BIOSYNTHESIS OF HYDRASTINE AND BERBERINE

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OF

HYDRASTINE AND BERBERINE

By

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SCOPE AND CONTENTS: The biosynthesis of hydrastine and of berberine was investigated by feeding radioactive tyrosine and dopamine to Hydrastis canadensis L. It was shown that both hydrastine and berberine are specifically derived from two molecules of tyrosine, but that the ratio of incorporation of the two molecules of radiotyrosine was not unity, but varied with time. Dopamine was also incorporated into hydrastine without randomization, but only one molecule was utilized. The results, which are consistent with Robinson's hypothesis of biogenesis, throw light on some of the individual steps of the biosynthetic path by which hydrastine and berberine are derived.

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TABLE OF CONTENTS

BIOGENESIS AND BIOSYNTHESIS OF ALKALOIDS

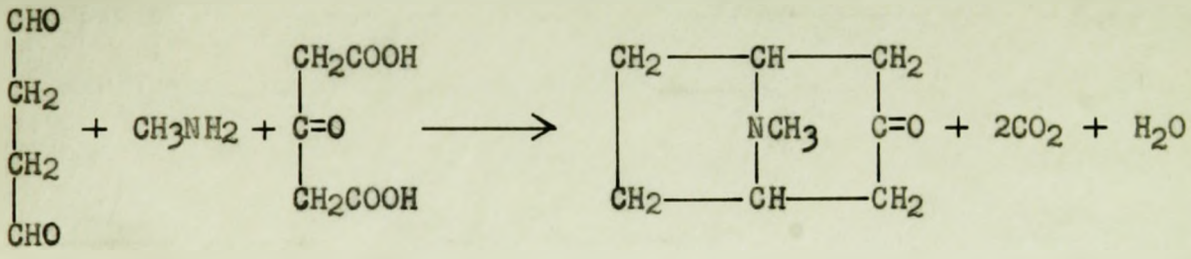
1. INTRODUCTION

The origin and the mode of synthesis of alkaloids in plants is a topic upon which much has been written in the chemical literature. Only in the last decade, however, when the use of radioactive isotopes (especially carbon -14) has become common, has the subject become amenable to direct experimental investigation. By the use of a radioactive tracer it is possible to gain direct information about the synthesis of an alkaloid in a plant, and to confirm or disprove the intervention of any particular postulated intermediate. Results obtained by direct experimentation of this type will be referred to as biosynthetic work in this thesis.

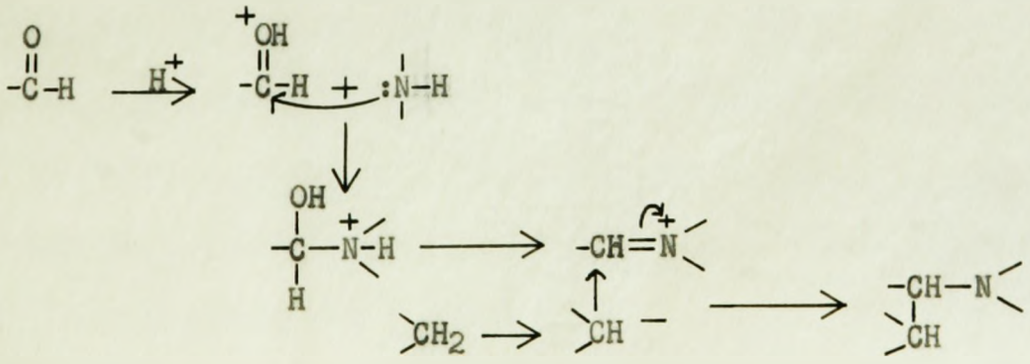
Although knowledge of the biosynthesis of alkaloids is only of recent standing, the theoretical consideration of this problem has occupied chemical thought for over fifty years. During that time many hypotheses have been advanced which bear on the possible mode of synthesis of alkaloids. These suggestions all lacked direct experimental support for the simple reason that, at the time of their conception, there was no known way of proving or disproving them experimentally. This theoretical treatment of the origin of alkaloids will be termed biogenesis. In spite of the initial lack of experimental proof the biogenetic approach was of great importance, since it has provided a basis on which the new tools of biosynthesis could be employed in the attack on the problem.

2. BIOGENESIS OF ALKALOIDS

The first general hypothesis of the biogenesis of alkaloids was put forward by Robinson (1917a). His ideas were developed and supplemented by himself (Robinson, 1934, 1936, 1948a, b) and others (e.g. Barger, 1934), and refined to such an extent that in his Jerusalem lectures Robinson (1955) was able to interpret the biogenesis of the great majority of known alkaloids on the basis of his hypothesis. Robinson's initial interest in this question began with his laboratory synthesis of tropinone from succindialdehyde, methylamine, and acetonedicarboxylic acid (Robinson, 1917b). The possibility occurred to him that tropinone might



also be synthesized in this comparatively simple manner in the plant, and that analogous reactions of this type might be responsible for the synthesis of other alkaloids. This reaction consists of the interaction of an aldehyde, an amine, and a carbon atom bearing an active hydrogen, and is now referred to as a Mannich reaction. It may be visualized in the following manner. Robinson suggested that the amines and aldehydes necess-



ary for these reactions were derived from amino acids. The amines could be obtained directly by decarboxylation, and the aldehydes either by direct oxidation of the amine or the amino acid, or else from the amino acid by way of the keto acid.

It should be pointed out here that the possibility that such a reaction was involved in alkaloid biogenesis had been noted earlier. Pictet (1906) synthesized tetrahydroisoquinolines by the reaction of substituted phenylethylamines with formaldehyde and other aldehydes, and postulated from this that formaldehyde had a role in the formation of alkaloids. Winterstein and Trier (1910) pointed out that if the formaldehyde were replaced by an aromatic aldehyde an intermediate would be formed which could easily give rise to papaverine.

These had been only isolated suggestions, however, and it remained for Robinson to put forward a broad general hypothesis concerning alkaloid biogenesis. He suggested that alkaloids were derived mainly from modified amino acids, and occasionally also from small molecules such as formaldehyde and acetoacetate which occurred naturally, or could be derived from known naturally occurring substances by simple chemical processes. He conceived the fundamental synthetic route to be by way of Mannich reactions e.g. the synthesis of tropinone as shown, or the synthesis of the papaverine skeleton as suggested by Winterstein and Trier. In addition to this main synthetic route various other simple reactions were necessary, according to Robinson, for the conversion of the intermediates obtained from the Mannich reactions into the alkaloids. The most important of these were

(i) methylation at O or N

(ii) hydroxylation of aromatic rings

(iii) aromatisation of alicyclic rings

On the basis of this hypothesis Robinson was able to suggest schemes for the biogenesis of the pyrrolidine group of alkaloids from glutamic acid or ornithine, the piperidine group from lysine, the isoquinoline group from phenylalanine and tyrosine, and the indole group from tryptophan. It should be stressed that in no case did Robinson claim either a specific precursor or a specific order of events within his schemes. His reasons for preferring his particular schemes were due to their high "degree of coincidence" when related to each other, and he repeatedly stressed that no particular order of processes could be assumed, particularly in regard to the incorporation of extra-skeletal groups such as methyl.

Robinson's hypothesis of biogenesis received support by another approach. Schöpf (1937) reviewed the work which had been carried out by himself and others on "syntheses under pseudophysiological conditions". These were chemical syntheses of alkaloids, using "cell possible" starting materials, and carried out at room temperature in dilute solution, and at a pH close to neutrality. Under such mild conditions Schöpf was able to carry out "in vitro" some of the reactions which Robinson had predicted as biogenetic pathways for the formation of alkaloids e.g. the synthesis of tropinone from succindialdehyde, methylamine, and acetonedicarboxylic acid. Schöpf reasoned that since these reactions did take place under such mild conditions it might be expected that they could take place in the same manner in the plant. By this time it was realized that most biochemical reactions were enzyme catalysed. However, Schöpf felt that some might take place spontaneously due to very reactive intermediates. Recent work has indicated that "Schöpf chemistry" has

little or no biochemical significance "in vivo". Nevertheless the reactions did support the possibility of Robinson's biogenetic schemes.

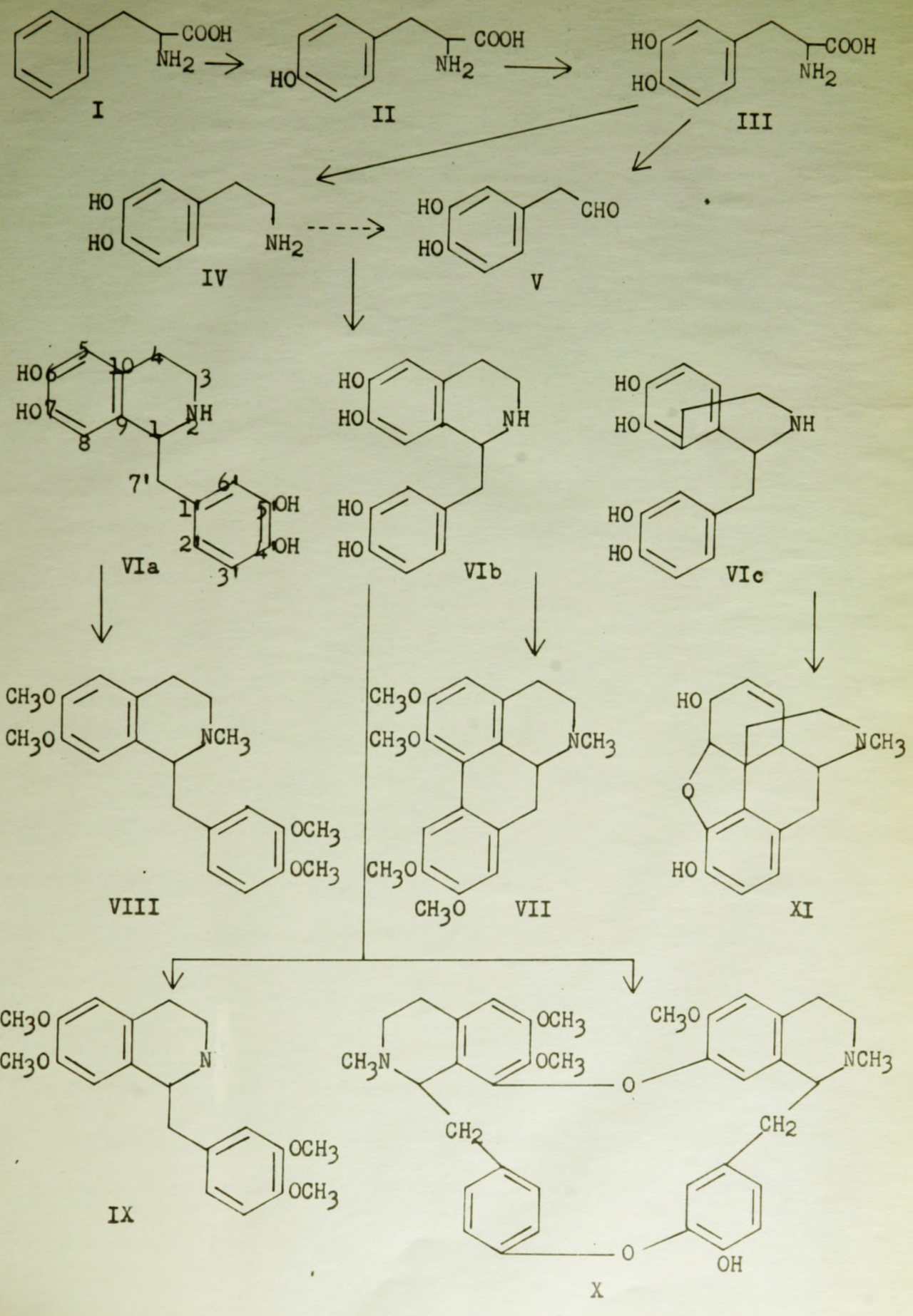
Although Robinson was able to suggest a derivation of many alkaloids from amino acids by means of the simple reactions that have been described, some alkaloidal structures were too complex to permit the formulation of their biogenesis in a simple manner. A suggestion which extended the scope of Robinson's biogenetic hypothesis was made by Woodward (1948) and later supported by Robinson (1948a). Woodward postulated that ortho-dihydroxy-aromatic rings could be cleaved to yield a hydroxyaldehyde, which could then undergo a further Mannich reaction. This "Woodward fission", originally put forward in connection with the biogenesis of strychnine, has been successfully applied to explain the biogenetic origin of such apparently unrelated alkaloids as ajmaline, cinchonine, and emetine amongst many others. Robinson's hypothesis, as refined over the last forty years, thus encompasses the biogenesis of a great number of alkaloids from amino acids as the main biogenetic precursors. The biogenetic ideas, which were first put forward in 1917 entirely on the basis of chemical analogy and without direct experimental support, which at that time was unobtainable, have stood the test of time and have, in principle, been confirmed by recent biosynthetic work (Battersby,1961).

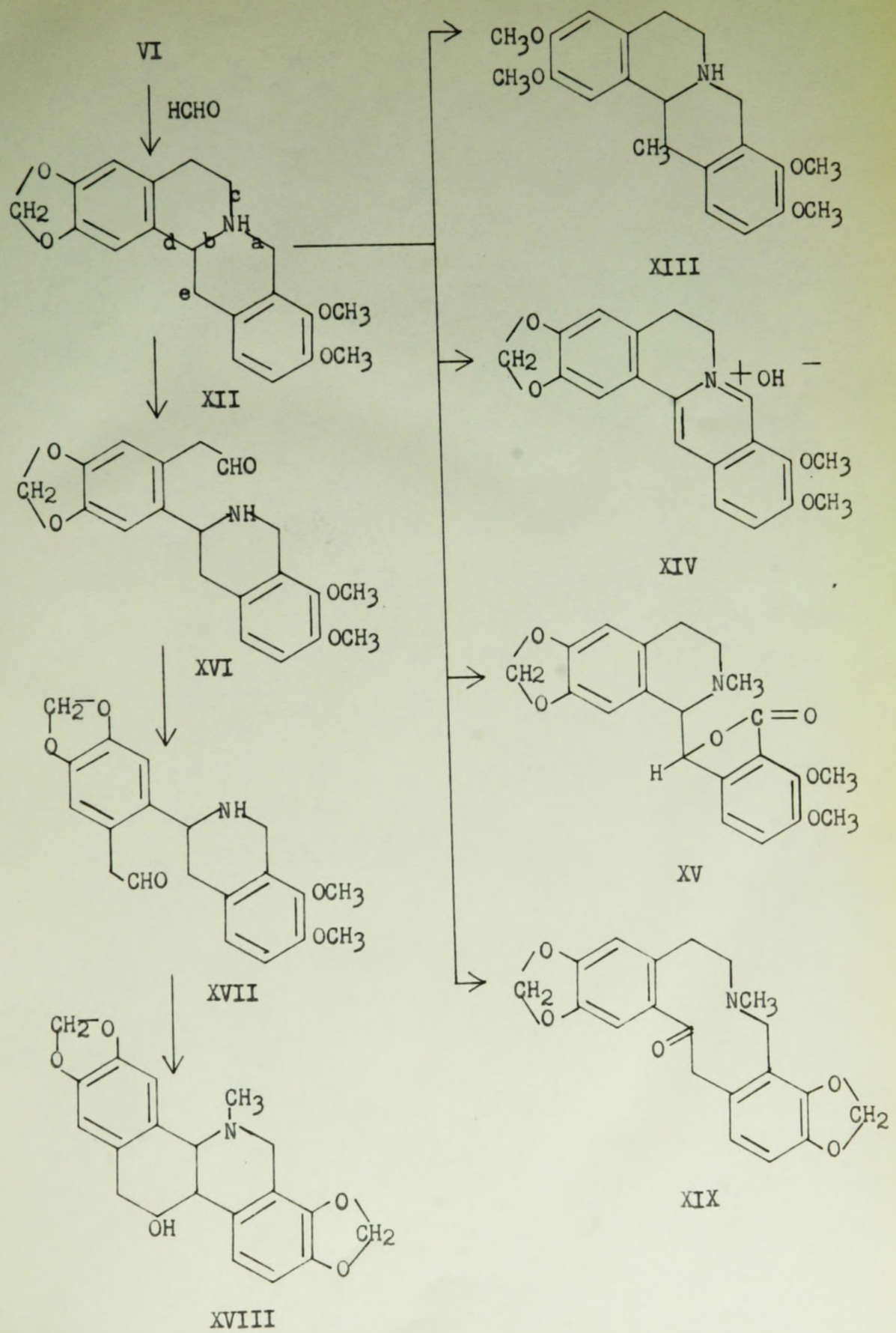
For many years Robinson's theory was the only general one which had been put forward to account for the biogenesis of alkaloids. Recently, however, a new hypothesis has been suggested (Wenkert, 1959; Wenkert & Bringi, 1959), based on recent knowledge of the biosynthesis of the aromatic amino acids. By 1959 it had been shown (Davis, 1955) that prephenic acid, the key intermediate in the biosynthesis of tyrosine and

phenylalanine, is derived from carbohydrate. Erythrose -4- phosphate and pyruvic acid, both metabolites of glucose, combine to yield sedoheptulose -7- phosphate, which cyclizes to shikimic acid. This reacts with another pyruvic acid unit to give prephenic acid. Wenkert proposed that the precursor of the pyrrolidine group of alkaloids and of others which contained a C_4 unit was not ornithine but erythrose -4- phosphate, while that of alkaloids containing a C_6-C_1 structure, such as lycorine and colchicine, was not tyrosine but shikimic acid, and that prephenic acid and not tyrosine or tryptophan was the precursor of alkaloids containing a C_6-C_2 and a C_6-C_3 skeleton, a family of alkaloids which includes the large number of isoquinoline and indole bases. This hypothesis thus postulates that all alkaloids are derivable, at least formally, from formaldehyde, acetate, pyruvate, erythrose, shikimate, and prephenate units, by routes that are analagous in every detail to well known enzymic reactions. This is in contrast to Robinson's view which derives alkaloids from formaldehyde, acetate, and amino acids. Clear-cut critical experiments to test the validity of one or the other of these two hypotheses are not easily designed. In most cases the "Wenkert precursor" would not only yield the product, but would also be predicted to yield the "Robinson precursor". Biosynthetic incorporation into an alkaloid of a "Wenkert precursor" does not therefore disprove the Robinson hypothesis. The work to be described in this thesis, however, deals with one of the few instances where conclusive differentiation of the two hypotheses is possible. The results to be reported unequivocally disprove Wenkert's hypothesis.

3. BIOGENESIS OF ISOQUINOLINE ALKALOIDS

According to Robinson the aromatic amino acids phenylalanine and/or tyrosine are the precursors of the isoquinoline alkaloids. Phenylalanine I could be converted to tyrosine II and then further to dihydroxyphenylalanine (DOPA) III by hydroxylation. Decarboxylation of III would give dopamine IV. The aldehyde V could be obtained either by direct oxidation of III or IV, or by way of the corresponding keto acid which could lose carbon dioxide immediately or at a later stage. Combination of IV and V in a Mannich reaction gives norlaudanosoline (VI a b c). By further elaboration of this compound in a variety of ways Robinson (1934, 1948b) and Barger (1934) were able to account for the origin of practically all of the isoquinoline alkaloids. Abstraction of two hydrogen atoms from positions 8 and 2', and union to form a ring, gives the skeleton of glaucine VII. Methylation gives laudanosine VIII, and further aromatisation of the isoquinoline ring gives papaverine IX, a member of the benzylisoquinolines. Methylation and dimerization by the formation of ether linkages leads to the bisbenzylisoquinolines of which oxyacanthine X is an example. Rotation of the isoquinoline ring around the 1-9 axis (VI c), and oxidative union of carbons 10 and 6' accounts for the skeleton of morphine XI. A further Mannich reaction between VI and formaldehyde leads, by introduction of the so-called "berberine bridge", to a further class of compounds. Canadine XII is obtained directly from VI. Methylation at (e) which might proceed via the dehydrocompound gives the skeleton of corydaline XIII, whereas partial aromatisation gives berberine XIV. Oxidation at (a) followed


I
II
III
IV
V
VIa
VIb
VIc
VIII
VII
XI
IX
X

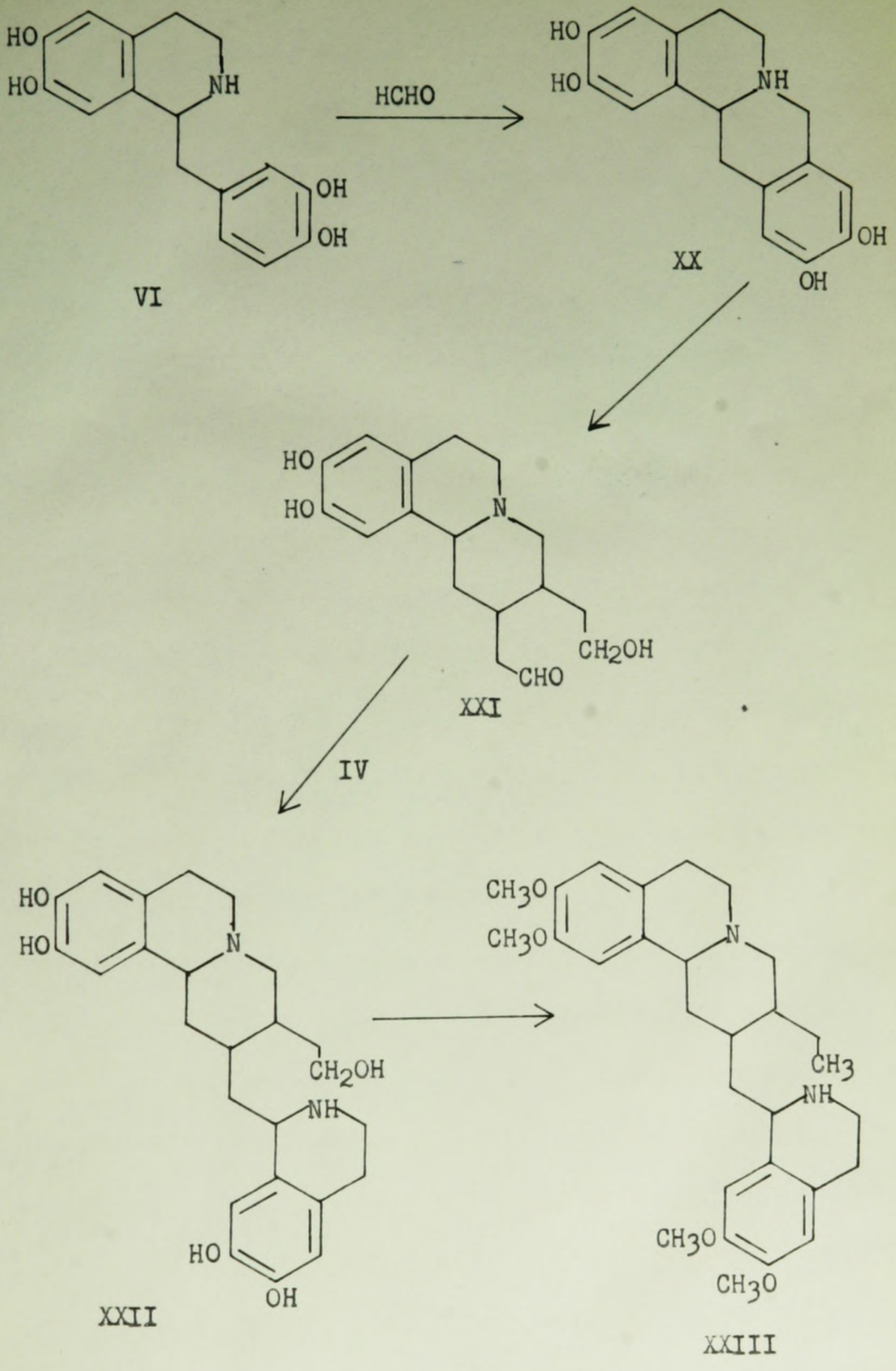

VI
HCHO
XII
XVI
XVII
XVIII
XIII
XIV
XV
XIX

by methylation gives hydrastine XV, whereas oxidation at (b) gives the skeleton of protopine XIX. Oxidation at (c) leads to yet another series of compounds: Rotation of the top ring in the intermediate XVI about bond (d) yields an equivalent structure XVII. Recyclization of the aldehyde group at (e) gives the skeleton of chelidonine XVIII.

A glance at this outline shows that this biogenetic scheme does indeed posess a high "degree of coincidence". This is even more pronounced when one realizes that each of the ring skeletons in the scheme is representative of a group of compounds which differ only in the position of substituents, and which may be accommodated by only minor modifications in the biogenetic route. Whatever the specific precursors or the exact reaction sequence, the hypothesis demands that two tyrosine equivalents are required to form these isoquinoline alkaloids. Whether phenylalanine, tyrosine, DOPA, or a combination of two of these were involved, at what stage hydroxylations took place, and at what stage exocyclic groups such as methyl groups were incorporated, were questions which Robinson did not attempt to answer.

The "Woodward fission", whose intervention is uncommon in schemes for the biogenesis of the isoquinoline alkaloids, is involved in the biogenesis of emetine (Robinson, 1948a). A Mannich reaction between norlaudanosoline VI and formaldehyde leads to XX, which undergoes a "Woodward fission" to give XXI. Another Mannich reaction of this compound with dopamine IV yields XXII. Methylation and reduction of XXII accounts for the structure of emetine XXIII.

The alternative hypothesis of the biogenesis of the isoquinoline alkaloids, due to Wenkert, in its original form (Wenkert, 1959) proposed


HO
HO
NH
OH
OH
VI
HCHO
HO
HO
NH
OH
OH
XX
HO
HO
N
CH2OH
CHO
XXI
IV
HO
HO
N
CH2OH
NH
HO
OH
XXII
CH3O
CH3O
N
CH3
NH
CH3O
CH3O
XXIII

a hydrated prephenic acid XXIV as the starting point (Fig. 1). When it was shown that a hydrated intermediate was not formed in the conversion of shikimate to prephenate (Levin & Sprinson, 1960) the hypothesis was modified to accomodate prephenate itself as the starting point (Fig. 2) (Wenkert, 1962). These postulated precursors could yield the phenylpyruvic acid derivative XXVIII and the phenylethylamine derivative IV, which, as in the earlier hypothesis, were proposed as the direct precursors of norlaudanosoline, and hence of the benzylisoquinolines, aporphines, morphine, and other "non-berberine bridged" structures. By N-formalation of the amine IV the anion XXIX would be produced, which on condensation with the phenylpyruvate XXVIII at two points would eventually yield the berberine-bridged skeletons of the protoberberines and the protopine alkaloids.

Unequivocal experimental differentiation of the classical and the carbohydrate hypothesis is not possible in the above cases. Demonstration of specific incorporation of two prephenate units into any of these alkaloids, as predicted by Wenkert, would not invalidate Robinson's amino acid hypothesis, since prephenate is a known precursor of the aromatic amino acids (Sprinson, 1960), which in turn could yield the amine IV and the keto acid XXVIII by known biochemical transformations. For the same reason Wenkert's hypothesis would not be invalidated by the demonstration that two tyrosine units were incorporated into these alkaloids, as predicted by Robinson.

A different situation obtains in the case of the phthalideisoquinoline bases. According to Robinson's views (Robinson, 1948b), this

Figure 1

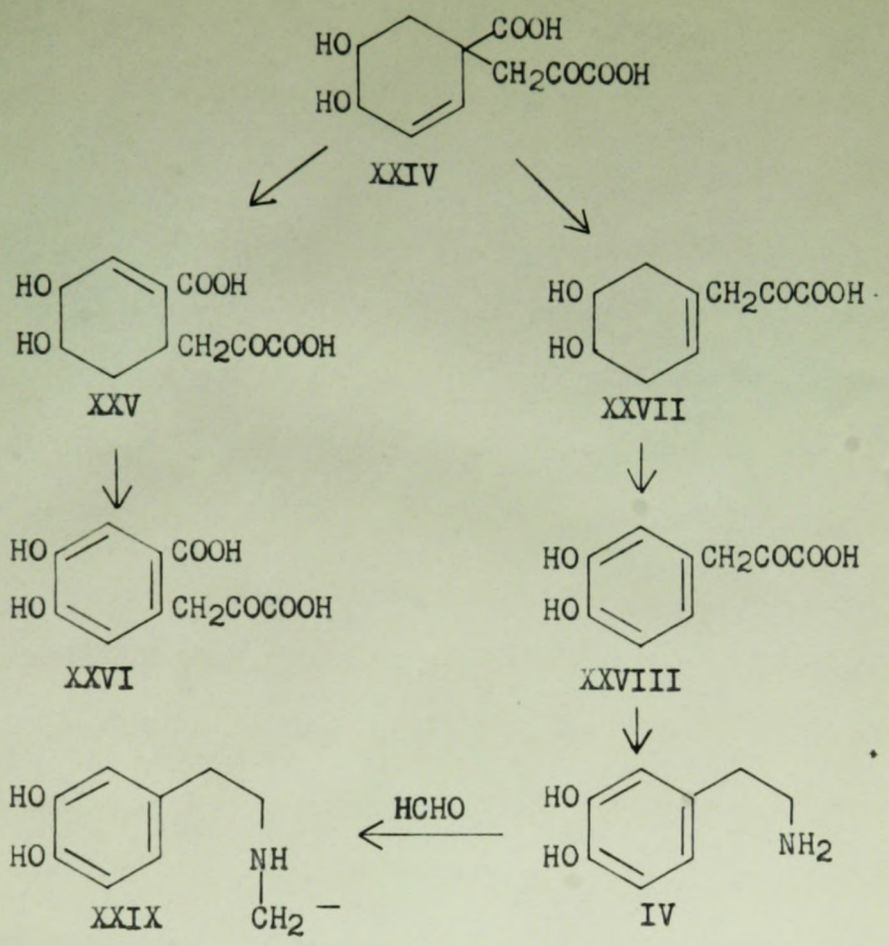
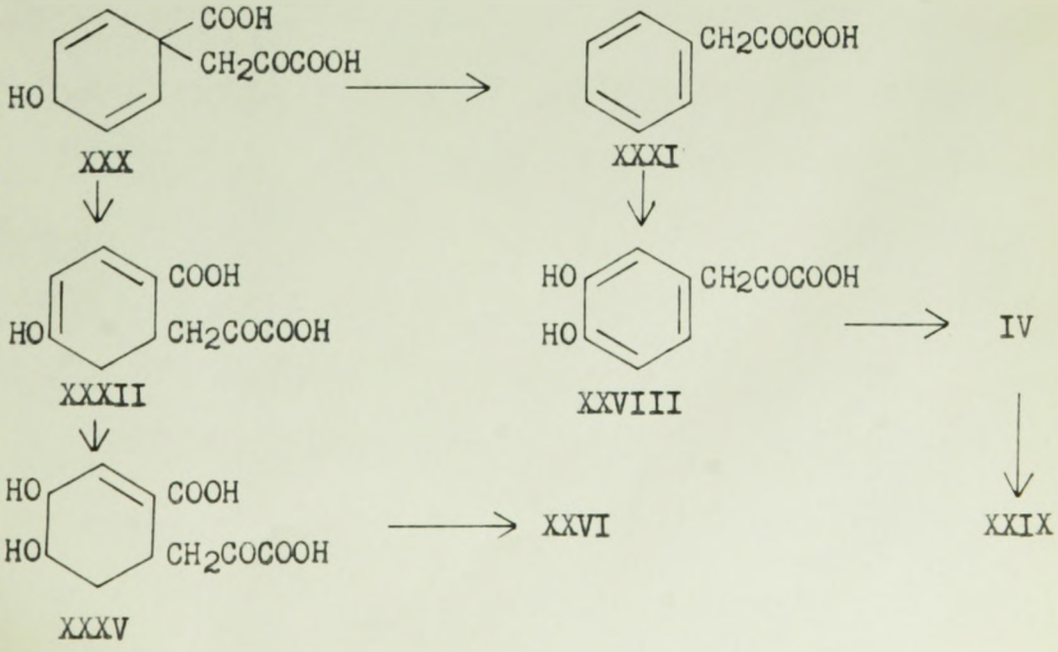


Figure 2



group is derived from two tyrosine equivalents by way of norlaudanosoline, followed by the insertion of a C_1 unit to form a berberine-bridged canadine-type intermediate. The latter might either yield berberine and thence hydrastine by further oxidation, or might serve as a common intermediate from which berberine and hydrastine are elaborated by separate oxidative pathways (Perkin & Robinson, 1910). According to this scheme two tyrosine units should be specifically incorporated into the hydrastine molecule.

Wenkert, on the other hand, postulates that in the biogenesis of the phthalideisoquinolines the amine IV reacts with another prephenic acid metabolite, a phenylpyruvate -o- carboxylic acid XXVI, which already contains the carbon atom from which the "one-carbon bridge" of hydrastine is to be derived. Whereas the amine, derived according to Wenkert from prephenate, could also be derived from tyrosine by a well known enzymatic reaction, there is no known metabolic process whereby the second fragment XXVI, also derived from prephenate, can be obtained from tyrosine. The enzymatic conversion of prephenate to tyrosine has been shown to be irreversible (Schwink & Adams, 1959).

Demonstration of specific incorporation of one tyrosine unit into the isoquinoline segment of the hydrastine molecule would clearly confirm Wenkert's hypothesis and invalidate Robinson's. Demonstration of specific incorporation of two tyrosine units into hydrastine, on the other hand, would establish the validity of the classical hypothesis, and unequivocally disprove Wenkert's scheme for the biogenesis of hydrastine.

4. BIOSYNTHESIS OF ISOQUINOLINE ALKALOIDS

When this work was begun no experimental evidence on the biosynthesis of the nucleus of the isoquinoline alkaloids was available.

In 1953 the origin of the extranuclear carbon atoms of protopine was investigated (Scribney & Kirkwood, 1953), and it was established that methionine, and to a lesser extent formate, could serve as precursors of the methylenedioxy group, whereas choline was not utilized for this purpose. Within the last three years, however, a number of biosynthetic results have become available.

In extracts of Berberis vulgaris L. radioactivity was qualitatively detected by autoradiography in a chromatographic spot attributed to berberine, after feeding of 2-^{14}C-phenylalanine (Beal & Ramstad, 1960). In Coptis japonica Makino DL-2-^{14}C-tyrosine gave rise to berberine containing radioactivity (Imaseki, Oneyama & Tajima, 1960). Degradation showed that tyrosine had not suffered complete breakdown since the methoxy groups and the methylenedioxy group contained no activity. However, no information was obtained on the labelling of the nucleus of the active berberine. Other workers showed (Kleinschmidt & Mothes, 1959) that activity from L-U-^{14}C-tyrosine was incorporated into papaverine, narcotine, and narcotoline in Papaver somniferum L. The distribution of carbon -14 in narcotoline suggested that it was derived from two tyrosine units, but the evidence was indicative rather than conclusive.

There have been two alkaloids on which a detailed investigation has been carried out. Battersby and Harper (1959) proved by unam-

biguous degradation of labelled papaverine, obtained from feeding DL-2-^{14}C-tyrosine to Papaver somniferum L., that two units of precursor were incorporated into the alkaloid without randomization. Independent work by Battersby and Harper (1958), Battersby, Binks and LeCount (1960), and by Leete (1959), has shown that in Papaver somniferum L. labelled morphine is biosynthesized from two units of 2-^{14}C-tyrosine without randomization. In this case too the results are supported by unambiguous degradation procedures. In addition to this work one other interesting experiment has been carried out in connection with morphine. Battersby and Binks (1960) synthesized specifically labelled ^{14}C-norlaudanosoline VI and fed it to Papaver somniferum L. They obtained active morphine, and noted that incorporation of radioactivity into morphine was more efficient from radionorlaudanosoline than from any other precursor tested to date. Specific incorporation was not proven, however.

The scanty knowledge available on the biosynthesis of the isoquinoline bases confirms postulated biogenetic patterns in a general way. The present study was undertaken to extend knowledge by obtaining data on the biosynthesis of berberine, a protoberberine alkaloid, and hydrastine, a phthalideisoquinoline base. The investigation of the mode of formation of the latter alkaloid was thought to be of diagnostic value in establishing the validity of one or the other of the biogenetic hypotheses of Robinson and Wenkert.

INCORPORATION OF RADIOACTIVE PRECURSORS INTO HYDRASTINE AND BERBERINE

1. INTRODUCTION

The Canadian Golden Seal, Hydrastis canadensis L. (Ranunculaceae), contains two major alkaloids, berberine of the protoberberine group, and hydrastine of the phthalideisoquinoline group. Biosynthetic experiments with this plant therefore would not only give information about the biosynthesis of alkaloids representative of these two groups, but would also distinguish between the hypotheses of Robinson and of Wenkert, as applied to the isoquinoline alkaloids. Plants of this species, which were from two to three years old, had wintered in the open, and had been allowed to grow in soil during the spring and early summer, were used for root-feeding experiments. Plants of a similar history but from three to four years old were used for wick-feeding experiments.

Incorporation of a number of labelled precursors into hydrastine and berberine was tested. U-^{14}C-glucose, 2-^{14}C-phenylalanine, 2-^{14}C-tyrosine, and 3-^{14}C-tyrosine were fed to Hydrastis plants by a root-feeding method (Experiment 1-8; Table 5), and 3-^{14}C-tyrosine and 1-^{14}C-dopamine hydrobromide were fed by a wick-feeding method (Experiment 9, 10; Table 5). In each case hydrastine and berberine were extracted from the roots and rhizomes and assayed for activity, and in one case (Experiment 4L) the alkaloids in the leaves and stems were also extracted and assayed (Table 7).

The results obtained showed that of all the compounds tested tyrosine (labelled in either the 2- or 3- position) was the most efficient precursor of the alkaloids. Wick-feeding resulted in better incorporation of radioactivity over a given period of time than did root-feeding. It was also shown that the stems and leaves contained only small amounts of alkaloids, and that the incorporation here was substantially lower than in the roots and rhizomes of the plant. Dopamine was almost as good a precursor of berberine as was tyrosine, but was a less efficient precursor of hydrastine. Glucose was a much less efficient precursor of both alkaloids, whereas incorporation of phenylalanine was so low as to be negligible. The specific radiochemical yields obtained in each experiment are shown in Table 1.

Table 1

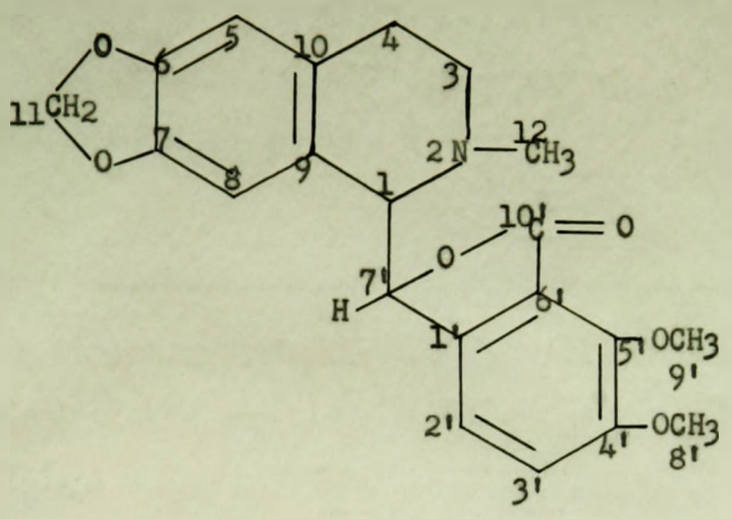
Experiment	Precursor	Specific Radiochemical Yield x 10^4	
		Hydrastine	Berberine
1	U-^{14}C-glucose	4.0	11
2	2-^{14}C-phenylalanine	1.4	1.7
3	2-^{14}C-tyrosine	68	100
4	2-^{14}C-tyrosine	100	240
4L	2-^{14}C-tyrosine	9.5	76
5	3-^{14}C-tyrosine	16.5	35
6	3-^{14}C-tyrosine	20	44
7	3-^{14}C-tyrosine	73	108
8	3-^{14}C-tyrosine	21	36
9	3-^{14}C-tyrosine	83	128
10	1-^{14}C-dopamine hydrobromide	14	74

$$\text{Specific Radiochemical Yield} = \frac{\text{Specific activity of product}}{\text{Specific activity of precursor}} \times 100$$

2. TYROSINE AS A SPECIFIC PRECURSOR OF HYDRASTINE

In order to demonstrate whether or not tyrosine was a specific precursor of hydrastine the radioactive alkaloid obtained from Experiment 4 was degraded according to the series of reactions described in Chapter V.

It was found that the activity was located exclusively at C_1 and C_3 of hydrastine XV. This showed that tyrosine was a specific precursor of



XV

hydrastine, and that two molecules of tyrosine were utilized per molecule of hydrastine. The ratio of radioactivity in C_1 and C_3 was not 1 : 1 but was found to be 2 : 3. This finding will be discussed later. Degradation of hydrastine from Experiment 3 was also undertaken. Although the sample was lost before the degradation was carried through all its stages, and complete confirmation of the above results was therefore not obtained, it was shown that carbon atoms 1' -10' and 12 of this sample contained no activity (Table 8).

In order to investigate a possible relationship between the ratio of incorporation of the two molecules of tyrosine and the period of growth in contact with tracer, 3-^{14}C-tyrosine was fed to Hydrastis for different periods of time (Experiment 5-8). The active hydrastine was isolated in each case and degraded as outlined in Chapter V. Since

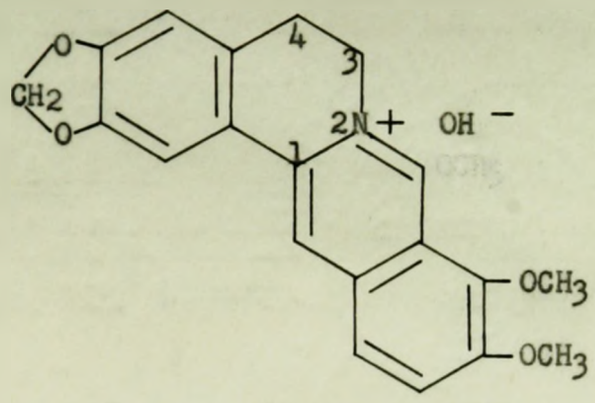
incorporation of two molecules of tyrosine into hydrastine had already been proven, it was sufficient in these experiments to separate the two labelled carbon atoms (4 and 7') from one another, and unnecessary to isolate them completely. The results of these experiments are summarized in Table 9, and at first sight no distinctive trend is apparent. In order to compare the effectiveness of two different methods of feeding, 3-^{14}C-tyrosine was fed to Hydrastis for 9 days (Experiment 9) by a wick-feeding method. A comparison of the specific activities of the alkaloids obtained from this experiment with those obtained from a root-feeding experiment of the same duration (Experiment 7), shows that the wick-feeding experiment gave better incorporation of the precursor into the alkaloids. Actually the difference is even more pronounced than is shown by a comparison of the specific activities, since plants of differing sizes were involved. A better comparison is obtained by consideration of the total activities of the alkaloids isolated in each case (Table 2).

Table 2

Experiment	Specific activity of hydrastine counts/min/mM	Total activity of hydrastine counts/min	Specific activity of berberine counts/min/mM	Total activity of berberine counts/min
7	4.96×10^4	1.55×10^4	7.32×10^4	3.60×10^4
9	5.76×10^4	6.38×10^4	8.90×10^4	17.9×10^4

3. TYROSINE AS A SPECIFIC PRECURSOR OF BERBERINE

Radioactive berberine was isolated in all the feeding experiments. Berberine obtained in Experiment 4 was degraded as described in Chapter V. It was found that activity was located exclusively at carbon atoms 1 and 3 of berberine XIV, and that in this case the ratio of incorporation was 1 : 1 (Table 11). This showed that tyrosine was a specific precursor of



XIV

berberine, and that two tyrosine units had been incorporated into berberine.

4. DOPAMINE AS A SPECIFIC PRECURSOR OF HYDRASTINE

In an attempt to find further intermediates of biosynthesis the incorporation of dopamine into hydrastine and berberine was tested. 1-^{14}C-Dopamine hydrobromide was fed to *Hydrastis* by a wick-feeding method for a period of ten days (Experiment 10). Radioactive hydrastine was extracted from the roots and rhizomes and was degraded as before. It was found that the activity was located exclusively at carbon atom 3. This showed that dopamine was a specific precursor of hydrastine, but that only one dopamine unit had been utilized in the biosynthesis of the alkaloid.

5. DOPAMINE AS A PRECURSOR OF BERBERINE

Radioactive berberine was obtained from the plants which had been fed the 1-^{14}C-dopamine hydrobromide. An attempt was made to degrade this in the same manner as was used previously, but a mistake in experimental work, as yet unexplained, caused the formation of an unknown compound in place of an expected degradation product. It is not yet known whether useful results can be obtained from this experiment.

THE BIOSYNTHESIS OF HYDRASTINE AND BERBERINE

1. THE VALIDITY OF THE BIOGENETIC APPROACH

The results of the feeding experiments carried out on *Hydrastis* showed that tyrosine was a direct precursor of both hydrastine and berberine, and moreover that two molecules of tyrosine were used per molecule of each alkaloid. By comparison, however, results showed that phenylalanine gave almost no incorporation into either alkaloid. This information is in agreement with Robinson's biogenetic scheme.

The evidence shows unequivocally, on the other hand, that Wenkert's scheme in its most recent modification (Wenkert, 1962), cannot be valid as a scheme for the biogenesis of hydrastine and berberine. There are two lines of evidence whereby this statement can be substantiated.

It has been pointed out that hydrastine is one of the few alkaloids by which one can differentiate between Robinson's and Wenkert's hypothesis. According to Wenkert both precursors (IV and XXVI) of the hydrastine molecule should be derived from prephenic acid. However, due to the irreversibility of the prephenate-tyrosine conversion, *only one* of these precursors IV *can* be derived from tyrosine. Thus the finding that both halves of the hydrastine molecule can be derived directly from tyrosine, is in direct opposition to Wenkert's scheme of biogenesis. Since, however, Robinson postulates that both halves of hydrastine are derived from tyrosine, these results are in agreement

with his hypothesis. This finding clearly invalidates Wenkert's scheme for the biogenesis of hydrastine, but not his scheme for berberine since in the latter case both the "Wenkert precursors" could logically be derived from tyrosine.

The second reason why Wenkert's biogenetic scheme cannot be valid rests on the fact that phenylalanine was not incorporated into either hydrastine or berberine. Since phenylalanine was fed to the plants at a time when, according to other experiments, active synthesis of the alkaloids was taking place, the results indicate that the conversion of phenylalanine to tyrosine is minimal in this plant. A similar observation has been reported on Salvia splendens Sello (McCalla & Neish, 1959). However, one of Wenkert's intermediates in the biogenesis of both hydrastine and berberine is phenylpyruvic acid (Fig.2), which is equivalent to phenylalanine by transamination. Thus the lack of incorporation of phenylalanine into these alkaloids is not compatible with Wenkert's hypothesis either for hydrastine or for berberine.

It must be emphasized that these results disprove Wenkert's hypothesis directly only for hydrastine and berberine. However, recent experimental work on the biosynthesis of alkaloids (Battersby, 1961) shows that alkaloids of the same group (differing only in extra-skeletal substituents) often are derived from the same precursor(s). Since both hydrastine and berberine are representatives of a much larger class of alkaloids of similar skeletal structure, the present results must be regarded as a strong argument against Wenkert's hypothesis for the biogenesis of many or all of the isoquinoline alkaloids.

2. BIOSYNTHESIS OF HYDRASTINE

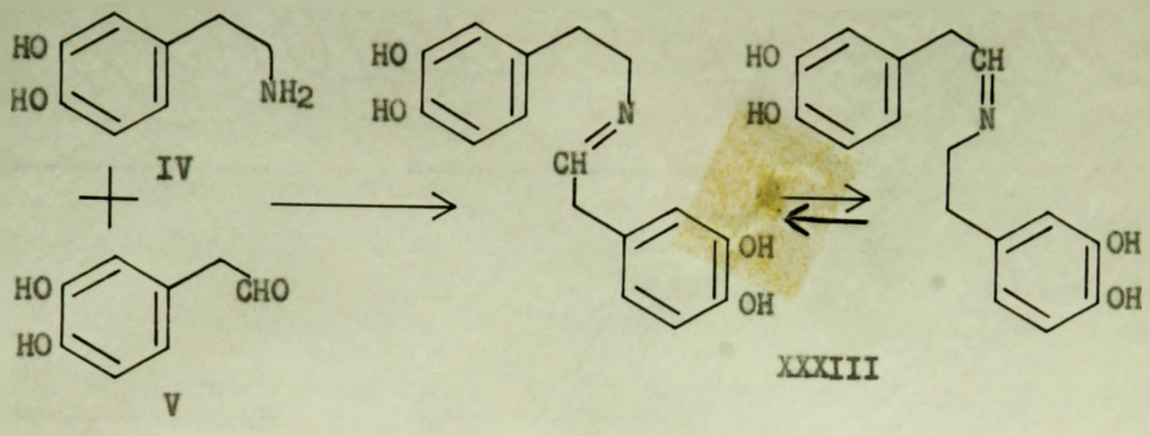
The results of the degradation of the active hydrastine from Experiment 4 showed that activity was confined to carbon atoms 1 and 3 of hydrastine, and thus that two moles of tyrosine were incorporated specifically into the alkaloid. The most interesting feature of these results, however, is the unequal labelling of carbon atoms 1 and 3, which have a relative activity of 39 and 60 respectively. This ratio indicates differential utilization of radioactive, but not of chemical, precursor for the two segments of the alkaloid molecule. Such a possibility has never been considered,[1] and it has hitherto always been tacitly assumed that biosynthetic utilization of two units of a single radioactive precursor to form the major portion of an end product, would inevitably be associated with equal labelling in the two segments of the latter. Reported work on papaverine (Battersby & Harper, 1959) and morphine (Battersby & Harper, 1958; Leete, 1959; Battersby et al, 1960) has tended to support this assumption, which, nevertheless, is not generally valid. Equal incorporation of radioactivity into the two segments of such a "dimeric" product is obligatory only if the "doubling" step in the biosynthetic sequence is of the type,

$$2A \longrightarrow A' - A'$$

that is, if two identical units of "monomeric" precursor combine to yield a true dimer as a biosynthetic intermediate (direct doubling); or if

[1] Since this work was completed (Gear & Spenser, 1961) similar results have been obtained in a study of the biosynthesis of morphine from carbon -14- dioxide (Rapoport, Levy & Stermitz, 1961).

the dimer produced by the combination of two dissimilar units of "monomeric" precursor becomes symmetrical (IV + V ⟶ XXXIII) (Rapoport et al, 1961).



If the doubling step were preceeded by one or more dissimilar structural modifications of either or both monomer units, such as

$$A \longrightarrow X \quad , \quad B \longrightarrow Y,$$

$$X + Y \longrightarrow X' - Y'$$

(indirect doubling), and if X' -Y' cannot become symmetrical, then the ratio of radioactivities in the two segments of the product will depend on a number of factors, the most important of which are described below.

(i) The number of metabolic steps in each branch of the sequence prior to "doubling" will be of great importance. If one branch of the sequence has more steps than the other, then the active carbon atom which passes through this branch will be diluted to a greater extent at all times during the feeding process.

(ii) The rate of each step in each branch will have an effect on the ratio.

(iii) The size of the metabolic pool of each branch intermediate is also a very important factor. If one of the intermediates has a large endogenous pool in the plant then the activity that joins this pool will be diluted to a large extent. Thus the ratio will initially favor the branch with the smallest pools. However, as time passes all active intermediates will have been cleared from the smaller pools, and the ratio will come to favor the branch with the larger pools, which will take longer to pass along all the active intermediates.

(iv) It is possible that branch or "dimeric" intermediates may be utilized for other metabolic processes (leakage). If this should happen only in one path then naturally less activity will pass through into the product and this will affect the ratio.

(v) Conversely there may be feed-in of branch intermediates from other non-radioactive precursors. This will dilute the activity in any path where it happens and thus affect the ratio.

(vi) Finally the ratio will be affected by the turn-over rate of the product. If there is no turn-over then all activity that gets into the product will accumulate. If there is turn-over then the ratio will vary continuously depending both on the rate of incorporation, and on the rate of turn-over.

It can be seen that these factors which cause the ratio to vary are of two kinds. Some (iv, vi) will always tend to force the ratio in one direction. Others (i, ii, iii, v) change with time, and may cause the ratio to favor one path at one stage in the feeding process, while at a later stage the other path may be favored. Thus it is difficult to

gain direct information about biosynthetic routes from such ratios, since several factors cause these ratios to vary, and may act at the same time, either independently or interdependently. In general, equal incorporation into both segments of the product will be approached in a single-dose feeding experiment at infinite time, provided there is no leakage or feed-in of branch precursors, no leakage of "dimeric" intermediates, and provided the product accumulates continuously. Unless these restrictions hold, any ratio of radioactive incorporation in the two segments of the product, including a fortuitous ratio of one, is possible. This ratio will vary continuously with time, and its numerical value in any single experiment will depend on the length of time allowed to elapse between initial administration of the tracer and harvesting of the plant.

The variation of this ratio with time was investigated by feeding specifically labelled tyrosine to Hydrastis plants for different periods of time (Experiment 4-9). In each experiment the hydrastine was isolated and degraded so as to separate the two active carbon atoms and to determine the ratio of C_1 to C_3 (or C_7' to C_4 in the cases where 3 -^{14}C-tyrosine was fed). If there was no leakage or feed-in of branch precursors, no leakage of "dimeric" intermediates, and the product accumulated continuously, a long-term experiment would be expected to lead to equal incorporation into both segments and a ratio of one. If alternatively the product did not accumulate continuously, the activity in each half of hydrastine would eventually reach zero. The ratios found at different times are summarized in Table 3, and although they show a definite trend with time, starting at a high value, dropping to a

Table 3.

Experiment	Precursors fed to plants	Duration of feeding (days)	Ratio of C_1 to C_3 or $C_{7'}$ to C_4
5	3-^{14}C-tyrosine	3	1.30
6	3-^{14}C-tyrosine	6	1.00
7	3-^{14}C-tyrosine	9	1.01
9	3-^{14}C-tyrosine (wick)	(9)	(0.75)
4	2-^{14}C-tyrosine	17	0.61
8	3-^{14}C-tyrosine	24	1.23

minimum, and then returning to a value greater than one, it does not appear to be possible to draw any definite conclusions about the biosynthesis of hydrastine from these figures. It should be noted that, since Experiment 9 was a wick-feeding experiment whereas all others were root-feeding experiments, the ratio of 0.75 obtained from it is not directly comparable with the other ratios. At the same time it is interesting to see that the value of 0.75 is between the values of 1.01 for Experiment 7 and of 0.61 for Experiment 4. This would appear to indicate that wick-feeding causes faster uptake of precursor, and that a nine-day wick-feeding experiment is equivalent to a root-feeding experiment of greater than nine but less than seventeen days. Unfortunately the figures obtained are not sufficient to give a complete picture of the change of the ratio with time. This uncertainty could be resolved by a further series of experiments in which 3-^{14}C-tyrosine were fed to Hydrastis for periods of less than three days, and of greater than twenty-four days.

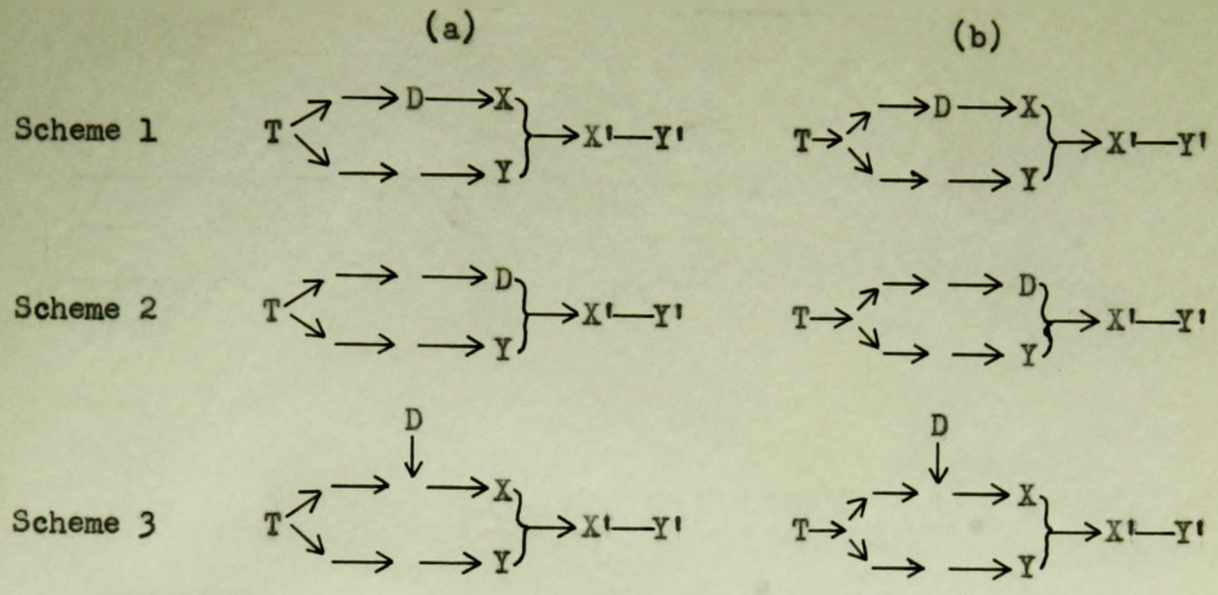
Although the major portions of each of the two segments of hydrastine (atoms 3-10, and 1, 1'-7' respectively) are now shown to be derived from a tyrosine unit, the two fragments differ in oxygen sub-

stitution (atoms 11, and 8'-9' respectively). In addition, a one-carbon unit (atom 10') is required to complete the skeleton of the alkaloid. Any or all of these modifications of the tyrosine units may take place either before or after the "doubling" step. However, the differential utilization of two tyrosine units in hydrastine biosynthesis implies that the "doubling" step of the anabolic sequence is preceded by one or more dissimilar structural modifications of either or both tyrosine units. It is likely therefore that tyrosine gives rise by independent pathways to two different "monomeric" metabolic intermediates, which join to form a "dimeric" precursor, from which the alkaloid is then derived by further steps.

The results obtained by the degradation of hydrastine from Experiment 10 showed that dopamine serves as a specific precursor of hydrastine, but that only one dopamine unit is utilized in the biosynthesis of the alkaloid (Spenser & Gear, 1962). Thus dopamine can serve as a precursor of one (that one containing C_3), but not of the other, of the "monomeric" intermediates; whereas tyrosine serves as a precursor of both the intermediate containing C_3 and the intermediate which provides C_1. Since no trace of activity was found in C_1 of the dopamine-derived hydrastine, the branch in which dopamine is involved must include an irreversible step.

Figure 3 shows the possible biosynthetic schemes with which the present evidence is compatible. These depend on whether tyrosine itself lies at the point of metabolic branching (Schemes 1a, 2a, 3a), or whether it gives rise to another compound (e.g. DOPA) which disproportionates

Figure 3

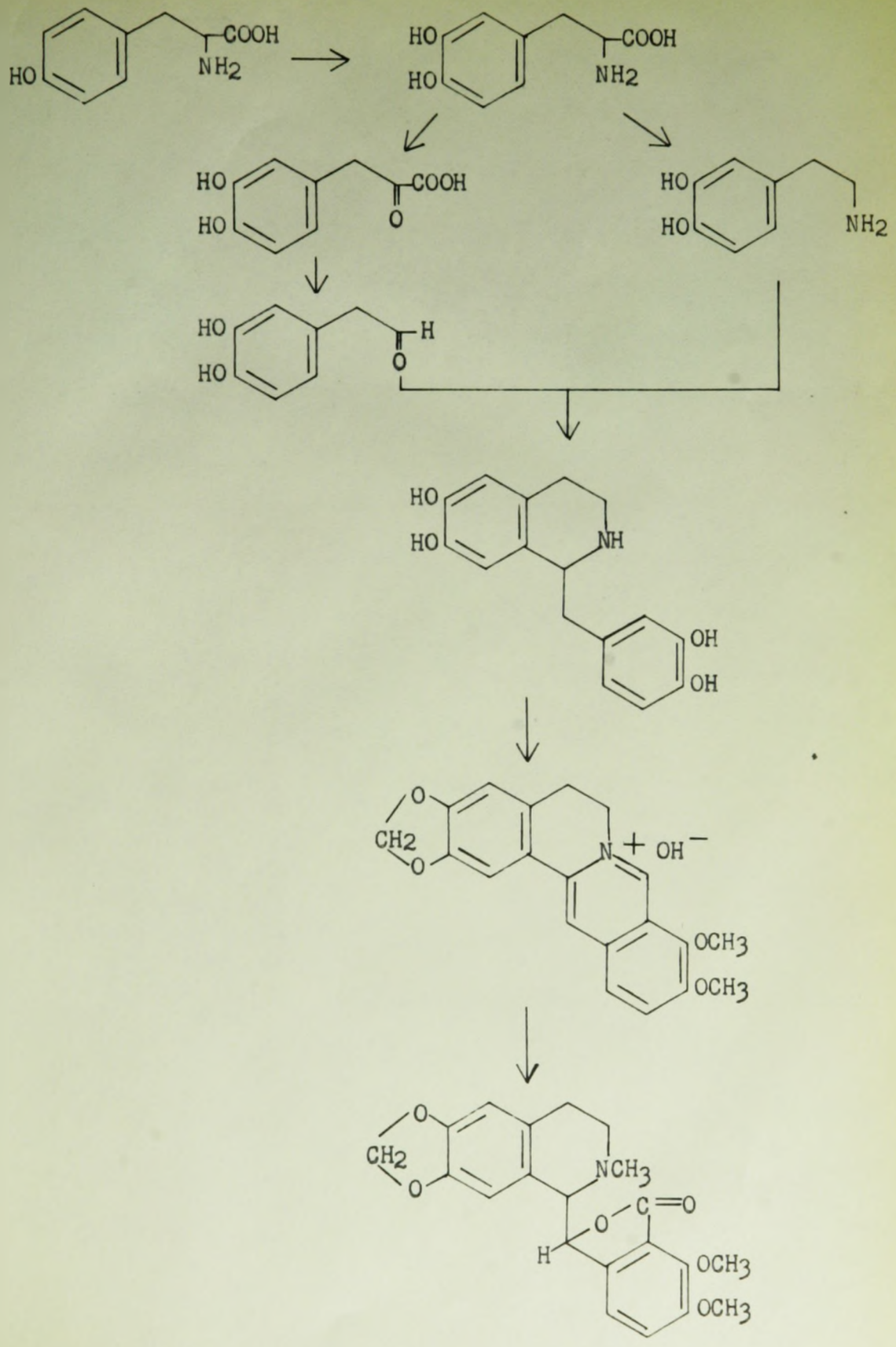


T = Tyrosine D = Dopamine
X, Y, = "monomeric" branch precursors involved in the doubling step
X'—Y' = "dimeric" product.

(Schemes 1b, 2b, 3b). Nor is it possible to decide from the present experiments whether dopamine is an obligatory stage of one branch (Schemes 1a, 1b), whether it is the "monomeric" intermediate of this branch involved in the "doubling" step (Schemes 2a, 2b), or whether it merely serves as an alternative source of a true intermediate in this branch (Schemes 3a, 3b).

An attractive possible scheme for the biosynthesis of hydrastine is shown in Figure 4. In this scheme DOPA lies at the point of metabolic branching and dopamine occupies one path. The occupant of the other path can be visualized as the keto acid or the aldehyde corresponding to DOPA. Combination of these two leads to norlaudanosoline, which by the introduction of a "berberine bridge" and appropriate methylation and oxidation could give berberine. Further oxidation of berberine could give hydrastine, which is always isolated with a lower specific activity than berberine.

Figure 4


HO
COOH
NH2
HO
HO
COOH
NH2
HO
HO
COOH
O
HO
HO
NH2
HO
HO
H
O
HO
HO
NH
OH
OH
O
CH2
O
N + OH −
OCH3
OCH3
O
CH2
O
NCH3
O
C=O
H
OCH3
OCH3

3. BIOSYNTHESIS OF BERBERINE

Degradation of the radioactive berberine obtained from Experiment 4 has shown that tyrosine is a specific precursor of berberine, and moreover that two molecules of tyrosine are incorporated into the alkaloid. However, the ratio of incorporation of these two units was 1:1, in contrast to the 3:2 ratio obtained for hydrastine in the same experiment. This does not necessarily indicate a separate biosynthetic path. Only if hydrastine and berberine are both formed directly and simultaneously from the same precursor, and if both accumulate continuously, would one expect to find identical ratios in both alkaloids. Under any other conditions these two ratios would be expected to differ. Although berberine has been isolated from the plants used in Experiment 5-9 it has not been degraded. Thus it is not yet known whether the ratio of incorporation of the two tyrosine moieties varies with time, either in the manner found for hydrastine or in a different manner, or whether the ratio retains the value one at all times.

Although it was found that dopamine served as a precursor of the berberine isolated from Experiment 10, the alkaloid has not been completely degraded. It remains to be seen therefore, whether dopamine is a specific precursor of berberine, and if so whether one or two molecules of dopamine are utilized in the biosynthesis of the alkaloid. Until this is known one cannot say whether berberine is formed in the same way as hydrastine (by a "split path"), or whether it is derived by a different scheme.

The question of whether dopamine is a direct precursor of these two alkaloids is one that should be answered by a comparison of the specific activities of the dopamine-derived and the tyrosine-derived alkaloids (Experiment 10 and 9 respectively). These values, related to C_3 in each case, are compared in Table 4. If dopamine were indeed on the direct route from tyrosine to hydrastine, the specific radiochemical yield with respect to C_3 of hydrastine should be higher on feeding radiodopamine than on feeding radiotyrosine. Table 4 shows that this is not the case. Taken at face value this result would appear to indicate that dopamine is not a direct intermediate in hydrastine biosynthesis. A different picture emerges in the case of berberine. On the assumption that the activity from dopamine is restricted to C_3, the specific radiochemical yield with respect to C_3 derived from dopamine is slightly larger than that calculated for berberine derived from tyrosine. (Should the activity from dopamine not be restricted to C_3, then it would be larger by a greater factor). It would appear therefore that dopamine lies directly on the route from tyrosine to berberine.

Clarification of this latter problem must await further work, in particular the investigation of the metabolic inter-relationship of the two alkaloids. It would be of great interest to feed radioactive hydrastine and berberine to different plants, in order to see whether both are formed from the same precursor, from different precursors, or whether one of them is formed from the other. It would also be of interest to investigate further other possible

Table 4

PRECURSOR		PRODUCT			
		HYDRASTINE		BERBERINE	
Chemical precursor	Specific activity counts/ min/mM	Specific activity with respect to C_3 counts/min/mM	Specific radiochemical yield with respect to C_3[1]	Specific activity with respect to C_3 counts/min/mM	Specific radiochemical yield with respect to C_3
3-^{14}C-tyrosine	6.95×10^8	3.28×10^4	4.72×10^{-3}	8.90×10^4	6.40×10^{-3}
1-^{14}C-dopamine	6.12×10^8	0.87×10^4	1.92×10^{-3}	4.50×10^4	7.35×10^{-3} [2]

[1] Specific radiochemical yield with respect to C_3

$$= \frac{\text{specific activity with respect to } C_3}{\text{specific activity of precursor}} \times 100$$

[2] Specific activity with respect to C_3 was calculated on the assumption that, by analogy with hydrastine, radioactivity in berberine is confined to C_3.

intermediates in the biosynthesis of hydrastine and berberine.

Compounds which might be expected to yield information are DOPA, dihydroxyphenylpyruvic acid, dihydroxyphenylacetaldehyde, norlaudanosoline, and analogous compounds containing methoxy or methylenedioxy groups.

THE CHEMISTRY OF THE DEGRADATION OF HYDRASTINE AND BERBERINE

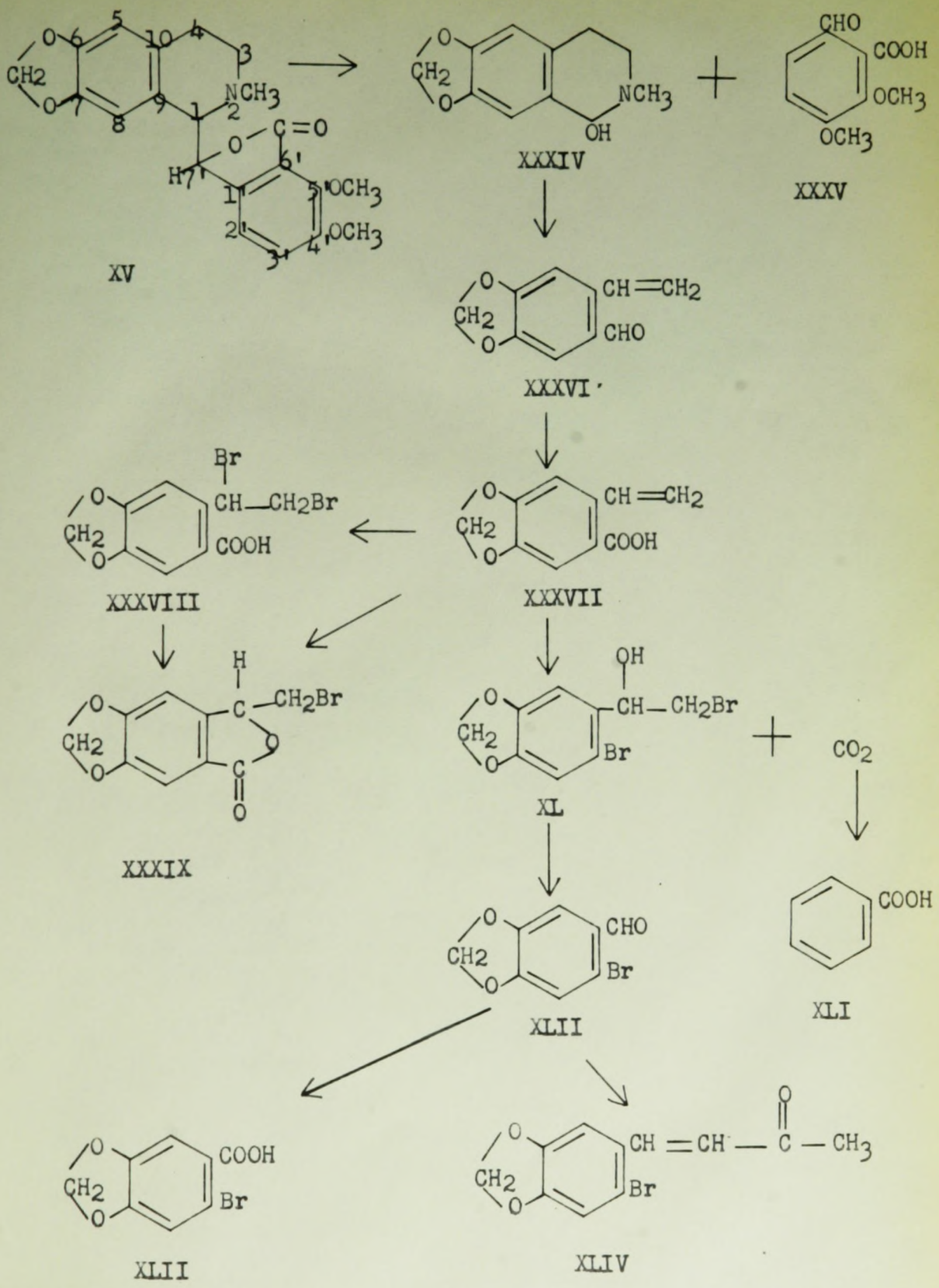
1. HYDRASTINE

In the first experiment on the biosynthesis of hydrastine from tyrosine, 2-^{14}C-tyrosine was used. If incorporation of this compound into the alkaloid were specific, hydrastine labelled at C_1 and/or C_3 would be expected. For this reason a degradation of hydrastine was devised which allowed the separation of these particular carbon atoms. A diagram of the reaction sequence to which the molecule was subjected in the course of degradation is shown in Figure 5.

The first stage in the degradation was the oxidation of hydrastine XV to give hydrastinine XXXIV and opianic acid XXXV. This reaction, which has been carried out with any one of a variety of oxidising agents(including air), was performed in good yield using 20% nitric acid. The hydrastinine still contained both C_1 and C_3.

To remove the nitrogen atom and N-methyl group the hydrastinine was subjected to a Hofmann reaction which yielded hydrastal XXXVI. Attempts to effect this conversion in a two-stage reaction by way of the quaternary methiodide (Brown & Newbold, 1952) gave overall yields which were quite low. It was found, however, that the reaction could be done in one step in a heterogeneous system (benzene-water), when yields of the order of 70% were general. Hydrastal was converted in good yield by silver oxide oxidation into 6-vinylpiperonylic acid XXXVII, a new compound, which still contained C_1 and C_3. The N-methyl

Figure 5


XV
XXXIV
XXXV
XXXVI
XXXVII
XXXVIII
XXXIX
XL
XLI
XLII
XLIII
XLIV

group was not isolated since it was not expected to contain activity.

With the intention of isolating C_1, attempts were made to decarboxylate the vinyl acid by heating in quinoline with a copper chromite catalyst. The carbon dioxide was readily isolated from this reaction, but the resulting methylenedioxystyrene was rather unstable and polymerized easily. This undesirable side reaction was partially retarded in the presence of hydroquinone, and the styrene was converted to the dibromo compound by bromine-water. However, yields were unsatisfactory and the product was extremely hard to purify.

Other attempts were made to isolate C_3 by the oxidation of the vinyl group of XXXVII. These were completely unsatisfactory. Vigorous oxidation to the carboxylic acid had to be avoided, since conversion to a phthalic acid derivative would have rendered C_1 and C_4 indistinguishable. Milder oxidation should have given the glycol, but this could not be isolated. Reaction with osmium tetroxide followed by sodium periodate, which should have given formaldehyde and an aromatic aldehyde, did appear to take place, but no definitive products were identified.

It was found that C_1 could be removed by bromodecarboxylation. Jones and Robinson (1917) had used this technique for the removal of the carboxyl group from piperonylic acid, using bromine-water in dilute sodium carbonate. Since, in the active degradation, the carbon dioxide from C_1 was to be collected and assayed for activity, these conditions were not applicable to the present problem. Attempts to carry out the reaction with bromine-water in acetone or carbon

disulphide did not lead to decarboxylation. Bromination of the vinyl compound XXXVII in carbon disulphide gave the dibromo derivative XXXVIII, which on recrystallization from acetone–water underwent spontaneous hydrolysis and lactonization to yield the lactone XXXIX. The same lactone was isolated directly when the vinyl compound was brominated in acetone solution. Although the lactone was a very stable compound it might have been expected to undergo alkaline hydrolysis and the haloform reaction in the presence of sodium hypoiodite. The first time this reaction was tried iodoform was indeed isolated in quantitative yield. However, despite many attempts, this reaction could not be repeated.

Finally it was found that the vinyl acid XXXVII could be converted in good yield to the bromohydrin XL by bromine–water in a solution of disodium hydrogen phosphate. The carbon dioxide (C_1) was liberated from solution by hydrochloric acid and collected as barium carbonate. The barium carbonate was then converted to benzoic acid XLI for reasons that are discussed in a later section.

It still remained to separate C_3 from the rest of the molecule. One would expect the bromohydrin XL to undergo an iodoform reaction, but many attempts showed that it did not do so. It was found, however, that oxidation of XL with potassium permanganate in pyridine–water gave 6-bromopiperonal XLII. The other fragment was not isolated, but since only C_3 was lost its activity could be found by comparing the activities of XL and XLII.

Since XLII was difficult to purify it was converted into a derivative. Oxidation with silver oxide gave 6-bromopiperonylic acid

XLIII in good yield. Another derivative which was prepared was 6-bromo-piperonylidene acetone XLIV. This was easily obtained by the interaction of XLII with acetone in dilute sodium hydroxide.

2. BERBERINE

In the degradation of berberine a series of reactions was used which had been originated by Freund and Fleischer (1913) (Figure 6). Berberine had been isolated from the plant as berberine hydrochloride XLV. This was allowed to react with benzylmagnesium bromide to give benzyldihydroberberine XLVI in good yield.

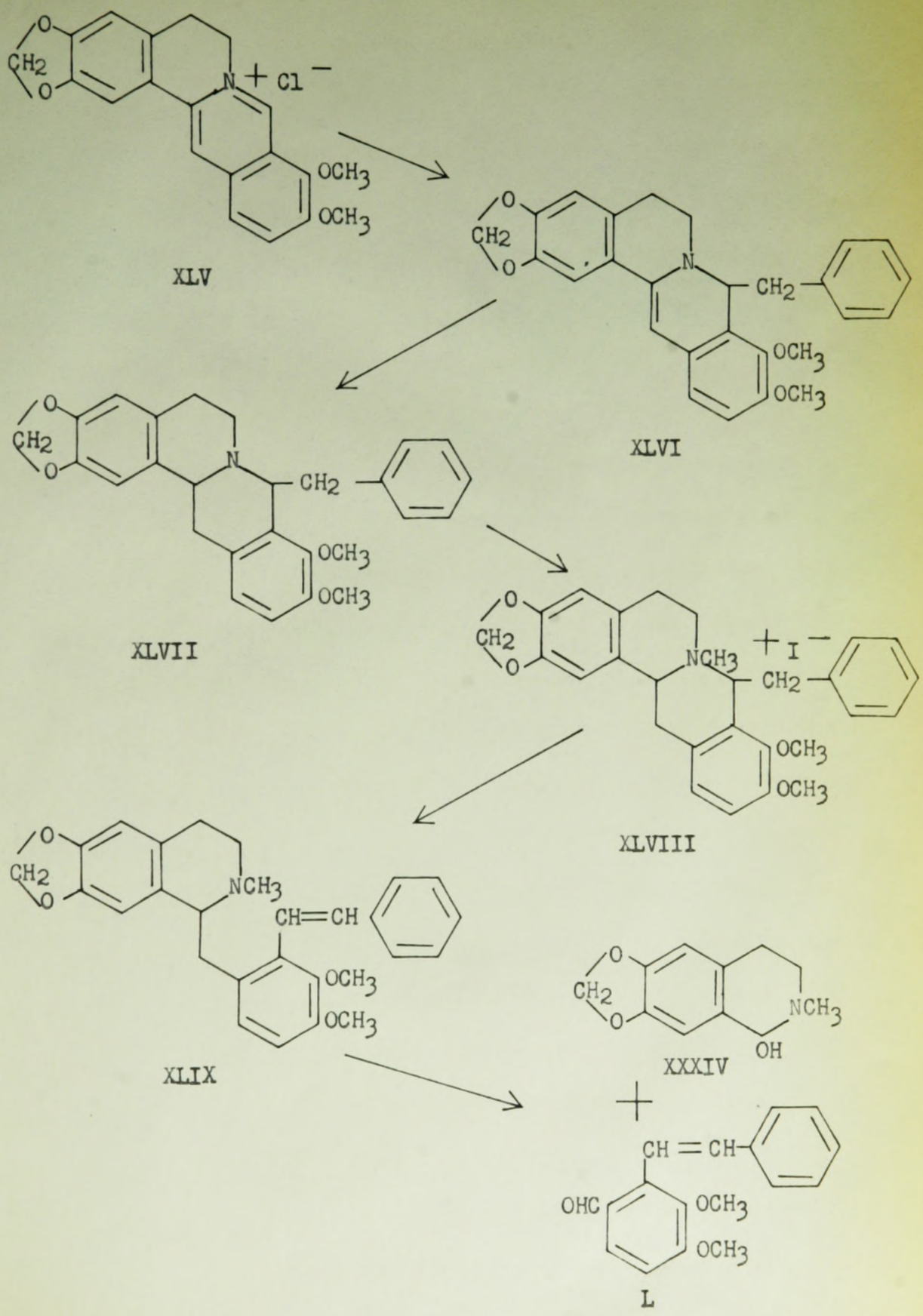
Freund and Fleischer (1913) had reduced this compound with tin and hydrochloric acid, but this reaction could not be repeated. Instead reduction by means of sodium borohydride was investigated, and was found to convert XLVI rapidly to benzyltetrahydroberberine XLVII in almost quantitative yield. When XLVII was heated in a sealed tube with methyl iodide the methiodide XLVIII was formed. This methiodide was then allowed to undergo a Hofmann reaction. Silver oxide and solid sodium hydroxide, as used by Freund and Fleischer (1913), gave poor yields of product. Methanolic potassium hydroxide, however, was found to give good yields of des-N-methylstyryltetrahydroberberine XLIX.

Oxidation of XLIX with sodium dichromate in acetic acid gave hydrastinine XXXIV and 3, 4-dimethoxy-2-styrylbenzaldehyde L. The hydrastinine, containing both predicted sites of radioactive carbon (C_1 and C_3), was degraded in the manner already described.

3. ISOLATION OF CARBON DIOXIDE AS BENZOIC ACID

The radioactive organic compounds used in this work were assayed in the form of very thin uniform films on aluminum planchettes, using a

Figure 6



thin-window Geiger-Mueller counter, by a technique described in Chapter V. Barium carbonate cannot be plated in the same manner, since no suitable solvent is available. Even if a solvent were available, the assumption made for organic compounds (that since carbon-hydrogen ratios do not differ greatly the self absorption has a constant dependence on sample thickness) would not hold for barium carbonate, and thus results from the two sources would not be comparable.

Barium carbonate can be counted, in the solid form, on a planchette as an infinitely thick layer; or the carbon dioxide can be liberated and counted in a gas counter. The disadvantage of the use of either of these methods in the present work would have been that, due to the different geometries of the systems, the results obtained would not have been directly comparable with the results from the assay of the organic compounds. In order to compare the two sets of results directly the counting efficiencies of the two systems would have had to be compared. This could be done, for example, by the combustion of a sample of an organic compound (the activity of which had been measured as a thin film on a planchette) and the measurement of its activity, either as solid barium carbonate or as gaseous carbon dioxide. Direct comparison of results from the different methods would then be possible.

A neater method was found to circumvent this problem. It is not known whether this method has been used by others, but as far as the author is aware, no one has reported the use of this method in biosynthetic studies of this type. The carbon dioxide was liberated from the barium carbonate, and allowed to react with phenylmagnesium bromide to yield benzoic acid XLI which contained C_1. The benzoic acid was then assayed in the same manner as all other radioactive organic

compounds, and the results obtained were directly comparable. The apparatus used to carry out the Grignard reaction on a small scale is described by Van Bruggen, Claycomb, and Hutchens (1950). The yields were only fair, but were adequate for the purposes of this experiment.

EXPERIMENTAL

1. ADMINISTRATION OF TRACER TO THE PLANTS

For root-feeding experiments small 2- and 3- year old plants of Hydrastis canadensis L., which had been wintered in the open, and allowed to grow in soil during the spring and early summer, were used. The plants were dug up before they had reached the flowering stage, and soil was removed by soaking the roots in water. Nine plants were inserted through holes in a wooden box, with their stems supported by glass wool plugs, and their roots dipping into 1500 ml of nutrient solution (Leete, Marion & Spenser, 1954) in a glass dish which was kept in the dark. An aquarium pump was used to bubble filtered air continuously through the solution, which was restored to its original volume using a mark on a small U tube, before samples were taken out for counting. The plants were kept in 75% shade by a framework of slats covered with cheesecloth. Before the start of the actual feeding experiment the plants were allowed to grow in nutrient solution under these conditions for 1 week, during which time they had completely recovered from transplanting. The nutrient solution was then replaced with 1500 ml of fresh nutrient solution containing the ^{14}C labelled compound, and the plants were allowed to grow in this system for the required period. The radioactivity of the nutrient solution and of the plant leaves was assayed daily. After a given time the plants were

harvested and rinsed with distilled water. Roots and rhizomes were separated from leaves and stems, and dried separately for several days in a vacuum desiccator (NaOH). The dried material was stored in this manner.

Wick-feeding experiments were carried out on large 3- and 4- year old Hydrastis canadensis L. plants which were almost at the flowering stage. In these experiments the plants were kept in soil under 75% shade. Wicks of mercerized cotton were passed through the stem of each plant, and the loose ends were allowed to dip into an aqueous solution of the ^{14}C labelled compound in a small container[1]. When the solution had been absorbed by the plant the containers were repeatedly refilled with water to ensure complete absorption of the tracer. After the required growing time the plants were harvested and stored as described earlier. The cotton wicks were extracted with methanol and with water, and the extract was assayed for activity.

Eight root-feeding experiments (1-8) and two wick-feeding experiments (9,10) were carried out. The details of these are summarized in Table 5. During each root feeding experiment samples of the nutrient solution were assayed at regular intervals. It was found that in all cases 95% of the activity disappeared from the nutrient solution within 40 hours. A typical set of results is given in Table 6. In an attempt to follow the uptake of radioactivity by the plant, pieces of leaf were assayed at different times. In general

[1] A drop of hydrochloric acid was added to the aqueous solution in order to dissolve tyrosine.

Table 5

Experiment	Compound fed	Weight fed (mg)	Specific activity counts/min/mM	Total activity fed counts/min	Number of plants	Period of growth in contact with tracer
			ROOT FED PLANTS			
1	U-^{14}C-glucose	18.02	8.75×10^8	8.77×10^7	9	17 days
2	2-^{14}C-phenylalanine	35.0	4.18×10^8	8.87×10^7	9	9 "
3	2-^{14}C-tyrosine	30.5	6.55×10^8	11.0×10^7	9	9 "
4	2-^{14}C-tyrosine	30.5	5.15×10^8	8.68×10^7	9	17 "
5	3-^{14}C-tyrosine	2.8	7.23×10^8	1.12×10^7	6	3 "
6	3-^{14}C-tyrosine	2.8	6.96×10^8	1.08×10^7	6	6 "
7	3-^{14}C-tyrosine	5.6	6.75×10^8	2.10×10^7	9	9 "
8	3-^{14}C-tyrosine	2.8	6.58×10^8	1.03×10^7	6	24 "
			WICK FED PLANTS			
9	3-^{14}C-tyrosine	2.8	6.95×10^8	1.07×10^7 [1/2]	4	9 "
10	1-^{14}C-dopamine hydrobromide	13.0	6.12×10^8	2.16×10^7	6	10 "

[1] The wicks used in this experiment contained negligible residual activity at the end of the feeding period.

[2] The solution initially fed in this experiment had a total activity of 3.40×10^7 counts/min. However, extraction of the wicks at the time of harvesting yielded a total activity of 1.24×10^7 counts/min, which was shown to be due to unchanged dopamine by chromatography in n-propanol/water : 7/3, and by radioassay of the chromatogram. The total activity of dopamine taken up by the plant was thus 2.16×10^7 counts/min.

Table 6

Activity of Nutrient Solution (Experiment 4)

Time	Total remaining activity of solution	% Remaining
0 hrs	8.65×10^7 counts/min	100.0
20 "	4.67×10^7 "	54.0
43 "	0.25×10^7 "	2.9
67 "	0.14×10^7 "	1.6
283 "	0.04×10^7 "	0.5

the leaves showed high activity 40 - 90 hours after the start of the experiment. Activity decreased to a low value after about 200 hours. Due to the obvious difficulty of plating leaf samples of uniform weight and thickness, or samples which represented a uniform percentage of the plants foliage, these observations have little quantitative significance.

2. EXTRACTION OF ALKALOIDS FROM PLANTS (Cromwell, 1955).

In each of the experiments the alkaloids, hydrastine and berberine, were extracted from the roots and rhizomes of the plants. A typical separation procedure (Experiment 3) is described.

The roots and rhizomes of well dried Hydrastis canadensis L. plants (9.3 g) were ground to a fine powder in a mortar. To extract hydrastine the powder was thoroughly moistened with 3 M ammonium hydroxide (10 ml), allowed to stand for 30 minutes, transferred to a Soxhlet thimble (Whatman, 35 x 80 mm), and extracted continuously with ether (125 ml) for 26 hours. The ether extract was transferred to a separatory funnel and washed, first with 3 M ammonium hydroxide (30 ml), and then with water (30 ml). The washings were rejected. The washed

ether solution was extracted with 10% hydrochloric acid (4 x 25 ml), and the combined acid portions were made strongly basic with 15 M ammonium hydroxide. This ammoniacal solution was extracted with ether (4 x 50 ml), the combined ether extracts were evaporated to dryness, and the residue was dissolved in a very small amount of hot ethanol. Hydrastine (173 mg; 1.9%) crystallized on cooling.

To obtain berberine the material remaining in the thimble was extracted continuously with 95% ethanol (125 ml) for 24 hours. The ethanolic extract was evaporated to dryness, and water (30 ml) was added to the residue, which was heated on the steam bath and filtered. The solid remaining after filtration, which contained amino acids, including radioactive tyrosine, was dissolved in 3% aqueous sodium hydroxide and stored. The filtrate was made strongly acidic with conc. hydrochloric acid and allowed to stand overnight, when berberine hydrochloride (205 mg; 2.2%) crystallized. The mother liquor from this crystallization, which contained some amino acids, was also stored.

In this way hydrastine and berberine were isolated from roots and rhizomes of the plants from each of the ten experiments. In one case (Experiment 4) the alkaloids were also isolated from the leaves and stems by the same procedure (Experiment 4L). Chemical and radioactive yields from all experiments are shown in Table 7.

Table 7

Experiment	Weight of plant material (g)	HYDRASTINE Weight (mg)	HYDRASTINE % yield	HYDRASTINE Specific activity counts/min/mM	BERBERINE HYDROCHLORIDE Weight (mg)	BERBERINE HYDROCHLORIDE % yield	BERBERINE HYDROCHLORIDE Specific activity counts/min/mM
1	36.7	553	1.5	0.35×10^4	679	1.9	1.0×10^4
2	25.7	463	1.8	0.06×10^4	511	2.0	0.07×10^4
3	9.3	173	1.9	4.48×10^4	205	2.2	6.54×10^4
4	14.5	205	1.4	5.17×10^4	380	2.6	12.20×10^4
4L	5.0	22	0.4	0.49×10^4	16	0.3	3.92×10^4
5	14.2	238	1.7	1.19×10^4	389	2.7	2.53×10^4
6	15.4	273	1.8	1.42×10^4	359	2.3	3.07×10^4
7	15.2	240	1.6	4.96×10^4	415	2.7	7.32×10^4
8	12.0	219	1.8	1.36×10^4	355	3.0	1.83×10^4
9	35.6	424	1.2	5.76×10^4	818	2.3	8.90×10^4
10	36.0	555	1.5	0.87×10^4	825	2.5	4.50×10^4

3. DEGRADATION OF HYDRASTINE

Conversion of hydrastine to hydrastinine and opianic acid

20% Nitric acid (2.5 ml) was added to hydrastine (136 mg) in a 10 ml beaker, and the mixture was kept at 41° in an incubator for 8.5 hours. The solution was cooled in an ice bath, and conc. aqueous sodium hydroxide was added at a rate such that the temperature did not rise above room temperature. A gummy mass separated, which, when the cold solution was stirred vigorously, first solidified and then broke up into finer particles which were filtered off to give hydrastinine (65 mg; 88%) melting at 110 - 113°. One crystallization from ethyl acetate raised the melting point to 116 - 117°. The filtrate remaining after hydrastinine had been removed was made strongly acidic with conc. hydrochloric acid and stored in the freezer overnight. The solid which separated when the frozen mixture was restored to room temperature was filtered off to give opianic acid (38 mg; 51%) melting at 141 - 144°. One crystallization from water raised the melting point to 144 - 145°.

Conversion of hydrastinine to hydrastal

Benzene (10 ml), 20% aqueous sodium hydroxide (10 ml), and methyl iodide (5 ml) were added to hydrastinine (101 mg) in a 100 ml round bottom flask. The flask was stoppered, the stopper was secured with tape, and the mixture was shaken for 24 hours. Water (10 ml) was added, a downward condenser was fitted, and the flask was heated on a steam bath for 30 minutes. Benzene and excess methyl iodide distilled off leaving an aqueous basic solution, from which hydrastal separated as an oil which solidified on cooling. After standing for 3 hours this solid was filtered off to give hydrastal (75 mg; 87%) melting at

75 - 78°. One crystallization from ethyl acetate raised the melting point to 78 - 79°.

Conversion of hydrastal to 6-vinylpiperonylic acid. (Gensler, Healy, Onschuus & Bluhm, 1956).

Hydrastal (106 mg) was suspended in a solution of potassium hydroxide (425 mg) in water (4.25 ml) in a 50 ml Erlenmeyer flask, and a solution of silver nitrate (650 mg) in 50% ethanol (8 ml) was added. After 5 minutes the mixture was heated at 80° on a water bath for 15 minutes, allowed to cool for 2.5 hours with occasional swirling, and filtered. The pH of the filtrate was adjusted to pH 1 by means of 50% nitric acid, and the mixture was then heated on a steam bath (with the addition of a little ethanol if necessary) until a clear solution was obtained. The solution was allowed to cool, and the solid was filtered off to give 6-vinylpiperonylic acid (95 mg; 82%) melting at 166 - 168°. One crystallization from aqueous ethanol raised the melting point to 168°. (Found: C, 62.4; H, 4.5. $C_{10}H_8O_4$ requires C, 62.5; H, 4.2%).

Conversion of 6-vinylpiperonylic acid to 6-bromo-α-hydroxyhomopiperonyl bromide.

6-Vinylpiperonylic acid (59 mg) was dissolved in 3% disodium hydrogen phosphate (15 ml) in a 50 ml Erlenmeyer flask by swirling for 1 minute, and the solution was cooled in an ice bath for 10 minutes. Saturated bromine-water (10 ml) was added dropwise over a period of 2 minutes, and the mixture was allowed to stand for 3 minutes. It was then filtered quickly under moderately reduced pressure, and the wet precipitate (A) was set aside. The filtrate was immediately transferred to a 50 ml round bottom flask which was attached to a gas line. Nitrogen,

which had been purified by passage through saturated barium hydroxide solution and through conc. sulphuric acid, was bubbled through the solution. The exit gases were passed through 1M aqueous sodium hydroxide (15 ml), which was protected from the atmosphere by a trap containing saturated barium hydroxide solution. Through a small dropping funnel in a side arm conc. hydrochloric acid (3 ml) was added to the flask containing the reaction filtrate. Nitrogen was passed at room temperature, and swept the liberated carbon dioxide into the sodium hydroxide trap. After 1 hour 17% barium chloride solution (3 ml) was added to the solution in the trap. The precipitate of barium carbonate (44 mg; 61%) was filtered off.[1] Meanwhile the wet precipitate (A) was transferred, along with the filter paper, to a 50 ml Erlenmeyer flask. Any material adhering to the funnel was washed into the flask with methanol, and enough methanol was added to dissolve the solid on warming. The mixture was filtered, and the filtrate was evaporated to dryness at the water pump. The residue was 6-bromo-α-hydroxyhomopiperonyl bromide (79 mg; 79%) melting at 148 - 153°. Two crystallizations from aqueous methanol raised the melting point to 157 - 158°.

[1] It was shown by several blank experiments that if this procedure were followed exactly, 3 mg of the barium carbonate so obtained came from extraneous sources, and would thus act as an inactive impurity if the barium carbonate derived from the reaction contained ^{14}C. A correction factor to account for this must be incorporated into any calculations which derive results from the count rate of this barium carbonate or of its derivatives.

Conversion of 6-bromo-α-hydroxyhomopiperonyl bromide to 6-bromopiperonal.

6-Bromo-α-hydroxyhomopiperonyl bromide (65 mg) was dissolved in pyridine (6 ml) in a 50 ml 2-necked round bottom flask. A small reflux condenser and a small dropping funnel were fitted. Powdered potassium permanganate (65 mg) was dissolved in water (4 ml) by warming on a steam bath, and a pellet of potassium hydroxide was added. The solution was poured into the dropping funnel and the last drops washed in with water (2 ml). The round bottom flask was warmed on a heating mantle until the contents boiled gently. The permanganate solution was then added dropwise, and washed in with water (2 ml). After boiling for a further 10 minutes the mixture was allowed to cool, and a few drops of ethanol were added to destroy any unreacted potassium permanganate. When quite cold the mixture was filtered. The filtrate was made strongly acidic with conc. hydrochloric acid, and then extracted with ether (4 x 25 ml). The combined ether extracts were evaporated to dryness in a 50 ml flask leaving a residue of 6-bromopiperonal (28 mg; 61%) melting at 105-110°. Recrystallization from aqueous ethanol gave a purer sample of 6-bromopiperonal, but it was found that the best way to purify this compound for the purposes of this experiment, was to convert it either to 6-bromopiperonylic acid or to 6-bromopiperonylidene acetone.

Conversion of 6-bromopiperonal to 6-bromopiperonylic acid.

A solution of potassium hydroxide (500 mg) in water (5 ml) was added to 6-bromopiperonal (28 mg) in a 50 ml round bottom flask. A solution of silver nitrate (750 mg) in 50% ethanol (10 ml) was added to this mixture which, after standing for 5 minutes, was heated on a

water bath at 85° for 30 minutes. The mixture was then allowed to stand for 2.5 hours and filtered. The filtrate was acidified with conc. hydrochloric acid, and then extracted with ether (3 x 30 ml). The combined ether extracts were evaporated to dryness, and the residue dissolved in hot water (30 ml). The solution was filtered and allowed to cool. The solid was filtered off to give 6-bromopiperonylic acid (25 mg; 83%) melting at 200 - 202°. One crystallization from water raised the melting point to 204 - 205°.

Conversion of 6-bromopiperonal to 6-bromopiperonylidene acetone.

6-Bromopiperonal (28 mg) was dissolved in acetone (3 ml) in a 25 ml Erlenmeyer flask. 3% Aqueous sodium hydroxide (2 ml) was added, and the solution was heated on a steam bath until a precipitate had formed and the excess acetone had evaporated. The solid was filtered off to give 6-bromopiperonylidene acetone (25 mg; 81%) melting at 148 - 151°. One crystallization from methanol raised the melting point to 152 - 154°.

Conversion of piperonal to 6-bromopiperonylidene acetone.

Piperonal (1.38 g) was dissolved in 95% ethanol (20 ml), and bromine-water was added until a brown oil and some crystals separated. The crystals were filtered off, washed well with water, and recrystallized from aqueous ethanol to give 6-bromopiperonal (390 mg; 19%) melting at 126 - 127°. A portion of this 6-bromopiperonal was converted, in the manner just described, to 6-bromopiperonylidene acetone melting at 152 - 154° and identical in all respects to 6-bromopiperonylidene acetone derived from the degradation of hydrastine. A mixed melting

point of the samples of 6-bromopiperonylidene acetone, prepared from the two different sources, showed no depression. This information serves as a check on the identity of the final product obtained from the degradation of hydrastine.

Preparation of approx. 0.25M phenylmagnesium bromide.

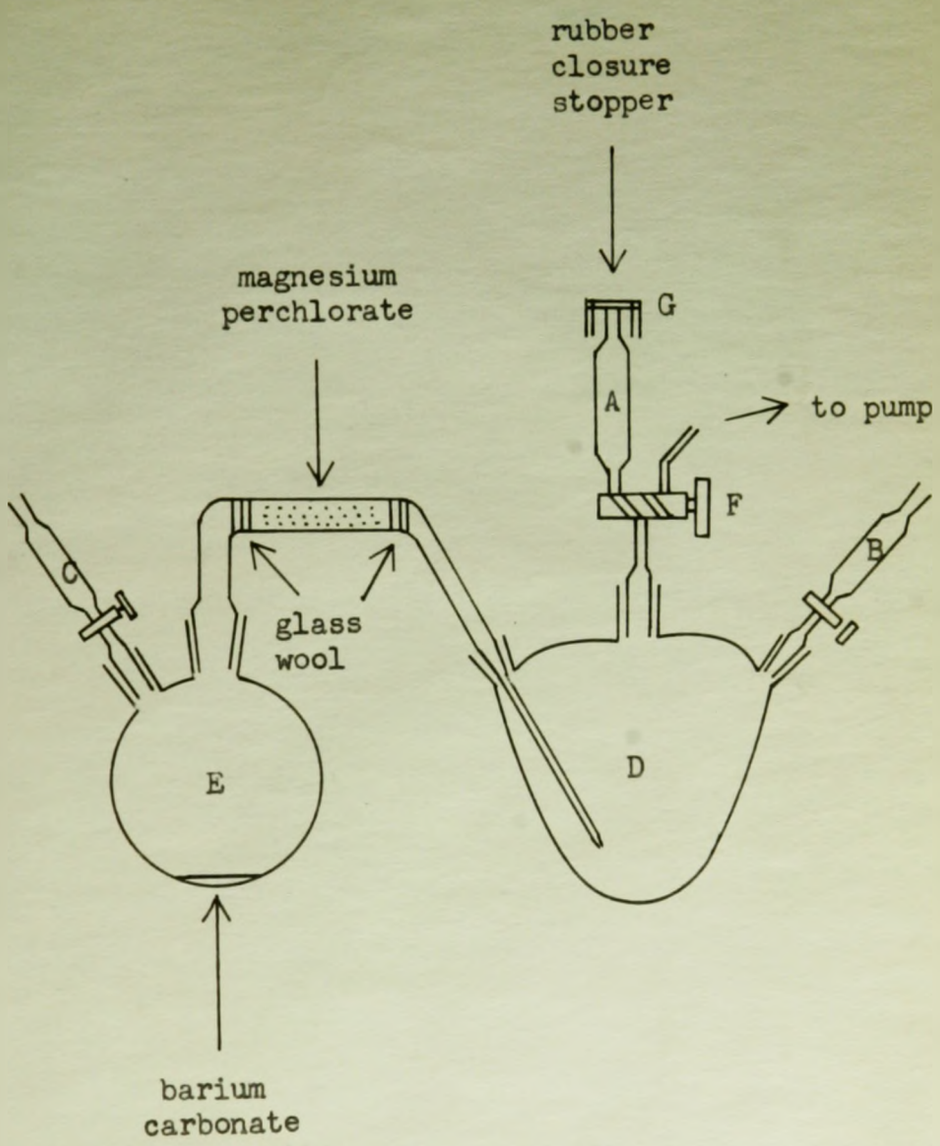
Magnesium turnings (885 mg), which had been ground in a mortar, and a crystal of iodine were introduced into a 200 ml 3-necked round bottom flask. The flask was fitted with a condenser protected with a drying tube ($CaCl_2$), a dropping funnel, and a nitrogen inlet; and the whole system was flushed with nitrogen which had been passed through saturated barium hydroxide solution followed by conc. sulphuric acid. Freshly distilled bromobenzene (5.0 g) in sodium-dried ether (15 ml) was introduced into the dropping funnel. The magnesium was agitated vigorously with a magnetic stirrer, and the ether solution was added at a rate such as to keep the reaction from becoming too vigorous when heat was applied from a glass-col mantle. After the addition was complete the mixture was stirred for 30 minutes. The nitrogen inlet was replaced by a tube which connected the flask to a small flat bottom flask of 150 ml capacity. More sodium-dried ether (150 ml) was added to the mixture, and when the solid had settled enough of the solution was decanted to fill the small flask. This flask was stoppered with a rubber closure stopper and stored at 10°. It contained a solution of phenylmagnesium bromide which was approximately 0.25M.

Conversion of barium carbonate to benzoic acid.

The apparatus used in this reaction is described by Van Bruggen

et al (1950), and is illustrated in Figure 7. All ground glass joints were greased with Apiezon except F which was greased with Dow-Corning high vacuum grease. Barium carbonate (64 mg) was placed in flask E. The apparatus was assembled and evacuated (including funnel A) by a water pump to as low a pressure as possible. 20% Hydrochloric acid was placed in funnels B and C. Using an all-glass hypodermic syringe with a 3 inch needle, phenylmagnesium bromide (8 ml) was withdrawn from the stock flask and inserted through the rubber closure stopper G into funnel A. The bottom of flask D was immersed in liquid air and a little hydrochloric acid was run into flask E from funnel C. The carbon dioxide which was liberated solidified on the bottom of flask D. A little of the phenylmagnesium bromide solution was then run into flask D. This at first vaporized the carbon dioxide, and then both froze as an intimate mixture. This procedure was repeated until all carbon dioxide and all of the phenylmagnesium bromide were frozen together in D. The liquid air was then replaced by an ice-water bath until the contents of D had melted. After 5 minutes the mixture was refrozen, and then allowed to melt again. The ice bath was removed and hydrochloric acid was added from B until the vigorous reaction ceased, showing that excess phenylmagnesium bromide had been destroyed. The system was evacuated again to remove any unreacted carbon dioxide, and then allowed to reach atmospheric pressure. The contents of D were rinsed out with ether into a separatory funnel and the water layer was extracted with ether (3 x 20 ml). The combined ether extracts were evaporated to dryness and the residue was dissolved in hot water, filtered, and cooled. The solid was filtered off to give benzoic acid

Figure 7


rubber
closure
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magnesium
perchlorate
G
A
to pump
F
B
C
glass
wool
E
D
barium
carbonate

(17 mg; 43%) melting at 119 - 121°. One crystallization from water raised the melting point to 122°.

Conversion of 6-vinylpiperonylic acid to 6-(β-bromo-α-hydroxyethyl)-piperonylic acid lactone.

6-Vinylpiperonylic acid (150 mg) was dissolved in acetone (10 ml) in a 50 ml Erlenmeyer flask. Saturated bromine-water (15 ml) was added dropwise to the solution over a period of 30 minutes, followed by water (10 ml) over a period of 5 minutes. The solid was filtered off to give 6-(β-bromo-α-hydroxyethyl)-piperonylic acid lactone (190 mg; 90%) melting at 134 - 134.5°. Recrystallization did not raise the melting point. (Found: C,44.5; H, 2.7; Br, 29.5. $C_{10}H_7O_4Br$ requires C, 44.6; H, 2.6; Br, 29.5%). This compound was also obtained in the following manner. 6-Vinylpiperonylic acid (54 mg) was dissolved in carbon disulphide (5 ml), excess bromine in carbon disulphide was added, and the mixture was allowed to stand overnight. Excess petroleum ether was added and the solid was filtered off to give 6-(α,β-dibromoethyl)-piperonylic acid (87 mg; 88%) melting at 158°. When this compound (70 mg) was recrystallized from aqueous acetone 6-(β-bromo-α-hydroxyethyl)-piperonylic acid lactone (40 mg; 74%) was obtained which melted at 134 - 134.5°. A mixed melting point of samples of the lactone prepared by the two different procedures showed no depression.

4. DEGRADATION OF BERBERINE (Freund & Fleischer, 1913).

Conversion of berberine hydrochloxide to benzyldihydroberberine

Magnesium turnings (720 mg) which had been ground in a mortar, and a crystal of iodine were introduced into a 100 ml round bottom flask. A solution of freshly distilled benzyl chloride (3.25 g) in sodium-dried ether (36 ml) was added, a condenser was fitted to the flask, and after the vigorous reaction had ceased the mixture was heated under reflux for 2 hours. The solution was then slowly added to berberine hydrochloride (800 mg) in sodium-dried ether (10 ml) in a 100 ml round bottom flask. When the initial reaction had subsided the mixture was heated under reflux for 2.75 hours. The contents of the flask were then extracted with 6 M hydrochloric acid (50 ml) in a separatory funnel and the aqueous layer was transferred to a 400 ml beaker. This solution was cooled in an ice bath, and 15 M ammonium hydroxide was added at a rate such that the temperature did not rise above room temperature. A gummy mass separated, which, when the cold solution was stirred vigorously, first solidified and then broke up into finer particles which were filtered off to give benzyldihydroberberine (770 mg; 92%) melting at 131 - 134°. One crystallization from methanol-acetone raised the melting point to 135 - 137°. Further recrystallization did not raise the melting point again, and at no time was benzyldihydroberberine obtained which had the reported melting point of 162° (Freund & Fleischer, 1913).

Conversion of benzyldihydroberberine to benzyltetrahydroberberine

Benzyldihydroberberine (768 mg) was suspended in methanol (100 ml) in a 250 ml round bottom flask. Sodium borohydride (2.0 g) was added carefully in small portions. A reflux condenser was fitted to the flask, and when the vigorous reaction had ceased the solution was heated under reflux for 15 minutes. The solution was allowed to cool, and acidified with 6 M hydrochloric acid while being stirred with a magnetic stirrer. The contents of the flask were then made basic with 15 M ammonium hydroxide and allowed to cool. The solid was filtered off to give benzyltetrahydroberberine (716 mg; 93%) melting at 162 - 165°. One crystallization from methanol-acetone raised the melting point to 165°.

Conversion of benzyltetrahydroberberine to benzyltetrahydroberberine methiodide

Benzyltetrahydroberberine (715 mg) was introduced into a heavy-walled hard glass tube (7 mm x 600 mm which had been sealed at one end), and washed in with methyl iodide (8 ml). The tube was cooled in ice and the open end was sealed. It was then kept at 100 - 105° for 5 hours. After cooling in ice the tube was opened, and the solid in it was filtered off to give benzyltetrahydroberberine methiodide (798 mg; 84%) melting at 203 - 208°. One crystallization from methanol which contained a little sodium hydroxide raised the melting point to 220°.

Conversion of benzyltetrahydroberberine methiodide to des-N-methyl-styryltetrahydroberberine

Benzyltetrahydroberberine methiodide (795 mg) was suspended in

methanol (2 ml) in a 50 ml round bottom flask. A solution of potassium hydroxide (2.0 g) in methanol (11 ml) was added, a condenser was fitted, and the mixture was heated under reflux for 1 hour. It was stored at 10° for 2 hours and the liquid layer was removed by decantation. The residue was dissolved in a minimum of hot methanol and stored in the freezer for several hours. The solid was filtered off to give des-N-methylstyryltetrahydroberberine (392 mg; 64%) melting at 121 - 123°. One crystallization from methanol raised the melting point to 123 - 124°.

Conversion of des-N-methylstyryltetrahydroberberine to hydrastinine and 2-styryl-3, 4-dimethoxybenzaldehyde

Des-N-methylstyryltetrahydroberberine (350 mg) was dissolved in glacial acetic acid (1 ml) in a 20 ml round bottom flask. A solution of sodium dichromate (271 mg) in 50% acetic acid (3 ml) was added, a condenser was fitted, and the solution was heated on a steam bath for 3.25 hours. The solution was cooled in an ice bath and water (4 ml) was added. After being stored at 10° for 3 hours the solution was decanted into a beaker, leaving an oily solid (A) which was set aside. The solution in the beaker was saturated with sodium carbonate, decanted from an oily residue into a separatory funnel, made strongly basic with conc. sodium hydroxide, and extracted with ether (2 x 20 ml). The combined ether extracts were evaporated to dryness in a 50 ml round bottom flask. The residue was suspended in water, and filtered off to give hydrastinine (105 mg; 64%) melting at 100 - 105°. One crystallization from ethyl acetate raised the melting point to 116 - 117°. Mean-

while the oily solid (A) was dissolved in a minimum of hot methanol, filtered, and stored in the freezer overnight. The solid was filtered off to give 2-styryl-3, 4-dimethoxybenzaldehyde (66 mg; 31%) melting at 64 - 67°. One crystallization from methanol raised the melting point to 68 - 70°.

5. DEGRADATION OF RADIOACTIVE ALKALOIDS

Hydrastine from Experiment 3

Hydrastine (115 mg; specific activity 4.48×10^4 counts/min/mM) from this experiment was oxidised with nitric acid to give hydrastinine (50 mg; 81%; specific activity 4.10×10^4 counts/min/mM) and opianic acid (15 mg; 22%; specific activity 0.06×10^4 counts/min/mM). In order to have sufficient hydrastinine to continue the degradation sequence the active hydrastinine (44.85 mg; specific activity 4.10×10^4 counts/min/mM) was mixed with an equal weight of inactive hydrastinine.*

Hydrastinine (89.7 mg; specific activity 4.10×10^4 counts/min/mM)* underwent a Hofmann reaction to give hydrastal (62 mg; 82%; specific activity 4.51×10^4 counts/min/mM).

Hydrastal (55 mg; specific activity 4.51×10^4 counts/min/mM) was oxidised with silver oxide to give 6-vinylpiperonylic acid. However, when the reaction mixture was acidified an excess of nitric acid was

* This gave a total of 89.7 mg with a specific activity half that of the original hydrastinine. The specific activity of all further degradation products was decreased correspondingly. In order to avoid confusion, however, the quoted specific activities of further degradation products are adjusted by taking into account this dilution factor, so that the figures quoted are directly comparable to the specific activity of the initial alkaloid.

inadvertently added. This caused oxidation and subsequent loss of product when the solution was heated. Thus this sample was damaged to such an extent that no further information could be obtained from it.

Hydrastine from Experiment 4

Hydrastine (144 mg; specific activity 5.17 x 10^4 counts/min/mM) from this experiment was oxidised with nitric acid to give hydrastinine (63 mg; 81%;specific activity 5.10 x 10^4 counts/min/mM) and opianic acid (40 mg; 51%; specific activity 0.04 x 10^4 counts/min/mM). In order to have sufficient hydrastinine to continue the degradation sequence the active hydrastinine (57.5 mg; specific activity 5.10 x 10^4 counts/min/mM) was mixed with an equal weight of inactive hydrastinine.

Hydrastinine (115 mg; specific activity 5.10 x 10^4 counts/min/mM)* underwent a Hofmann reaction to give hydrastal (79 mg; 81%; specific activity 5.16 x 10^4 counts/min/mM).

Hydrastal (72 mg; specific activity 5.16 x 10^4 counts/min/mM) was oxidised by silver oxide to give 6-vinylpiperonylic acid (67 mg; 86%; specific activity 5.02 x 10^4 counts/min/mM).

6-Vinylpiperonylic acid (59 mg; specific activity 5.02 x 10^4 counts/min/mM) was bromodecarboxylated to give barium carbonate (43 mg; 61%) and 6-bromo-α-hydroxyhomopiperonyl bromide (76 mg; 76%; specific activity 3.20 x 10^4 counts/min/mM). Active barium carbonate (22 mg) was diluted to 64 mg with inactive barium carbonate, and the mixture was converted to benzoic acid (17 mg; 43%; specific activity 2.00 x 10^4 counts/min/mM).*

6-Bromo-α-hydroxyhomopiperonyl bromide (65 mg; specific activity

3.20 x 10^4 counts/min/mM) was oxidised by potassium permanganate to 6-bromopiperonal, which was further oxidised by silver oxide to give 6-bromopiperonylic acid (23 mg; 47%; specific activity 0.14 x 10^4 counts/min/mM).

The results of the degradation of hydrastine samples from Experiments 3 and 4 are summarized in Table 8.

Table 8

Experiment 3

Compound	Specific activity counts/min/mM	Relative specific activity[1]
Hydrastine	4.48 x 10^4	100.0
Hydrastinine	4.10 x 10^4	91.6
Opianic acid	0.06 x 10^4	1.3
Hydrastal	4.51 x 10^4	100.5

Experiment 4

Compound	Specific activity counts/min/mM	Relative specific activity[1]
Hydrastine	5.17 x 10^4	100.0
Hydrastinine	5.10 x 10^4	98.8
Opianic acid	0.04 x 10^4	0.8
Hydrastal	5.16 x 10^4	100.0
6-Vinylpiperonylic acid	5.02 x 10^4	97.1
6-Bromo-α-hydroxy-homopiperonyl bromide	3.20 x 10^4	62.0
Benzoic acid	2.00 x 10^4	38.7
6-Bromopiperonylic acid	0.14 x 10^4	2.7

[1]Relative specific activity

$$= \frac{\text{specific activity of degradation product}}{\text{specific activity of alkaloid}} \times 100$$

Hydrastine from Experiment 5

Hydrastine (180 mg; specific activity 1.19 x 10^4 counts/min/mM) from this experiment was oxidised by nitric acid to give hydrastinine (84 mg; 86%; specific activity 0.50 x 10^4 counts/min/mM) and opianic acid (45 mg; 46%; specific activity 0.65 counts/min/mM).

Hydrastine from Experiment 6

Hydrastine (181 mg; specific activity 1.42 x 10^4 counts/min/mM) from this experiment was oxidised by nitric acid to give hydrastinine (87 mg; 89%; specific activity 0.70 x 10^4 counts/min/mM) and opianic acid (47mg; 48%; specific activity 0.69 x 10^4 counts/min/mM).

Hydrastine from Experiment 7

Hydrastine (178 mg; specific activity 4.96 x 10^4 counts/min/mM) from this experiment was oxidised by nitric acid to give hydrastinine (83 mg; 86%; specific activity 2.44 x 10^4 counts/min/mM) and opianic acid (44 mg; 45%; specific activity 2.46 x 10^4 counts/min/mM).

Hydrastine from Experiment 8

Hydrastine (150 mg; specific activity 1.36 x 10^4 counts/min/mM) from this experiment was oxidised by nitric acid to give hydrastinine (73 mg; 90%; specific activity 0.60 x 10^4 counts/min/mM) and opianic acid (39 mg; 48%; specific activity 0.74 x 10^4 counts/min/mM).

Hydrastine from Experiment 9

Hydrastine (350 mg; specific activity 5.76 x 10^4 counts/min/mM) from this experiment was oxidised by nitric acid to give hydrastinine (166 mg; 88%; specific activity 3.28 x 10^4 counts/min/mM) and opianic acid (95 mg; 50%; specific activity 2.46 x 10^4 counts/min/mM).

The results of the degradation of hydrastine samples from Experiments 5 - 9 are summarized in Table 9.

Table 9

Experiment	HYDRASTINE		HYDRASTININE		OPIANIC ACID	
	Specific activity counts/min/mM	Relative specific activity	Specific activity counts/min/mM	Relative specific activity	Specific activity counts/min/mM	Relative specific activity
5	1.19×10^4	100	0.50×10^4	42.0	0.65×10^4	54.5
6	1.42×10^4	100	0.70×10^4	49.0	0.69×10^4	49.0
7	4.96×10^4	100	2.44×10^4	49.0	2.46×10^4	49.5
8	1.36×10^4	100	0.60×10^4	44.0	0.74×10^4	54.0
9	5.76×10^4	100	3.28×10^4	57.0	2.46×10^4	42.7

Hydrastine from Experiment 10

Hydrastine (450 mg; specific activity 0.87 x 10^4 counts/min/mM) from this experiment was oxidised by nitric acid to give hydrastinine (215 mg; 89%; specific activity 0.87 x 10^4 counts/min/mM) and opianic acid (96 mg; 35%; specific activity 0.0084 x 10^4 counts/min/mM).

Hydrastinine (197 mg; specific activity 0.87 x 10^4 counts/min/mM) underwent a Hofmann reaction to give hydrastal (148 mg; 89%; specific activity 0.83 x 10^4 counts/min/mM).

Hydrastal (126 mg; specific activity 0.83 x 10^4 counts/min/mM) was oxidised by silver oxide to give 6-vinylpiperonylic acid (112 mg; 82%; specific activity 0.87 x 10^4 counts/min/mM).

6-Vinylpiperonylic acid (97 mg; specific activity 0.87 x 10^4 counts/min/mM) was bromodecarboxylated to give barium carbonate (83 mg; 83%) and 6-bromo-α-hydroxyhomopiperonyl bromide (125 mg; 76%; specific activity 0.89 x 10^4 counts/min/mM). Barium carbonate (82 mg) was converted to benzoic acid (20 mg; 40%; specific activity 0 counts/min/mM).

6-Bromo-α-hydroxyhomopiperonyl bromide (51 mg; specific activity 0.89 x 10^4 counts/min/mM) was oxidised by potassium permanganate to 6-bromopiperonal. This was reacted with acetone to give 6-bromo-piperonylidene acetone (30 mg; 71%; specific activity 0.015 x 10^4 counts/min/mM).

The results of the degradation of hydrastine from Experiment 10 are summarized in Table 10.

Table 10

Compound	Specific activity counts/min/mM	Relative specific activity
Hydrastine	0.87×10^4	100.0
Hydrastinine	0.87×10^4	100.0
Opianic acid	0.0084×10^4	1.0
Hydrastal	0.83×10^4	96.0
6-Vinylpiperonylic acid	0.87×10^4	100.0
6-Bromo-α-hydroxyhomopiperonyl bromide	0.89×10^4	102.0
Benzoic acid	0	0
6-Bromopiperonylidene acetone	0.015×10^4	1.7

Berberine from Experiment 4

In order to have sufficient alkaloid to complete the degradation sequence active berberine hydrochloride (319.6 mg; specific activity 10.50×10^4 counts/min/mM) was mixed with inactive berberine hydrochloride (639.2 mg) and recrystallized to give berberine hydrochloride (935 mg; specific activity 3.50×10^4 counts/min/mM). Berberine hydrochloride (894 mg; specific activity 3.50×10^4 counts/min/mM) then underwent a Grignard reaction with benzyl chloride and magnesium to give benzyldihydroberberine (876 mg; 94%).

This benzyldihydroberberine (876 mg) was reduced by sodium borohydride in methanol to give benzyltetrahydroberberine (637 mg; 72%; specific activity 3.60×10^4 counts/min/mM).

Benzyltetrahydroberberine (632 mg; specific activity 3.60×10^4

counts/min/mM) was converted by methyl iodide to benzyltetrahydroberberine methiodide (677 mg; 81%).

This benzyltetrahydroberberine methiodide (677 mg) underwent a Hofmann reaction to give des-N-methylstyryltetrahydroberberine (326 mg; 62%; specific activity 3.65 x 10^4 counts/min/mM).

Des-N-methylstyryltetrahydroberberine (320 mg; specific activity 3.65 x 10^4 counts/min/mM) was oxidised by sodium dichromate in acetic acid to give hydrastinine (114 mg; 76%; specific activity 3.40 x 10^4 counts/min/mM), and 2-styryl-3, 4-dimethoxybenzaldehyde (69 mg; 36%; specific activity 0.03 x 10^4 counts/min/mM).

Hydrastinine (110 mg; specific activity 3.40 x 10^4 counts/min/mM) underwent a Hofmann reaction to give hydrastal (79 mg; 85%).

This hydrastal (79 mg) was oxidised by silver oxide to give 6-vinylpiperonylic acid (44 mg; 51%). In order to have sufficient 6-vinylpiperonylic acid to continue the degradation sequence, the active 6-vinylpiperonylic acid (32.3 mg) was mixed with an equal weight of inactive 6-vinylpiperonylic acid.

6-Vinylpiperonylic acid (64.6 mg) was bromodecarboxylated to give barium carbonate (59 mg; 89%) and 6-bromo-α-hydroxyhomopiperonyl bromide (91 mg; 83%; specific activity 1.72 x 10^4 counts/min/mM*). Active barium carbonate (59 mg) was converted to benzoic acid (12 mg; 33%; specific activity 1.75 x 10^4 counts/min/mM*).

6-Bromo-α-hydroxyhomopiperonyl bromide (72 mg; specific activity 1.72 x 10^4 counts/min/mM) was oxidised by potassium perman-

ganate to 6-bromopiperonal. This was reacted with acetone to give 6-bromopiperonylidene acetone (31 mg; 52%; specific activity 0.07×10^4 counts/min/mM).

The results of the degradation of berberine from Experiment 4 are summarized in Table 11.

Table 11

Compound	Specific activity counts/min/mM	Relative specific activity
Berberine hydrochloride	3.50×10^4	100
Benzyltetrahydroberberine	3.60×10^4	103
Des-N-methylstyryltetrahydroberberine	3.65×10^4	104
Hydrastinine	3.40×10^4	97
2-Styryl-3, 4-dimethoxybenzaldehyde	0.03×10^4	1
6-Bromo-α-hydroxyhomopiperonyl bromide	1.72×10^4	49
Benzoic acid	1.75×10^4	50
6-Bromopiperonylidene acetone	0.07×10^4	2

Berberine from Experiment 10

Berberine hydrochloride (802 mg; specific activity 4.50×10^4 counts/min/mM) underwent a Grignard reaction with benzyl chloride and magnesium to give benzyldihydroberberine (710 mg; 85%).

This benzyldihydroberberine was reduced by sodium borohydride in methanol to give benzyltetrahydroberberine (490 mg; 69%).

This benzyltetrahydroberberine (485 mg) was converted by

methyl iodide to benzyltetrahydroberberine methiodide (397 mg; 61%).

This benzyltetrahydroberberine methiodide (397 mg) underwent a Hofmann reaction to give a yellow compound (109 mg) which was not des-N-methylstyryltetrahydroberberine, melted at 180 - 185°, and appeared to contain all of the original activity. (Specific activity of the crude compound, assuming the molecular weight to be the same as that of des-N-methylstyryltetrahydroberberine, was 4.31×10^4 counts/min/mM).

6. PROCEDURE FOR THE ASSAY OF ACTIVITY

All radioactive samples were assayed in the following manner. A weighed amount of the compound was transferred to an aluminum planchette and dissolved in two drops of a 1% solution of collodion in dimethylformamide. (For berberine hydrochloride benzyl alcohol was used instead of dimethylformamide.) The solution was covered with a circle of lens tissue which caused the drop to spread evenly over the surface of the planchette, and was evaporated under an infra-red lamp. The planchette was counted by a thin-window counter in a low background automatic sample changer (Nuclear Chicago, model C 115), and the counts were recorded either on a scaler (Nuclear Chicago, model 161 A), or on an electronic counter (Beckman-Berkeley, model 7060).

To ensure that all impurities were removed from a compound prior to counting, a small amount of each radioactive compound was mixed with a weighed amount of a pure inactive sample of the same compound and recrystallized. This recrystallized sample was then

counted. At least three separate planchettes were assayed of each radioactive compound in order to ensure the precision of the results. A sample calculation of the specific activity of a compound is shown below.

Radioactive berberine hydrochloride (7.3 mg), isolated in Experiment 9, was mixed with inactive berberine hydrochloride (28.6 mg) to give a total of 35.9 mg which was recrystallized to 29 mg. The recrystallized berberine hydrochloride (1.06 mg) was plated on a planchette which, when empty, weighed 749.1 mg. When dry the planchette and its contents weighed 756.9 mg. Thus the total weight of its contents was 7.8 mg or 1.047 mg/cm^2 (since the area of the planchette was 7.45 cm^2). This thickness corresponds to a self absorption of 18.43% (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). Over a period of 27.77 minutes this planchette gave 1150, 1170, 1144, 1179, 1102, and 1137 counts, or an average value of 1147 counts. Over the same period the background was 77 counts. Thus the activity of the planchette over this period was 1070 counts or 38.6 counts/min. Since self absorption was 18.43% the corrected count-rate for this planchette is ($\frac{100}{81.57}$ x 38.6 = 47.3 counts/min). Since the molecular weight of berberine hydrochloride (plus two moles of water of crystallization) is 407.5, and this value of 47.3 counts/min was obtained from 1.06 mg, then the specific activity of the berberine hydrochloride is ($\frac{47.3 \times 407.5}{1.06}$ counts/min/mM). However, this sample that was counted was a diluted sample of the original berberine hydrochloride, and so the result must be multiplied by the dilution

factor of $\frac{35.9}{7.2}$. Therefore the specific activity of the original berberine hydrochloride is given by ($\frac{47.3 \times 407.5}{1.06} \times \frac{35.9}{7.2} =$ 8.94×10^4 counts/min/mM). Duplicate samples yielded specific activities of 8.90×10^4 and 8.86×10^4 counts/min/mM respectively. The average value was thus 8.90×10^4 counts/min/mM and this was taken to be the specific activity of berberine hydrochloride isolated from Experiment 9. The probable error in the specific activities quoted varies depending on the activity of the sample involved. However, a sufficiently large number of counts was taken to ensure that in no case did the error rise above $\mp 2\%$. In most cases it was closer to $\mp 1\%$.

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