N-methyl-$^{14}$C]codeine.—

Total $^{14}$C activity isolated as codeine = $2.58 \times 10^6 \times 69.6 \times 72.7$ dis/min.

\[
\frac{\text{\% recovery}}{21.2} = \frac{2.58 \times 69.6 \times 72.7 \times 10^6}{21.2 \times 0.00456 \times 2.2 \times 10^9} = 63\%
\]

(ii) Estimate of $^3$H incorporation (contamination) into Codeine.—

Total $^3$H activity isolated as codeine = $68 \times 69.6 \times 72.7$ dis/min

\[
\text{Total } ^3\text{H activity fed} = 0.0335 \times 2.2 \times 10^9 \text{ dis/min.}
\]

\[
= \frac{68 \times 69.6 \times 72.7 \times 10^2}{21.2 \times 0.0335 \times 2.2 \times 10^9} = 2.20 \times 10^{-2}\%
\]

i.e. $2.2 \times 10^{-2}\%$

163.
l-Bromocodeine Fraction.- The l-bromocodeine (66.6 mg.) isolated in the initial separation showed, by volumetric count, the total $^3$H activity present to be $151 \times 10^5$ dis/min and $^{14}$C activity $155 \times 10^3$ dis/min. The alkaloid was mixed with radioactive codeine (73.9 mg.) and chromatography on basic silica plates gave l-bromocodeine (57.9 mg.), total $^3$H activity being $95 \times 10^5$ dis/min and $^{14}$C activity $48 \times 10^2$ dis/min (by volumetric count). The recovered alkaloid (57.9 mg.) was mixed with radioactive material (20.4 mg.; total 78.3 mg.) and was chromatographed on a column of alumina in chloroform. Crystallisation (of recovered material) to constant specific activity from ethanol gave l-bromocodeine, m.p. 160-162°C (lit., $^{104}$ 161-162°C), having $^3$H s.a; $259 \times 10^3$ dis/min/mg, i.e. $979 \times 10^5$ dis/min/mmol. and estimated $^{14}$C activity 32 dis/min/mg. The 6-0-acetyl-l-bromocodeine derivative was formed and crystallisation from ethanol gave material, m.p. 191-192°C (lit., $^{109}$ 192-193°C), and molar specific activity $968 \times 10^5$ dis/min/mmol.

(i) Recovery of [2-$^3$H]-l-bromocodeine.-
Total $^3$H activity isolated as l-bromocodeine=$259\times10^3\times78.3\times74.3$ dis/min. $\frac{57.9}{57.9}$ recovery $=\frac{259\times78.3\times74.3\times10^5}{57.9\times0.0335\times2.2\times10^9}$ $= 35.2\%$

(ii) Estimate of $^{14}$C incorporation (contamination) into l-bromocodeine.-
Total $^{14}$C activity isolated as l-bromocodeine=$32\times78.3\times74.3$ dis/min. $\frac{57.9}{57.9}$ Estimated max. $\%$ incorporation $=\frac{32\times78.3\times74.3\times10^2}{57.9\times0.00456\times2.2\times10^9}$ $= 3.21 \times 10^{-2}\%$

i.e. $<0.03\%$
Phenolic Alkaloids.- The phenolic alkaloids (164 mg.) were treated directly with pyridine (5 ml.) and acetic anhydride (2 ml.) and heated on a water bath. Partial separation of diacetyl-1-bromomorphine and diacetylmorphine was achieved using p.l.c. on alumina plates in chloroform-benzene (1:1). The separation was not good, and after hydrolysis purification was by p.l.c. on basic silica plates developed in methanol.

Morphine Fraction.- Separation of diacetyl-derivatives gave diacetylmorphine (56.1 mg.) which contained both $^3$H and $^{14}$C activity (t.l.c. showed a trace of diacetyl-1-bromomorphine). After basic hydrolysis the recovered morphine (42.4 mg.) was mixed with radioinactive 1-bromomorphine (43.0 mg.) and chromatographed on basic silica plates developed in methanol. The recovered morphine (16.0 mg.) contained mainly $^{14}$C activity and was diluted with radioinactive alkaloid (53.1 mg; total 69.1 mg.). Crystallisation to constant specific activity was from methanol and gave morphine, m.p. 247-248°C (lit., 247-248°C), having $^{14}$C s.a. 880 dis/min/mg., i.e. $25.1 \times 10^4$ dis/min/mmol. and estimated $^3$H s.a. 56 dis/min/mg. The diacetyl derivative of the morphine was formed and crystallised from ethyl acetate, m.p. 171-172°C (lit., 171-172°C), and molar specific activity $24.5 \times 10^4$ dis/min/mmol.

(ii) Incorporation of Codeine into Morphine.-
Total $^{14}$C activity isolated as morphine = $880 \times 69.1 \times 67.8$ dis/min.

$\%$ incorporation = $\frac{880 \times 69.1 \times 67.8 \times 10^2}{16.0 \times 0.00456 \times 2.2 \times 10^9}$

= 2.57%
(ii) Estimate of $^3$H incorporation (contamination) into morphine.

Total $^3$H activity isolated as morphine = $\frac{56x69.1x67.8}{16.0}$ dis/min.

Estimated max.% incorporation = $\frac{56x69.1x67.8x10^2}{16.0x0.0335x2.2x10^9}$

= $2.2x10^{-2}$

1-Bromomorphine Fraction.-- The initial separation of the diacetyl-derivatives gave a faster moving fraction, diacetyl-l-bromomorphine (33.3 mg.), which from t.l.c. contained some diacetyl-morphine. The fraction was hydrolysed using base, then mixed with radioinactive morphine (30.1 mg.). Chromatography on basic silica plates gave pure l-bromomorphine (20.4 mg.) (pure by t.l.c.) which contained mainly $^3$H activity and a trace of $^{14}$C activity. The alkaloid was rechromatographed on basic silica plates and the recovered material (18.6 mg.) was diluted with radioinactive l-bromomorphine (43.9 mg.; total 62.5 mg.). The total alkaloid was dissolved in 0.5N hydrochloric acid (ca. 4 ml.), then after addition of sodium hydrogen carbonate to adjust to pH 7 the solution was set aside for ca. 1 hr. The micro-crystalline material formed was removed, and after drying in vacuo at 100°C for 3 hr., its specific activity was determined in the usual way. "Crystallisation" to constant specific activity was carried out in this way and gave l-bromomorphine with $^3$H s.a. 712 dis/min/mg. The diacetyl derivative was formed and crystallised from methanol to constant specific activity m.p. 206-207°C (lit. 206°C), and molar specific activity 15.2 x $10^4$ dis/min/mmol. which corresponds for l-bromomorphine to 417 dis/min/mg. The estimated $^{14}$C s.a. was 29 dis/min/mg.

(i) Incorporation of [2-$^3$H]-1-Bromocodeine into l-Bromomorphine.

Total $^3$H activity isolated as l-bromomorphine = $\frac{417x62.5x77.5}{15.6}$ dis/min.
\[
\text{% incorporation} = \frac{417 \times 62.5 \times 77.5 \times 10^2}{18.6 \times 0.0335 \times 2.2 \times 10^9} = 0.15\%
\]

(ii) Estimate of \(^{14}\text{C}\) incorporation (contamination) into 1-bromomorphine.-

Total \(^{14}\text{C}\) activity isolated as 1-bromomorphine = \(29 \times 62.5 \times 77.5\) dis/min. 18.6

Estimated max. \% incorporation = \(29 \times 62.5 \times 77.5\)
\[\frac{18.6 \times 0.00456 \times 22 \times 10^9}{18.6 \times 0.00456 \times 22 \times 10^9} = 40.04\%
\]

(B) Experimental details associated with Chapter III.

Trifluoroacetic acid was used generally without special purification. In one experiment (see Chapter III) the acid was rigorously dried by distillation from phosphorus pentoxide.

**Thebaine Methotrifluoroacetate.** - Thebaine (3.76 g.) was heated under reflux in methanol (50 ml.) containing an excess of methyl iodide for 1 hr. The solution was evaporated and the residue dissolved in methanol and treated with silver trifluoroacetate (2.5 g.) in methanol (20 ml.). The precipitated silver iodide was filtered off and the filtrate was evaporated to give thebaine methotrifluoroacetate (4.5 g.). Recrystallisation from ethanol-ether gave material (4.1 g.), m.p. 199\(^\circ\)C, darkening from 185\(^\circ\) (Found: C, 60.3; H, 5.9. \(C_{22}H_{24}F_{2}NO_{5}\) requires C, 60.1; H, 5.5%).

**7,8-Dehydrometathebaine Metho-salts.** - (a) A solution of thebaine methotrifluoroacetate (1 g.) in trifluoroacetic acid (3 ml.) was kept at room temperature for 3 hr. then evaporated. The residue, in water, was converted into the chloride form by ion-
-exchange with De-acidite-FF SRA 70 resin (Permutit Co.Ltd.). Evaporation of the aqueous solution gave 7,8-dehydrometathebainone methochloride as a yellow resin (0.7 g.).

(b) Thebaine methotrifluoroacetate (500 mg.) was kept in concentrated hydrochloric acid (5 ml.) for 3 hr. at room temperature. The acid was evaporated off in vacuo over potassium hydroxide and phosphorus pentoxide at room temperature. The residual methochloride was freed from traces of acid by addition and evaporation of methanol.

Material from preparations (a) and (b) had identical spectroscopic properties: $\nu_{\text{max.}}$ (Nujol) 1660 cm$^{-1}$, $\lambda_{\text{max.}}$ (EtOH) 304 nm (ε 8650) shifting to 322 (ε 5680) and 440 nm (ε 4570) upon addition of sodium ethoxide. Fleischhacker et al. gave$^{93}$, for the corresponding perchlorate, $\nu_{\text{max.}}$ 1660 cm$^{-1}$, $\lambda_{\text{max.}}$ (H$_2$O) 312 nm (ε 9500). Treatment of this amorphous methochloride with Reinecke's salt, NH$_4$[Cr(NH$_3$)$_2$(CNS)$_4$].H$_2$O, in water gave the corresponding metho-salt which crystallised from water - 5% ethanol as small pink prisms, m.p. 167°C (Found: C, 42.2; H, 4.8. C$_{23}$H$_{28}$CrN$_7$O$_5$S$_4$.H$_2$O requires C, 42.6; H, 4.6%).

Metathebainone metho-salts.- 7,8-Dehydrometathebainone methochloride (0.3 g.) in methanol (20 ml.) was hydrogenated (uptake 21.7 ml. of H$_2$; calc. 21.1 ml.) using 10% palladised charcoal as catalyst. Evaporation of the solvent after filtering through celite gave metathebainone methochloride $\lambda_{\text{max.}}$ (EtOH) 294 nm. (ε 11,000) as a green amorphous solid. An aqueous solution of the latter was poured through an ion-exchange column to give the corresponding iodide salt, metathebainone methiodide;

168.
\( \nu_{\text{max.}} 1660 \text{ cm}^{-1} \lambda_{\text{max.}} \text{ (EtOH) 295 nm. (€ 11,000)}. \) The salt could not be obtained crystalline but its u.v. spectrum compared well with that of an authentic sample of metathebainone methiodide, \( \nu_{\text{max.}} 1660 \text{ cm}^{-1}, \lambda_{\text{max.}} \text{ (EtOH) 294 nm.} \) (€ 13,700), prepared by treatment of metathebainone with methyl iodide.
Appendix
Crystallisation of Codeine

<table>
<thead>
<tr>
<th>Crystallisation</th>
<th>$^3$H spec.activity</th>
<th>$^{14}$C spec.activity</th>
<th>$^3$H/$^{14}$C ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>152</td>
<td>177</td>
<td>8.61:1</td>
</tr>
<tr>
<td>2</td>
<td>162</td>
<td>238</td>
<td>6.81:1 (a)</td>
</tr>
<tr>
<td>3</td>
<td>187</td>
<td>264</td>
<td>7.08:1</td>
</tr>
<tr>
<td>4</td>
<td>138</td>
<td>217</td>
<td>6.34:1 (a)</td>
</tr>
</tbody>
</table>

Crystals and mother liquors combined, columned and recrystallised.

1 | 301 | 275 | 10.92:1 |

All material recombined and further diluted with radioactive codeine (146.6 mg.)

<table>
<thead>
<tr>
<th>-x10²</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>210</td>
<td>28.5</td>
</tr>
<tr>
<td>2</td>
<td>192</td>
<td>30.2</td>
</tr>
<tr>
<td>3</td>
<td>156</td>
<td>26.4</td>
</tr>
<tr>
<td>4</td>
<td>161</td>
<td>27.2</td>
</tr>
<tr>
<td>5</td>
<td>150</td>
<td>26.5</td>
</tr>
<tr>
<td>6</td>
<td>148</td>
<td>25.3</td>
</tr>
<tr>
<td>7</td>
<td>146</td>
<td>25.6</td>
</tr>
<tr>
<td>8 Picrate</td>
<td>146</td>
<td>25.9</td>
</tr>
<tr>
<td>formed and regenerated</td>
<td>149</td>
<td>26.2</td>
</tr>
</tbody>
</table>

(a) Averaged values from two determinations.

Table 1
Analysis of the $^{3}\text{H}/^{13}\text{C}$ ratio for the isolated codeine:-

If $x_1$ represents the $^{3}\text{H}/^{13}\text{C}$ ratio then:

\[
\begin{align*}
\bar{x}_1 &= \frac{\sum x_1}{n_1} = \frac{45.62}{8} = 5.702 \\
\bar{x}_1^2 &= \frac{\sum x_1^2}{n_1} = \frac{260.18}{8} = 32.522.
\end{align*}
\]

And $S^2(x_1) = \frac{\sum_{i=1}^{n_1} (x_1 - \bar{x}_1)^2}{n_1 - 1} = 0.093$.

Where $S$ is the Standard deviation of $x_1$.

The average $^{3}\text{H}/^{13}\text{C}$ value obtained for the codeine precursor ($\bar{x}_2$) (before feeding) was 5.32. This was based on only two determinations and it will be assumed that the standard deviation is the same as that determined for the codeine isolated from the plant i.e. 0.093. To determine if the average value of the $^{3}\text{H}/^{13}\text{C}$ ratio for the isolated codeine is significantly different from that of the precursor material the t-distribution test will be used:

It is assumed that $S(x_1) = S(x_2) = 0.093$. $S_p$ is the pooled estimate of the standard deviations.
The value of $t$ is given by the equation:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

for 8 degrees of freedom.

$$= \frac{5.702 - 5.32}{0.0926 \sqrt{\frac{1}{8} + \frac{1}{1}}}$$

$$= 3.88$$

From tables $t_8$ (P=0.05) 5% confidence limit = 2.31

$t_8$ (P=0.01) 1% confidence limit = 3.36

$t_8$ (P=0.005) 0.5% confidence limit = 3.69.

Thus even at the 0.5 confidence limit the averaged $^3$H/$^{14}$C ratio for the precursor and isolated codeine are significantly different.
Crystallisation of Morphine

<table>
<thead>
<tr>
<th>Crystallisation</th>
<th>$^3$H spec.activity $\frac{\text{dis/min/mg}}{\times 10^2}$</th>
<th>$^{14}$C spec.activity $\frac{\text{dis/min/mg}}{\times 10^2}$</th>
<th>$^3$H/$^{14}$C ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>419</td>
<td>62.2</td>
<td>6.73</td>
</tr>
<tr>
<td></td>
<td>381</td>
<td>55.3</td>
<td>6.88</td>
</tr>
<tr>
<td>2</td>
<td>290</td>
<td>48.5</td>
<td>5.98</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>53.9</td>
<td>5.93</td>
</tr>
<tr>
<td>3</td>
<td>176</td>
<td>32.0</td>
<td>5.50</td>
</tr>
<tr>
<td></td>
<td>158</td>
<td>28.7</td>
<td>5.50</td>
</tr>
<tr>
<td>4</td>
<td>163)</td>
<td>29.7</td>
<td>5.51 )</td>
</tr>
<tr>
<td></td>
<td>) 162</td>
<td>) 29.0 )</td>
<td>) 5.56</td>
</tr>
<tr>
<td></td>
<td>) 159</td>
<td>) 28.4 )</td>
<td>)</td>
</tr>
<tr>
<td></td>
<td><strong>46.2 \times 10^5</strong></td>
<td><strong>82.7 \times 10^4</strong> dis/min/mmol.</td>
<td></td>
</tr>
</tbody>
</table>

Diacetyl derivative formed:

<table>
<thead>
<tr>
<th></th>
<th>$\frac{\text{dis/min/mmol.}}{\times 10^4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82.7 \times 10^4 dis/min/mmol.</td>
</tr>
</tbody>
</table>

Table 2

Analysis of the $\frac{^3}{^14}$H/$^1$C ratio for the isolated morphine:

$$\sum x_3 = 33.44 \quad \sum x_3^2 = 186.40$$

174.
\[
\bar{x}_3 = \frac{\sum x_3}{n_3} = \frac{33.44}{6} = 5.573
\]
\[
\frac{\sum x_3^2}{n_3} = \frac{186.40}{6} = 31.066
\]
Thus \[S^2(x_3)^{111} = \frac{186.40 - (33.44)^2}{6} = 0.006\]
i.e. \[S(x_3) = 0.077\].

The pooled estimate of the standard deviations (Sp) for the precursor and isolated morphine are given as follows:

\[
Sp = \sqrt{\frac{5(0.077)^2 + 1(0.093)^2}{6}} = 7.98 \times 10^{-2}
\]
Using equation (2)
\[
t_6 = \frac{5.573 - 5.32}{0.0798/\sqrt{6} + 1/1} = 2.93
\]
From tables \[t_6 (P = 0.05) 5\% confidence limit = 2.45.\]
\[t_6 (P = 0.01) 1\% confidence limit = 4.32.\]
Thus the \(^3\)H/\(^1\)H ratio for the isolated morphine is significantly different from that of the precursor at the 5\% confidence limit. However, the two ratios are not significantly different at the 1\% confidence limit.
Difference between $^{3}H/^{14}C$ ratios for isolated codeine and morphine:

The pooled estimate of the standard deviations $S_p$ is 0.0798 (as before)

And

$$t_{12} = \frac{5.702 - 5.573}{0.0798/\sqrt{12} + \frac{1}{6}}$$

$$= 3.01$$

From tables $t_{12}$ ($P = 0.05$) 5% confidence limit = 2.18

$$t_{12} (P = 0.01) 1\% \text{ confidence limit} = 3.05.$$  

Thus the $^{3}H/^{14}C$ ratio for both the isolated codeine and morphine are significantly different at the 1% confidence limit.

Thus after metabolism the $^{3}H/^{14}C$ ratio of the isolated codeine and morphine differ from each other, and both are at variance with the ratio determined for the codeine precursor. In each case the ratio determined for the isolated alkaloids is greater than that determined for the precursor and this is most likely to have occurred by loss of $^{14}C$ activity. A possible explanation is that, after injection of the codeine precursor and after subsequent mixing, some de-N-methylation occurs giving rise to an equilibrium between codeine and de-N-methylated codeine. Some of the codeine pool, which would form the major fraction of the equilibrium, could then be metabolised to give morphine. The morphine itself may then undergo some de-N-methylation leading to a small loss of $^{14}C$ activity. Thus at the time of work-up the $^{3}H/^{14}C$ ratio for the metabolites would be greater than for the precursor. This of course presupposes that the precursor material undergoes some sort of "mixing" process with the plant alkaloids already present. The complete mixing process will take a finite time, and it must be assumed that this is exceeded by the period of time allowed for feeding experiment.
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