ROLE OF BETAINE IN TRANSMETHYLATION REACTIONS IN THE BARLEY PLANT

ORIGIN OF THE METHYLENEDIOXY GROUPS OF THE ALKALOID PROTOPINE

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by

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TITLES: Role of Betaine in Transmethylation Reactions in the Barley Plant

Origin of the Methylenedioxy Groups of the Alkaloid Protopine

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SCOPE AND CONTENTS: The role of the labile methyl groups of betaine in the transmethylation reactions in the barley plants was investigated using carbon-14 methyl labelled betaine. The N-methyl groups of N-methyl tyramine, hordenine and choline were found to arise from betaine. Betaine was also administered to castor bean seedlings and the alkaloid ricinine isolated. It was found that its N- and O-methyl groups did not arise from betaine methyl.

The origin of the methylenedioxy and N-methyl groups of the alkaloid protopine was also investigated by the tracer technique. Carbon-14 methyl labelled L-methionine, carbon-14 methyl labelled choline and carbon-14 labelled sodium formate were fed to Dicentra species and the extent and position of labelling of the protopine molecule determined by degradation.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. S. Kirkwood for helpful direction and discussion in guiding this research. He is also indebted to the Rockefeller Foundation for the financial support of this work.

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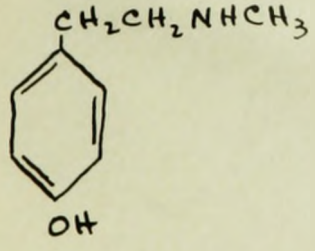
GENERAL INTRODUCTION

Transmethylation in the animal body is now a well understood process. Kirkwood and Marion (1) have shown that similar processes occur in the plant kingdom. They have shown with the aid of certain barley strains which elaborate the alkaloid hordenine that its methyl groups arise from formate by the stepwise methylation of tyramine.

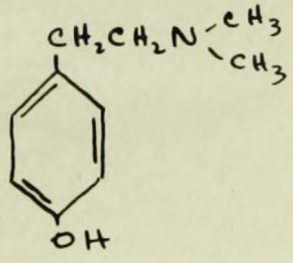
In this laboratory the role of L-methionine, choline, formate and bicarbonate in the transmethylation scheme in the barley plant and the castor bean plant has been investigated. It was shown by Dubeck and Kirkwood (2) and by Matchett et al (3) that choline did not transfer its labile methyl groups to the alkaloids ricinine and hordenine. This is in contrast to the well known availability of these groups in the labile methyl pool in animals. Bjerrum (4) has shown that in the three month old tobacco plant the N-methyl group of the alkaloid nicotine does arise from choline. There is considerable evidence in the literature that choline must first be oxidized to betaine before transmethylation will occur in the animal body (5,6,7). Presumably the same scheme is followed in the plant kingdom. Since very young plants were used in the investigations in this laboratory and Bjerrum used older tobacco plants, it is logical to postulate that the choline oxidase system required for the oxidation of choline to betaine is not present in the younger plants. To check this possibility the role of

betaine in transmethylation in the barley and castor bean plants, was investigated. Carbon-14 methyl labelled betaine was synthesized and fed to sprouting barley and to castor bean seedlings. In the barley plant the N-methyl groups of N-methyl tyramine, hordenine and choline were found to arise from betaine. It was found that betaine was not as efficient a methyl donor as methionine (3). On the other hand the N and O-methyl groups of the alkaloid ricinine in the castor bean did not arise from betaine methyl.

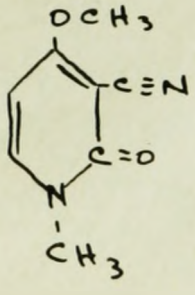
There is a rapidly growing body of evidence that all biological methylation processes and many other single carbon synthesis have a common pattern. It was decided, therefore, to investigate the possibility that the methylenedioxy group which occurs widespread in the plant kingdom might arise from labile methyl. Carbon-14 methyl labelled L-methionine, carbon-14 methyl labelled choline and carbon-14 labelled sodium formate were synthesized and fed to young Dicentra hybrids. The alkaloid protopine was isolated and the methylenedioxy groups and the N-methyl group degraded to formaldehyde and methyl iodide respectively. It was found that the methylenedioxy and N-methyl groups were active, but this did not account for the total activity in the molecule.



N-Methyl tyramine



Hordenine



Ricinine

HISTORICAL INTRODUCTION

(A) Occurrence of Betaine in Nature

Betaine was first isolated from the plant Solanaceae lycium by Husemann (8) in 1864. In 1869 Scheibler (9) isolated it from the sugar beet plant Beta vulgaris and hence the name betaine. He characterized the compound and assigned it the empirical formula $C_5H_{13}O_3N$ (10). Waller and Plimmer (11) reported that the immature beet plant contained 2.5 per cent by dry weight of betaine and that ripe plants only contained one per cent. Its occurrence in plants has since been reported to be widespread; barley 0.04 per cent, peas 0.016 per cent, horse bean 0.173 per cent, malt germ 0.2 per cent, and wheat germ 0.05 - 0.2 per cent (12). Its occurrence in following plants has also been reported, but the concentrations have not been stated; lupine seeds (13), tobacco leaves (14), mushrooms (15), and red pepper pericarp (16).

Betaine has also been reported in the animal kingdom. Wilson (17) and Kutscher and Ackermann (18) have reported isolating betaine in small quantities from the scallop, periwinkle, selachii, cyclostomes, crustacea, mussels and sponges. Higher animals also contain varying amounts of betaine. Guggenheim (12) reported the isolation of betaine from beef liver (0.015 per cent) and from hog liver (0.001 per cent). Since then Ackermann and Wasmuth (19) have isolated it from calf thymus (0.01 per cent).

It appears that betaine occurs widespread in both the plant and animal kingdoms.

(B) Role of Betaine in Methylation Phenomena

(a) Methylation processes

Biological methylation as a metabolic process was first proposed by Basedow (20) in 1846. This arose in connection with a number of fatalities which occurred as a result of mold action on wallpapers containing arsenic. He proposed that the poisoning was due to the formation of volatile cacodyl oxide I by the molds, but he presented no evidence

$$(CH_3)_2As - O - As(CH_3)_2$$

I

to support his theory. Gosio (21) obtained the first experimental evidence for biological methylation when he exposed potato mash containing arsenious oxide to the air. A volatile gas with a garlic-like odour was evolved. He thought this gas was an alkyl arsine. In 1931 Challenger (22) repeated Gosio's experiments and proved that the gas was trimethyl arsine II.

$$(CH_3)_3As$$

II

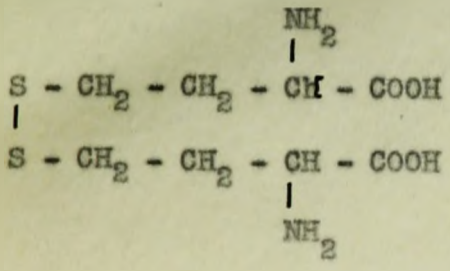
Challenger and his coworkers also discovered the biological methylation of inorganic tellurium and selenium compounds which are transformed to dimethyl telluride III and dimethyl selenide IV respectively (23,24).

$(CH_3)_2Te$ III

$(CH_3)_2Se$ IV

Up until 1939 all work on biological methylation was done with

the aid of molds. In 1939 du Vigneaud and his colleagues investigated the process in rats. They found (25,26,27) that homocystine V after conversion to homocysteine VI could replace methionine VII in the diet of the white rat only in the presence of choline VIII or betaine IX. It was suggested that the methyl group was transferred from the nitrogen of choline or betaine to the sulfur of homocysteine (transmethylation) to give methionine and that the reaction might be reversible, methionine acting as a donor of methyl groups to a choline precursor. Du Vigneaud



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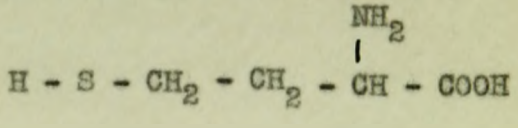
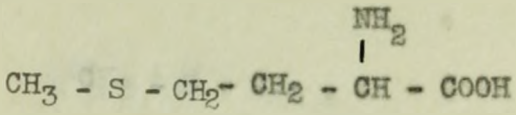


VI



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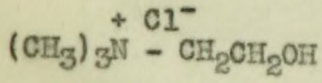
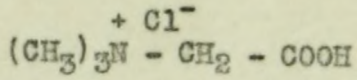
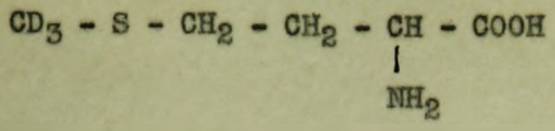


VIII

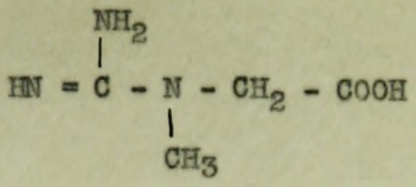


IX

tested his "transmethylation" hypothesis by feeding methionine labelled with deuterium in the methyl group to white rats which were kept on a methionine-choline free diet (28). The fed deuteriomethionine X contained 83.6 atom per cent of deuterium in the methyl group. An animal which was kept on this diet for 94 days was killed and the choline and

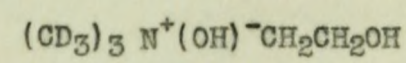


X



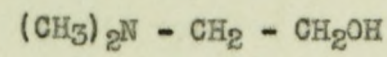
XI

and creatine XI isolated from the tissues. The atom percentage of deuterium in the methyl groups of tissue choline was found to be 74.2, for the tissue creatine 73.0. This clearly indicated that these reactions are true transmethylations (the methyl group being transferred as a whole). Du Vigneaud et al (29) administered trideuteriocholine XII to rats maintained on a methionine-choline free diet containing homocysteine. Deuterium was found in methyl of the tissue creatine and methionine. The methyl groups of choline can therefore take part in the transmethylation scheme in the animal body.



XII

The relation of mono and dimethylaminoethanol XV, XIII to choline and to transmethylation reactions has been investigated (30). When the dimethyl compound was fed to young rats on a methyl-free basal diet



XIII

containing homocysteine growth was not as good as when choline was fed under the same conditions. However, deuteriodimethylamino ethanol XIV

$$(CH_2D)_2\ N\text{-}CH_2\text{-}CH_2\text{-}OH$$

XIV

under similar conditions was readily converted into deuteriocholine and then into creatine by transmethylation. These results suggest that dimethylaminoethanol does not take part directly in transmethylation but it can accept methyl groups supplied by methionine or some other methyl donor in the body, thus giving rise to choline and accounting for the limited growth-producing power. If so, choline must release only one methyl group in transmethylation. Similar results were found in experiments in which deuteriomethylaminoethanol XV was fed.

$$CD_3\ NH - CH_2 - CH_2OH$$

XV

(b) Chemical evidence for transmethylation from betaine

The transfer of a methyl group from some methylated compound, such as choline or betaine, was first suggested by Reisser (31) to explain the production of creatine and methylated derivatives of selenium and tellurium in animals. Willstatter (32) found that betaine when

$$(CH_3)_3\ N^+ - CH_2 - COO^- \qquad\qquad (CH_3)_2\ N - CH_2\ COOCH_3$$

XVI

heated forms methyl dimethylaminoacetate XVI. Challenger and Higgenbottom (33) and Challenger et al (34) found that sodium sulfite,

organic disulfides, sodium selenite and sodium tellurite when heated with betaine yielded dimethyl sulfide, methyl alkyl or methyl aryl sulfide, dimethyl selenide and dimethyl telluride respectively. It was suggested by Challenger (35) that these pyrogenic reactions might proceed as follows:

$$(CH_3)_3 N^+CH_2COO^- + Na_2SeO_3 \longrightarrow (CH_3)_2NCH_2 - COONa + CH_3SeO_3Na$$

The sodium methyl selenite would then be reduced and methylated again by another molecule of betaine to produce dimethyl selenide IV.

The mobility of the N-methyl group of betaine is probably due to the "onium pole" structure of the molecule. A comparison of transmethylators, choline VIII and betaine IX with that of non-transmethylators dimethylglycine XVII, sarcosine XVIII and the aminoethanols XIII, XIV indicates that a positively charged nitrogen is a requirement

$(CH_3)_2N - CH_2 - COOH$ XVII

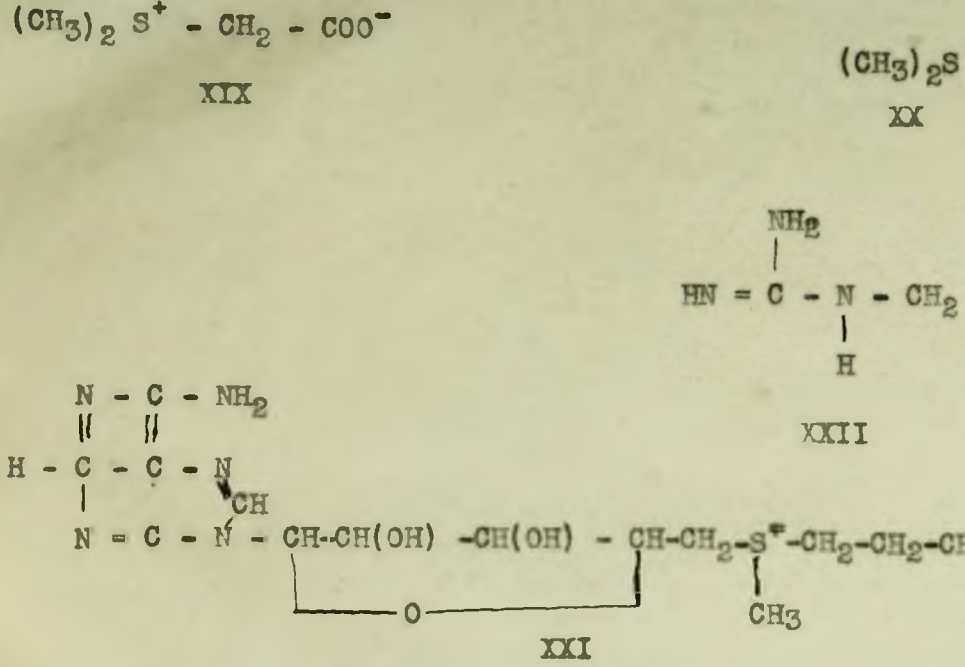
$CH_3 - NH - CH_2 - COOH$ XVIII

of the transmethylation process. The bond strengths of the methyl groups attached to the positively charged nitrogen are lower and more favourable toward elimination (enzymatic or otherwise) of a methyl group than those attached to an uncharged atom. The Hoffman type elimination reaction in which organic fragments are liberated from sulphonium and ammonium compounds illustrates the energetically favourable conditions for the breaking of carbon bonds from the "onium" grouping.

The mobility of the methyl group of α thetins has been

observed (36). When thetin anhydride XIX is heated with aniline or p-toluidine, dimethyl sulfide XX is produced. Dimethyl thetin XIX which is analogous to betaine is 10 - 20 times more effective than betaine in transmethylation in rat liver or kidney homogenates (37).

Recently Cantoni (38) has shown t at the active form of methionine is S-adenosyl methionine XXI which arises by reaction with adenosine triphosphate. He has shown that this active form of methionine will methylate quanidoacetic acid XXII to creatine XI in the presence of an enzyme known as a "methylpherase". This work



XIX

XX

XXII

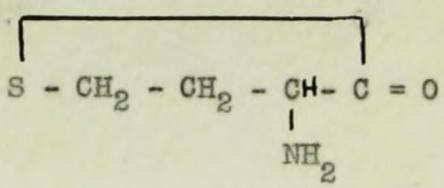
XXI

clearly indicates that transfer of a methyl group is dependent on being attached to a positively charged "onium pole".

(c) Betaine as a source of labile methyl in the animal body

Du Vigneaud's feeding experiments (28) with white rats which were kept on a methionine-choline-free diet containing homocysteine and betaine showed that betaine was able to support growth, probably

by taking part in the transmethylation reactions in the body. Stetten (39) showed that on administration of nitrogen-15 labelled betaine to rats the concentration of nitrogen-15 in the glycine of the tissue protein was almost as high as when isotopic glycine was fed. This proved beyond any doubt that demethylation of betaine had occurred in the animal body. The fate of the removed methyl group however, was not established, but Stetten believed it to be captured by ethanolamine (arising from the reduction of glycine) thus yielding choline which was found to contain a small amount of nitrogen-15. Du Vigneaud et al (40) fed betaine labelled with nitrogen-15 and with deuterium in the methyl groups to white rats. Analysis of the methyl groups of the choline and creatine isolated from the tissues showed that they arose from betaine. Deuteriomethyl groups from dietary betaine appeared in the tissue choline almost as rapidly as they appeared from dietary deuteriocholine, thus indicating that betaine is a very efficient methyl donor. Borsook and Dubnoff (41) using rat liver homogenates have shown that homocysteine VI, homocysteine V, and homocysteinethiolactone XXIII were



XXIII

methylated to methionine by betaine or choline. The aerobic synthesis of methionine from choline was almost as effective as that with betaine, but anaerobic formation of methionine from choline is either slight or does not occur at all. These results indicate that choline must be

oxidized to betaine before transmethylation will take place.

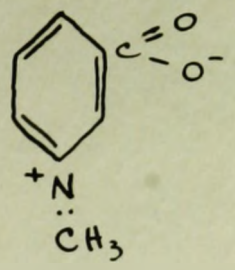
In du Vigneaud's feeding experiments the methyl group of dietary methionine appeared more rapidly in creatine than did those of dietary betaine (42). This was also the case with the methyl group of sarcosine XVIII which was found to arise from methionine and betaine (43). Possibly the transmethylation of the methyl group of betaine to form creatine or sarcosine involves transmethylation first to methionine and then to creatine or sarcosine.

(d) Fate of betaine molecule other than the methyl groups

When du Vigneaud and his colleagues (40) performed their experiments with betaine labelled with deuteriomethyl groups and nitrogen-15 they found very little of nitrogen-15 in the tissue choline indicating that the betaine molecule was not converted as a whole to choline as postulated by Stetten (39). Muntz (44) isolated nitrogen-15 labelled dimethylglycine from rat liver homogenates which were incubated with nitrogen-15 labelled betaine. Recently Mackenzie et al (45) have shown that both methyl groups of dimethyl glycine are oxidized to formaldehyde in rat liver homogenates. It would appear that the methyl groups of betaine are metabolized by at least two different pathways. One is used for transmethylation and the remaining two are oxidized to carbon dioxide, perhaps via formaldehyde. The remainder of the molecule becomes glycine, which is metabolized by pathways which cannot be discussed here.

(e) Enzyme systems involved in transmethylation from betaine

As yet nothing concrete is known concerning the enzyme or enzymes involved in the transfer of the labile methyl of betaine to a methyl acceptor. Ciusa and Nebbia (46) administered betaine with thiamine to humans and found that the excretion of trigonelline XXIV was abnormally high, indicating a methylation process.

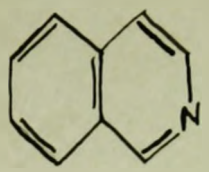


XXIV

Borsook and Dubnoff (37) reported the partial purification of a "betaine transmethylase" which catalyzed the methylation of homocysteine to methionine in rat liver slices. They were, however, unable to separate it from dimethylthetin XIX transmethylase. The enzyme action was not inhibited by cynanide, azide, arsenate or arsenite. It was found that the enzyme was unstable at a pH of 4.5. Recent work by Stekol et al (47) indicates that the extent of incorporation of the methyl group of betaine into tissue choline and creatine is reduced in the folic acid deficient rat. The function of thiamine and of folic acid in these processes is as yet very obscure.

(C) Occurrence of Methylenedioxy Groups in Nature

Methylenedioxy groups have been found to occur widely in the plant kingdom. They occur in the plant alkaloids, particularly in those with an isoquinoline nucleus XXV.



XXV

Dicentrine XXVI, chelidonine XXVII, corycavine XXVIII, narcotine XXIX and sanguinarine XXX are but a few of the alkaloids which fall in this class (48). Piperine XXXI is one of a few which has no isoquinoline nucleus, but has one methylenedioxy group (48). Many essential oils contain compounds which have a methylenedioxy group. Safrole XXXII found in oil of camphor (49), myristicin XXXIII in nutmeg and mace oil (50), apiole XXXIV in parsley seed (51) and apiolaldehyde XXXV which is found in dills (52) all contain this group. The presence of methylenedioxy groups in barley lignin has been reported by Sarker (53).

The alkaloid protopine XXXVI used in these experiments belongs to the isoquinoline group. It was first isolated by Hesse (54) from opium in 1912. Danckwortt (55) characterized it and

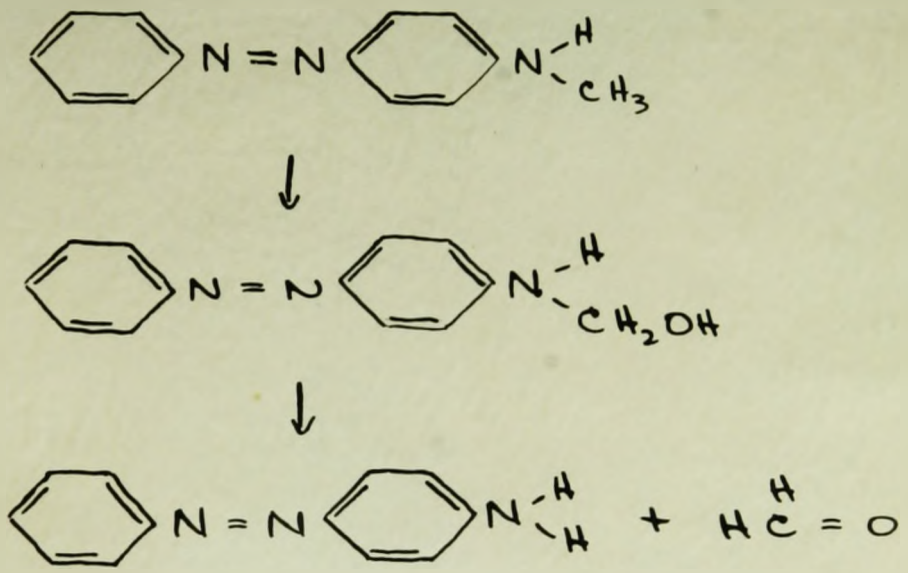
stated that the alkaloid contained two methylenedioxy groups and one N-methyl group. However it was Perkin who determined its structure (56). The alkaloid was first synthesized by Haworth and Perkin in 1926 (57).

(D) Origin of Methylenedioxy Groups

Little is known on the origin of the methylenedioxy groups in nature. Browne and Phillips (58) suggested that methylenedioxy and methoxyl groups found in nature did not arise from formaldehyde but from dissacharides by successive oxidation, hydrolysis and reduction. However, they gave no experimental evidence to back up their theory. Dawson (59) suggested that methoxyl, methylimino and methylenedioxy groups in nature might arise from sources which have labile methyl available, similar to that in the animal body.

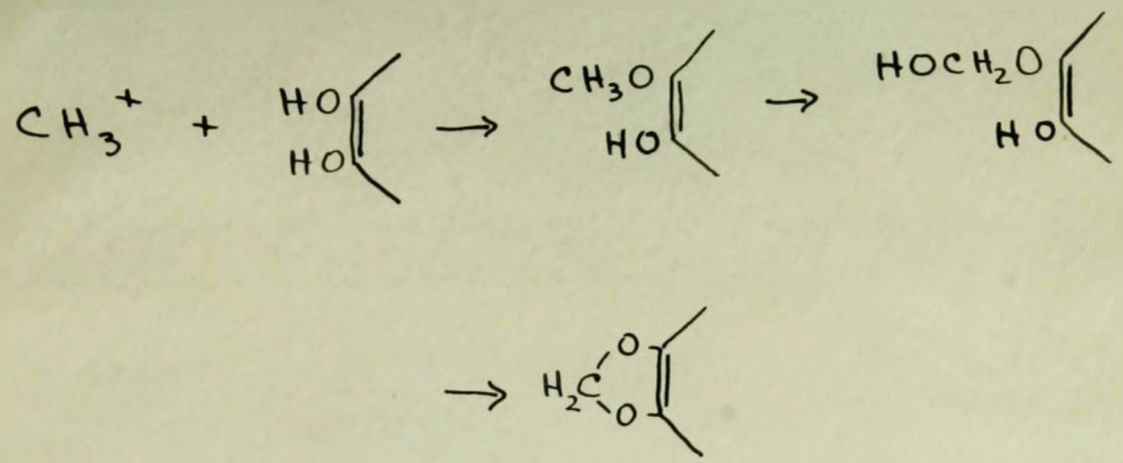
Recent work on oxidation of methyl groups and metabolism of carcinogenic dyes in the animal body has thrown some light on the probable origin of methylenedioxy groups. Mackenzie and du Vigneaud (60), and Mackenzie et al (61) labelled sarcosine dimethylaminoethanol, dimethylglycine and methanol with carbon-14 in the methyl groups and then incubated the radio compounds with rat liver homogenates. From the homogenates they were able to isolate formaldehyde labelled with carbon-14. When N-methyl carbon-14 labelled dyes such as 4-monomethylaminoazobenzene are fed to rats the carbon-14 is found in the β carbon of serine and in the methyl group of choline (62,63). After incubating these dyes in rat liver

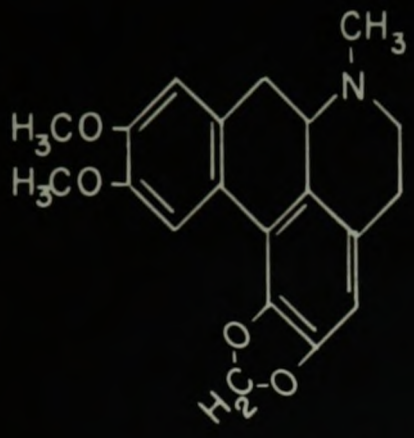
homogenates Mueller and Miller (64) isolated formaldehyde labelled with carbon-14. Miller and Miller (65) warmed a suspension of denatured normal liver protein in a solution of 4-aminoazobenzene and formaldehyde, and found that appreciable binding of the dye to the tissue protein had occurred. Since the products of demethylation of azo dyes are a primary amine and formaldehyde it appears that the reaction is an oxidative process rather than transmethylation.



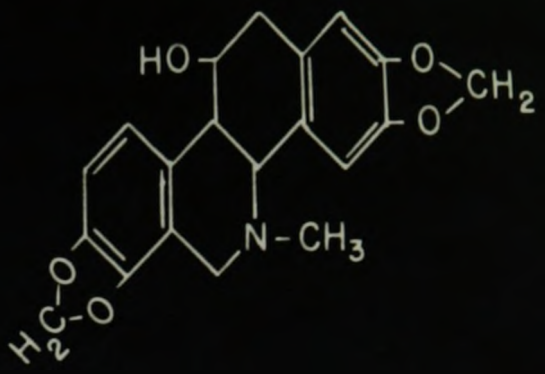
The great tendency for hydroxymethyl groups to dissociate to formaldehyde or to condense with compounds containing active hydrogen to form methylene derivatives is well known (66). Thus methylenedioxy groups in nature might arise from the oxidation of compounds which have an

O-methyl group adjacent to a phenolic hydroxyl. There are many such alkaloids found in nature. The morphine and sinomenine alkaloids (67) which are made up with a phenanthrene nucleus XXXVII are but a few which contain a phenolic hydroxyl adjacent to a methoxyl group. If this is the route of synthesis of these groups then methionine, choline and betaine would be expected to act as precursors since they are labile methyl precursors. The following scheme in the formation of methylenedioxy groups is postulated.

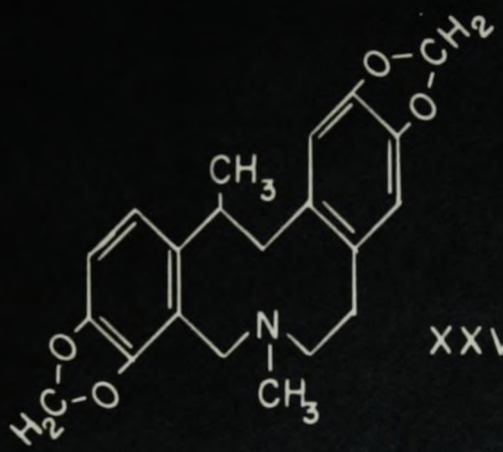




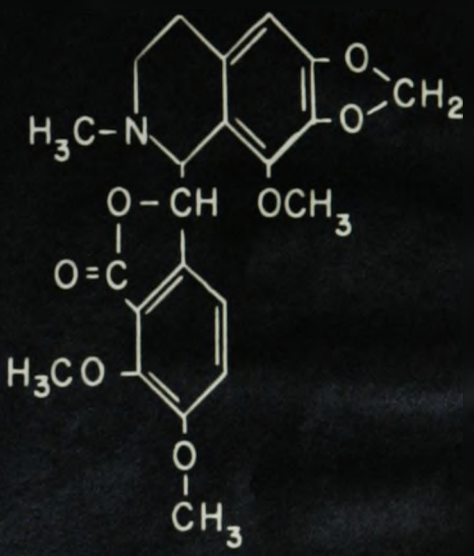
XXVI



XXVII



XXVIII



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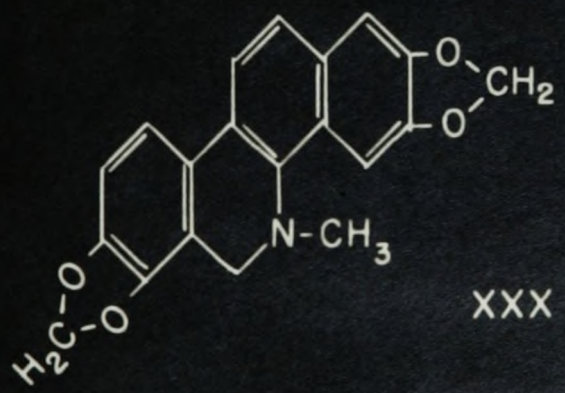

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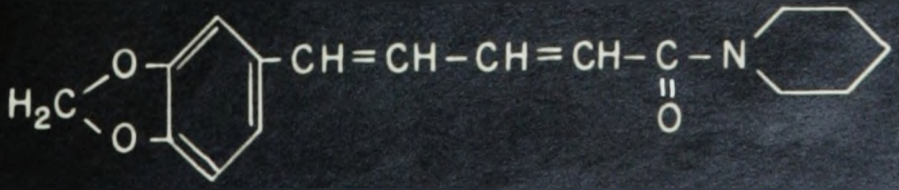
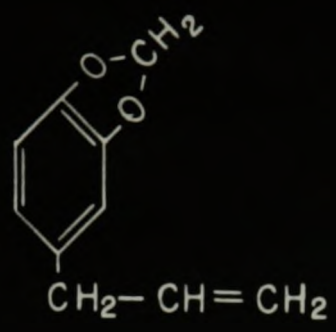


XXXI



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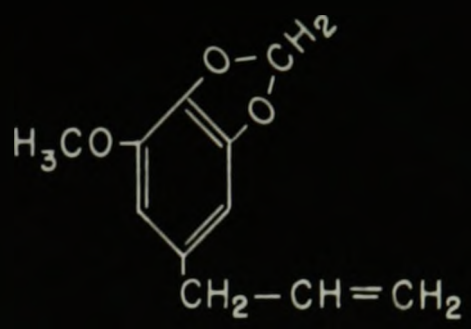

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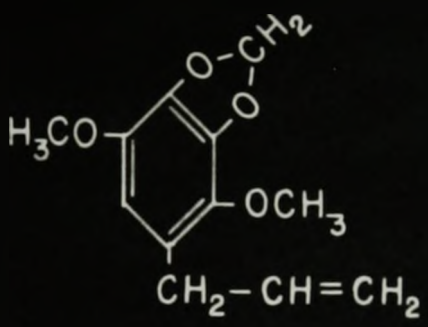

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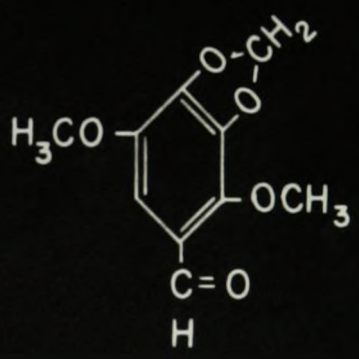

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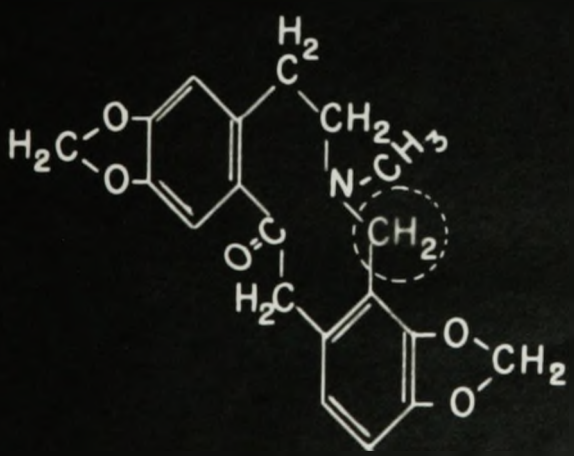

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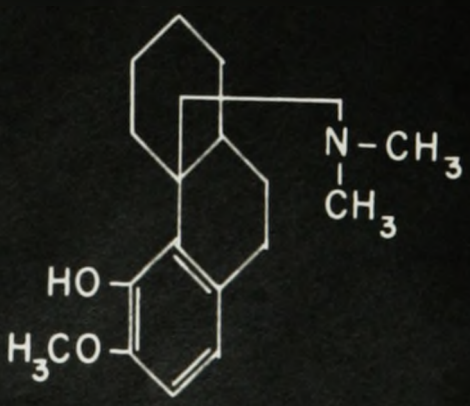

XXXVII

EXPERIMENTAL

(A) Measurement of Carbon-14 Activity

All carbon-14 activity was measured using a Nuclear Instrument "Q" gas counter. All substances counted as infinitely thin layers were dried on lens paper supports. This technique resulted in a much greater reproducibility in counting identical activities in different chemical forms. Although some of the activity was absorbed by the lens paper, this was not important since all results were relative. Protopine and choline and their degradation products were counted as infinitely thin layers. Hordenine and N-methyl tyramine were counted as infinitely thin layers, after the active material was diluted with inactive carrier. Appropriate corrections were then made and all specific activities reported as counts per minute per millimole (c.p.m./mM) for carrier free material.

(B) Synthesis of Carbon-14 Labelled Compounds

(a) Synthesis of carbon-14 methyl labelled L-methionine

Carbon-14 methyl labelled L-methionine was prepared from carbon-14 labelled methyl iodide and S-benzyl L-homocysteine using the procedure outlined by Melville (68).

The S-benzyl L-homocysteine was prepared by the method described by du Vigneaud and Patterson (69). D-L-homocysteine was converted to S-benzyl D-L-homocysteine which in turn was formylated to N-formyl

S-benzyl D-L-homocysteine. This formyl derivative was resolved with the aid of brucine into the D- and L-isomers and then reconverted to S-benzyl D-homocysteine and S-benzyl L-homocysteine.

(b) Synthesis of carbon-14 labelled sodium formate

The carbon-14 labelled sodium formate was synthesized by a procedure described by Dubeck (70). Carbon-14 labelled sodium carbonate was hydrogenated at 70°C. and 100 atmospheres pressure in the presence of palladium catalyst.

(c) Synthesis of carbon-14 methyl labelled choline

Carbon-14 methyl labelled choline was prepared by the method described by Ferger and du Vigneaud (71). Carbon-14 labelled methyl iodide was allowed to react with an excess of ethanolamine and the resulting choline was isolated as the reineckate salt. The choline was purified by employing the method of Kapfhammer and Bischoff (72).

(d) Synthesis of carbon-14 methyl labelled betaine

Carbon-14 methyl labelled betaine was prepared by a method described by Ferger and du Vigneaud (71). Carbon-14 labelled methyl iodide and the sodium salt of N-dimethyl glycine were used.

The sodium salt of N-dimethyl glycine was prepared by a method described by Anslow and King (73). An aqueous solution of monchloro-acetic acid containing 8.25 gm. of the acid in 8.2 ml. of water was slowly added with stirring to 100 ml. of 16.5 per cent solution of

dimethylamine in water. After refluxing the solution for four hours, an excess of baryta was added and the excess dimethylamine removed under vacuum. The barium was then precipitated with a stoichiometric amount of sulphuric acid and the filtrate concentrated to a small volume. On the addition of an equal volume of alcohol and hydrochloric acid the salt of N-dimethylglycine crystallized out. The free acid was prepared by adding excess silver carbonate and then removing the silver with hydrogen sulfide. Evaporation of the solution gave large crystals of N-dimethylglycine which were readily soluble in absolute methanol and crystallized upon careful addition of diethyl ether. Yield, 63 per cent. M.P. 178 - 179°C. corr.

The apparatus used in the preparation of betaine is shown in Figure I. A 0.25 ml. sample of carrier methyl iodide was distilled through tip (a) into trap (B) which was cooled with liquid air. After removing vessel (A), tip (a) was sealed and the entire system evacuated and sealed (F). Break-seal (c) on sample tube (C) containing one-fifth of a millicurie of carbon-14 labelled methyl iodide was broken with a magnetic breaker, allowing the contents to mix with the carrier methyl iodide in (B). The mixed methyl iodide sample was alternately condensed from (B) to (C) to ensure complete mixing. Finally the entire sample was frozen into (B), kept at liquid air temperature while seal (E) was broken and nitrogen allowed to enter the system. Tip (a) was reopened and trap (A), containing 520 mg. of the sodium salt of N-dimethylglycine in 10 ml. of 75 per cent alcohol, replaced. Trap (A) was kept at the temperature of dry ice-acetone. As nitrogen

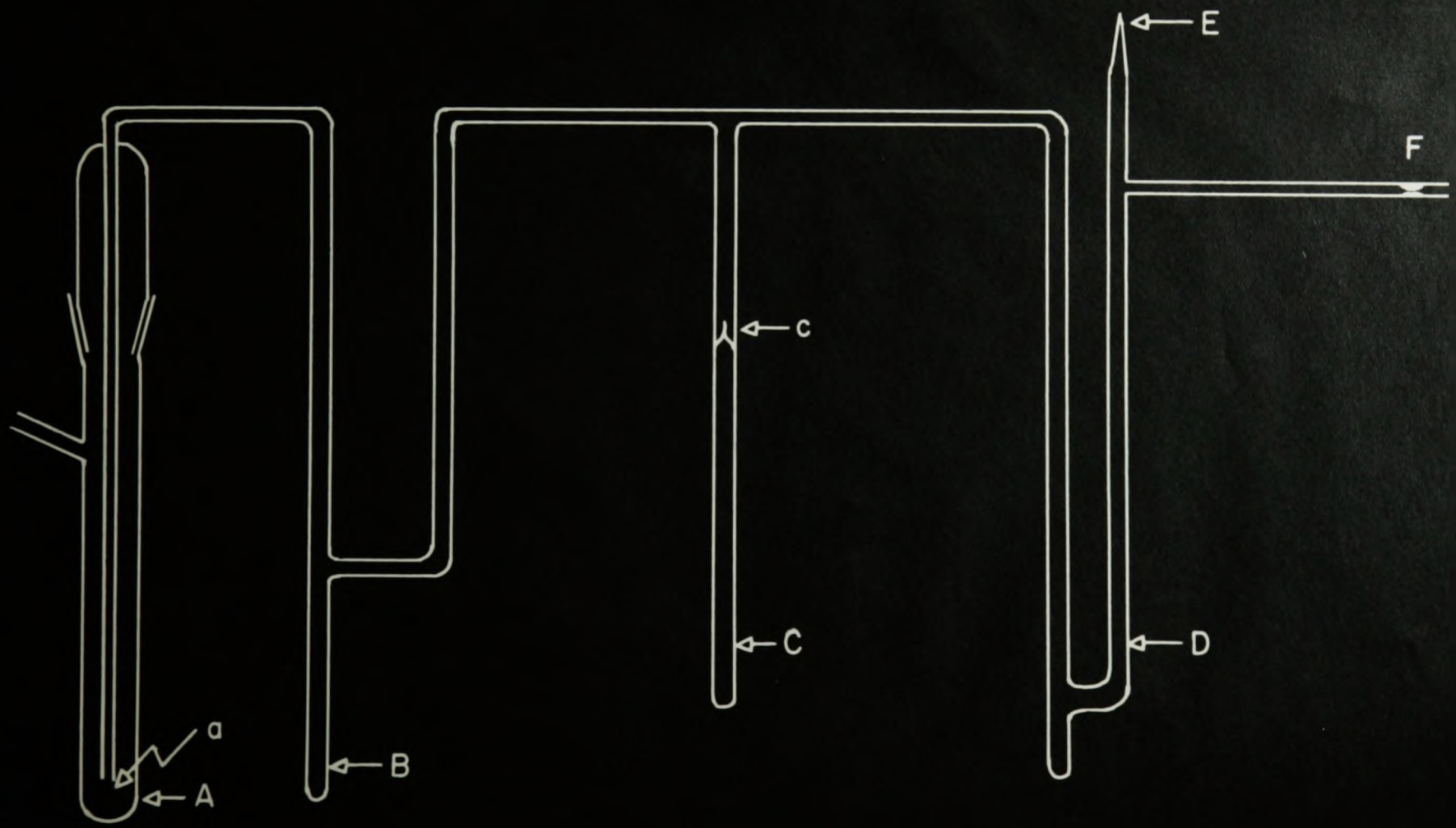

FIG. 1

APPARATUS FOR THE SYNTHESIS OF C^{14} METHYL LABELLED BETAINE

was allowed to bubble through the solution trap (B) was slowly raised to 40° until all the methyl iodide had been swept into trap (A). After flushing for two hours the contents of (A) were transferred to a Carius tube which was then sealed and heated at 70° for 45 minutes. The solution was evaporated to a small volume, made acidic with 0.1N hydrochloric acid and saturated ammonium reineckate solution added. The betaine reineckate which precipitated was filtered off and thoroughly washed to remove any unreacted material. The reineckate was then decomposed by the method of Kapfhammer and Bischoff (72). The resulting solution was finally evaporated to dryness, the betaine taken up with hot ethanol and crystallized out by the addition of ether. Yield, 70 per cent. M.P. 237-9°. Total activity 1.1 x 10^8 c.p.m.

(C) Growth of Plant Material

(a) Growth of barley plants

Charlottetown No. 80 barley which has been shown by Leete *et al* (74) to elaborate both N-methyl tyramine and hordenine was used in this experiment.

A 720 gm. sample of the barley seeds was evenly divided among twelve 20 x 25 cm. Pyrex trays. The seeds were watered twice daily and on the sixth day of germination carbon-14 methyl labelled betaine was added, dissolved in water. The barley was harvested on the 11th day day of germination, the roots separated from the stems and both dried in an oven at 110°C. before working up for the desired material.

(b) Growth of castor beans

A 360 gm. sample of R. communis beans was evenly divided among six 20 x 25 cm. pyrex trays. They were then covered with a thin layer of vermiculite (a form of mica which is a non-nourishing plant support medium) and kept well dampened by watering twice daily. These seedlings were placed in a light-tight cabinet maintained at 27°C. Weevers (75) has shown that under these conditions castor seedlings have the greatest alkaloid producing power. Growth in the absence of light also eliminates photosynthesis which could cause appreciable random labelling of the ricinine molecule. After allowing the young seedlings to grow for four days carbon-14 methyl labelled betaine was administered with the daily water ration. The young plants were harvested when they were 12 days old. The roots were then removed and the cotyledons and hypocotyls dried in an oven at 110°C.

(c) Growth of Dicentra Hybrids

Dicentra hybrids (Dicentra exima (Kerr-Gawl) Torr. X Dicentra Oregana (Eastwood) were used in the investigation of the origin of methylenedioxy groups. These Dicentra hybrids were found by the author to produce considerable amounts of protopine under the condition of these experiments. Three young seedlings were planted in a garden in the open and allowed to grow for one month. At the end of the one month growth period the three young hybrids were removed from the soil, the roots washed and then placed in a culture solution (76). These three young hybrids were fed respectively carbon-14 methyl labelled

L-methionine, carbon-14 methyl labelled choline and carbon-14 labelled sodium formate. These labelled materials were administered dissolved in the liquid culture solution in which the plants were growing. After the plants had been in contact with the labelled material for one week they had translocated activity to their topmost leaves. They, then, were removed from the solutions and dried in an oven at 110°C.

(D) Isolation Techniques

(a) Isolation of N-methyl tyramine, hordenine and choline

N-methyl tyramine, hordenine and choline were extracted from the barley roots, while the stems yielded gramine and choline. The extractions were done using a procedure outlined by Kirkwood and Marion(77). The dried root material was extracted in a continuous methanol extractor for 24 hours. The methanol was evaporated under reduced pressure, the residue taken up with 200 ml. of 2 N sulphuric acid and extracted three times with an equal volume of ether. N-methyl tyramine and hordenine were continuously extracted for 48 hours with ether from the aqueous layer which had been made basic with ammonia. After ether extraction, this basic solution was treated with one-sixth its volume of methanol to prevent foaming and methanol and ammonia removed under reduced pressure. The solution was then made slightly acidic with dilute hydrochloric acid, filtered and the choline precipitated by the addition of a saturated solution of ammonium reineckate to the filtrate. The ether extract of the residue taken up by sulphuric acid,

containing the phospolipid choline, was evaporated to dryness, the residue dissolved in 1 N potassium hydroxide in 30 per cent ethanol and the mixture refluxed for four hours on the steam bath. After hydrolysis of the phospolipid choline the solution was made acidic with dilute hydrochloric acid and extracted twice with petroleum ether. The aqueous portion was filtered, and the choline precipitated by the addition of ammonium reineckate to the filtrate.

The stems were also extracted with methanol for 24 hours. The methanol was evaporated, the residue taken up with 2 N sulfuric acid and extracted with petroleum ether. The aqueous layer was made alkaline with sodium hydroxide and the gramine extracted with ether. The aqueous layer was then made acidic with dilute hydrochloric acid, filtered and the free choline precipitated by the addition of ammonium reineckate. The acid-ether extract which contained the phospolipid choline was hydrolyzed and converted to the reineckate as before. All the choline reineckate precipitates were combined, decomposed by the method of Kapfhammer and Bischoff (72), and the choline then precipitated as the chloroplatinate. It was found that betaine chloroplatinate was soluble in 95 per cent alcohol while choline chloroplatinate was not. To make doubly sure that betaine was separated from the choline, the choline chloroplatinate was decomposed with hydrogen sulphide and the choline reprecipitated as the reineckate in basic solution. Yield of choline chloroplatinate, 373 mg. from 140 gm. of plant material.

N-methyl tyramine and hordenine were separated by the method described by Leete et al (74). The ether extract was evaporated to dryness, the residue taken up with 200 ml. of benzene and the solution passed through an alumina column. Hordenine was eluted with a 10 per cent solution of methanol in benzene. The N-methyl tyramine was then eluted by washing the column with pure methanol. The hordenine and N-methyl tyramine fractions were evaporated to dryness, the residues transferred to spaith bulbs and the contents of each sublimed at 125°C. and 0.03 mm. pressure. Yield of N-methyl tyramine was 61 mg. M.P. 128 - 129°C. corr. Yield of hordenine was 73 mg. M.P. 118 - 119°C. corr.

(b) Isolation of ricinine

The ricinine was extracted from the dried stems and cotyledons by using a modification of the procedure outlined by Weevers (75). The dried plant material was thoroughly crushed and continuously extracted with methanol for 48 hours. The methanol extract was evaporated on a steam bath to 500 ml. and the residue washed into a beaker with one liter of hot water. The aqueous solution was allowed to cool to 40°C. and a saturated solution of lead acetate added to precipitate the amino acids. This precipitate was immediately filtered off and thoroughly washed with hot water. The excess lead was precipitated with hydrogen sulfide, the lead sulfide filtered off, the filtrate then evaporated to a volume of 80 ml. and extracted for 24 hours with chloroform in a

liquid-liquid extractor. The chloroform was evaporated under reduced pressure, the residue dissolved with hot methanol and the ricinine allowed to crystallize at 0°C. The ricinine was then purified by sublimation in a spaith bulb at 195°C. and 0.03 mm. pressure. Yield, 2.12 gm. or 3.0 per cent of dried material. M.P. 199 - 200°C.

(c) Isolation of protopine

The protopine from each of the plants was isolated according to the procedure described by Manske (78). The dried plants were extracted with methanol in a soxhlet extractor and 500 mg. of carrier protopine added. The methanol extract was evaporated to a small volume, made acidic with dilute hydrochloric acid, the remainder of the methanol removed, and the solution extracted with chloroform. The aqueous layer was made alkaline with potassium hydroxide and extracted with chloroform. The chloroform fraction was dried over sodium sulfate and evaporated to dryness under reduced pressure. The residue was washed with petroleum ether on a buchner funnel, taken up with methanol, evaporated to a small volume and made acidic with concentrated nitric acid. The protopine nitrate which crystallized out was filtered off and washed with ice cold methanol. To recover the free protopine the nitrate salt was dissolved in chloroform and shaken with a 10 per cent solution of potassium hydroxide. On evaporation of the chloroform fraction to a small volume, protopine crystallized out. The protopine from each plant was purified to constant specific activity by alternate recrystallization of the free base and the nitrate salt from methanol.

Yield was about 75 per cent based on the carrier protopine added. M.P. 203 - 205° corr.

(D) Degradation Techniques

(a) Degradation of hordenine

Hordenine was degraded by a modification of the procedure outlined by Leete et al (74). These authors reported that the α and β carbon atoms of the alkaloid underwent a Tiffeneau rearrangement (79) during the conversion of p-vinylanisole to homoanisaldehyde. However this rearrangement did not affect these results since it was only necessary to show that there was no activity in the hordenine molecule other than in the methyl groups.

The active sample of hordenine was diluted with 2.0020 gm. of inactive carrier. This was recrystallized until constant activity was attained. A 1.9936 gm. sample of the hordenine was dissolved in 25 ml. of methanol along with 10 ml. of methyl iodide and the mixture refluxed on the steam bath for 30 minutes. After the reflux period, the methanol was evaporated to a small volume and the hordenine methiodide allowed to crystallize out. The methiodide was filtered off and washed with cold methanol-ether solution. Yield, 96 per cent. M. P. 231 - 232°C. corr.

The hordenine methiodide was then converted to O-methyl hordenine methochloride. A 2.0000 gm. sample of the methiodide was dissolved in 16 ml. of 10 per cent sodium hydroxide and 11 ml. of dimethylsulfate were slowly added with stirring. After all the dimethylsulfate had been

added, the solution was stirred for an additional five hours. The resulting O-methyl hordenine methiodide was precipitated as the reineckate in basic solution. Any unreacted material would stay in solution. The reineckate salt was then filtered off, washed thoroughly, dried and decomposed by the method described by Kapfhammer and Bischoff (72). The O-methyl hordenine methochloride was recrystallized from methanol-ether. Yield, 90 per cent. A 1.1060 gm. sample of the compound was dissolved in 25 ml. of methanol along with fresh silver oxide made from 1.0 gm. of silver nitrate and sodium hydroxide. After stirring for three hours at room temperature in the dark, the silver chloride filtered off and the filtrate introduced into a sublimation bulb. The solution was evaporated almost to dryness under reduced pressure and the sublimation bulb connected to a high vacuum system through two traps, the first cooled to 0°C. in ice and the second to -70°C. in dry ice-acetone. The bulb was heated in an air bath to 140°C. at which temperature decomposition took place and the p-vinyl anisole distilled, most of it condensed in the first trap, while the trimethylamine condensed in the second trap. To the second trap methyl iodide was added and the tetramethylammonium iodide converted to the reineckate. The reineckate salt was purified to constant specific activity by the method of Kapfhammer and Bischoff (72). Yield, 81 per cent. The p-vinylanisole was taken up with ether, stirred with 1.5 gm. of yellow mercuric oxide and 1.5 gm. of iodine, for three hours. The mercuric oxide was then filtered off and the filtrate

shaken with aqueous sodium thiosulfate to remove excess iodine. The ether solution of the resulting homoanisaldehyde was evaporated to a small volume and stirred with a solution of 3 gm. of sodium bisulfite in 10 ml. of water. The bisulfite addition product was filtered off and stirred with 2 ml. of a solution of 0.20 gm. of hydroxylamine hydrochloride and 0.20 gm. of sodium carbonate in water. The oxime precipitated out as colorless crystals and was recrystallized from hot water. Yield, 10 per cent on original starting material. M.P. 119 - 200°C. corr.

(b) Degradation of N-methyl tyramine

The N-methyl tyramine was converted to hordenine methiodide by refluxing 1.600 gm. in 25 ml. of methanol, 9 ml. of methyl iodide and 1.00 gm. of sodium bicarbonate for two hours. The hot solution was filtered, evaporated to a small volume and allowed to cool when hordenine methiodide began to crystallize out. The hordenine methiodide was converted to O-methyl hordenine methochloride and degraded in the same manner as described for hordenine. Yield of tetramethylammonium reineckate, 75 per cent. Yield of homoanisaldehyde oxime, 8 per cent based on starting material.

(c) Degradation of choline chloroplatinate

To determine the location of the labelled carbon in the choline molecule, the salt was degraded by the method described by Lintzel and Monasterio (80). A 370 mg. sample of choline chloroplatinate was

placed in a three-necked flask along with 15 ml. of 20 per cent potassium hydroxide. The flask was connected to two traps each filled with 1 N hydrochloric acid. The flask was heated with a "glasscol" heater as air was passed through the solution. A saturated solution of potassium permanganate was slowly added dropwise until oxidation was complete as indicated by a permanent green color. The traps were then removed, the contents transferred to a flask and the hydrochloric acid removed under reduced pressure. The trimethylamine hydrochloride was taken up with water and ammonium reineckate added to the solution. The trimethylammonium reineckate was purified by the method of Kapfhammer and Bischoff (72) until constant activity was attained. Yield, 63 per cent of theoretical.

(d) Degradation of protopine

The specific activity of the N-methyl group was determined by degrading a portion of the alkaloid according to the methylimino determination of Pregl (81). The resulting methyl iodide was trapped in trimethylamine. The apparatus used in this procedure is shown in Figure 2. A 50 mg. sample of the alkaloid was placed in reaction vessel (A) along with 70 mg. of ammonium iodide, 2 drops of 10 per cent gold chloride solution and 15 ml. of constant boiling hydriodic acid. Trap (B) contained a 5 per cent solution of sodium thiosulfate and cadium sulfate, while trap (C) and (D) contained a 5 per cent ethanolic solution of trimethylamine at dry ice acetone temperature. Traps (C) and (H) contained dilute sulphuric acid. Stopcock (E) was

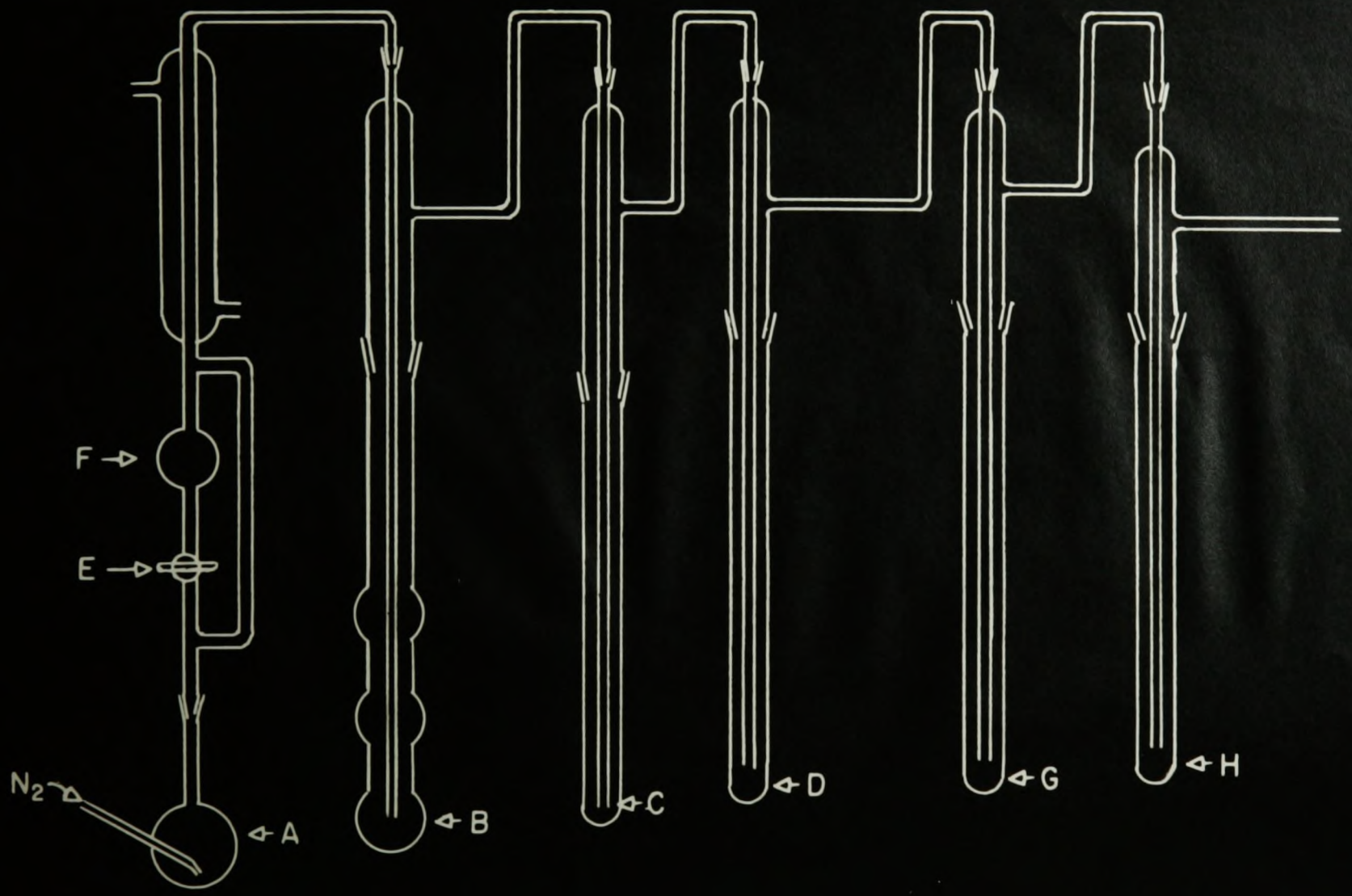

FIG. 2
APPARATUS FOR DEGRADATION OF N-METHYL GROUP

closed and nitrogen allowed to slowly bubble through the solution. To cleave the N-methyl group reaction vessel (A), was heated with a "glasscol" heater until all the hydriodic acid was distilled into trap (F). The residue remaining in (A) was thermally decomposed at 320°C. for thirty minutes, flask (A) cooled and the acid allowed to run back into (A). This was repeated twice more to ensure that a maximum yield of the N-methyl group was obtained. The contents of traps (C) and (D) were removed, transferred to a flask, 0.5 cm. of potassium hydroxide added and evaporated to dryness. Any formaldehyde which may have come over due to hydrolysis of methylenedioxy group would be evaporated off at this point. The residue was taken up with water, made acidic with dilute hydrochloric acid and the tetramethylammonium chloride precipated as the reineckate salt. The sample of tetramethylammonium reineckate was purified to constant specific activity by repeated decomposition and reprecipitation according to the procedure of Kapfhammer and Bischoff, (72). Yield of N-methyl group was 90 per cent of theoretical.

The specific activity of the methylenedioxy groups was obtained by hydrolysis of a separate portion of the alkaloid with 20 per cent sulphuric acid. Gadamer (82) reported the estimation of methylenedioxy groups by hydrolyzing with concentrated sulphuric acid in the presence of phloroglucinol. However, this was unsatisfactory for our purposes in that the yields were low and the nature of the resulting compound uncertain. Sarker (53) reported an improved method for the

estimation of the number of methylenedioxy groups in lignin. He hydrolyzed the groups to formaldehyde with 28 per cent sulphuric acid and obtained the formaldehyde as the dimedone derivative. The procedure followed in this work was a slight modification of Sarker's. A 100 mg. sample of the alkaloid and 70 ml. of 20 per cent sulphuric acid were placed in a flask connected by a condenser to another flask containing 170 mg. of dimedone in 20 ml. of water. The flask containing the alkaloid was heated by a "glasscol" heater until water began coming over. At this point more water was contimually added by means of a dropping funnel to maintain the concentration of the acid at 20 per cent. The receiver containing the dissolved dimedone was warmed slightly to ensure that all the formaldehyde which came over reacted with the dimedone. Heating was continued until 400 ml. of water had been distilled over into the receiver. The receiving flask was then cooled and the formaldehyde methone filtered off. The yield was 60 per cent of the theoretical. M. P. 190 - 191^{o}C. corr.

RESULTS AND DISCUSSION

The results of the betaine experiments are shown in Table I. These results indicate that betaine methyl takes part in the transmethylation scheme in the barley plant. Since the activity of the hordenine is twice that of the N-methyl tyramine, this indicates that tyramine is methylated to hordenine by a stepwise process and the two steps are not far separated with respect to time.

It is postulated that betaine methyl is utilized via methionine in the animal body (43). A comparison of the results obtained by Matchett et al (3) for methionine with these results shows that the total activity of the betaine fed was slightly more than twice that of methionine fed. However, the activity of the hordenine from the betaine experiment was one-fourth the activity of the hordenine from the methione experiment. It is reasonable to postulate, therefore, that the methyl group of betaine which is found in hordenine, is transferred directly to methionine or by some other intermediate or intermediates to methionine. It is also possible that such young plants depend on sources other than betaine for their labile methyl supply. The utilization of betaine methyl may not be developed until these sources have been exhausted and photosynthetic processes have been put into full operation. To check these possibilities it would be necessary to investigate the role of enzyme systems involved in the transfer of betaine methyl.

Matchett *et al* (3) have also shown that choline methyl arises from methionine. The results obtained in this work indicate that betaine

TABLE I

SPECIFIC ACTIVITIES OF BETAINE EXPERIMENTS

BETAINE ADMINISTERED		ALKALOID ISOLATED	SPECIFIC ACTIVITY c.p.m. per mM.		
WGT. MGM.	TOTAL ACTIVITY c.p.m.		Original Substance	Trimethyl Amine	Homo-Anisaldehyde Oxime
42.3	1.2×10^7	Ricinine[a]	$< 6.0 \times 10^2$		
		N-methyl tyramine	$4.5 \pm 0.1 \times 10^3$	$4.8 \pm 0.1 \times 10^3$	0
60.0	1.7×10^7	Hordenine	$9.4 \pm 0.1 \times 10^3$	$8.5 \pm 0.1 \times 10^3$	0
		Choline	$1.95 \pm 0.1 \times 10^4$	$1.81 \pm 0.1 \times 10^4$	

[a] not degraded

ansfers its methyl to choline. The transfer is probably via
nine. The same results could have been obtained by the two step
tion of betaine. However, if choline arises by the two step reduc-
. of betaine, then its activity would be expected to be much higher
an that obtained in this experiment. Du Vigneaud et al (40) showed
hat the reduction of betaine to choline was not significant in the
animal body. The results reported in Table I indicate that this is
also true for plants.

The fact that betaine did not transfer its labile methyl to that of the N and O-methyl group of the alkaloid ricinine in the castor bean plant is in contrast with the results obtained for the barley plant. Ciamician and Ravenna (83) failed to isolate betaine from the bean plant, although they found traces of choline. They did not state, however, the type of bean plant they had investigated. If betaine is not present in R. communis then the "methylpherase" required for the transfer of the labile methyl group would not be expected to be present in the plant. On the other hand, the young plants may not depend on betaine for their labile methyl supply, because of the large methionine supply that the growing seedlings have at their disposal. This is evident from the large endosperm which the young plants have in their possession. Labile betaine methyl may not be utilized until the supply of labile methyl in the endosperm is exhausted.

The results of the experiments on the origin of the methylenedioxy group are shown in Teble II. The results indicate that methionine serves as a precursor of both methylenedioxy and N-methyl groups of the alkaloid

TABLE II

SPECIFIC ACTIVITIES OF PROTOPINE EXPERIMENTS

METABOLITE ADMINISTERED			SPECIFIC ACTIVITY C.P.M. per mM.		
	Weight (mgm)	Activity c.p.m.	Protopine	Methyl-Enedioxy Group	N-Methyl Group
L-Methionine	82	1.2×10^7	$8.8 \pm 0.1 \times 10^3$	$1.5 \pm 0.1 \times 10^3$	$1.9 \pm 0.1 \times 10^3$
Sodium formate	10	5.0×10^7	$1.3 \pm 0.1 \times 10^3$		
Choline chloride	60	3.0×10^7	0		

protopine. Formate also contributes to the activity of the molecule, but not to as great an extent as methionine. Formate has been shown to be a labile methyl precursor in the barley (1) and tobacco plants (84). However, the activity of the protopine from the formate run was so low that significant activities for the degradation products were not obtainable. These results indicate that the methyl group of the methionine is transferred to a hydroxyl group, the resulting methoxyl oxidized to hydroxymethyl and then ring closed with an adjacent hydroxyl group. The second step in this scheme is similar to that postulated by Miller and Miller (65) to occur in the metabolism of the N-methyl groups of carcinogenic azo dyes. The ease with which hydroxymethyl groups react with active hydrogen is well known (66). If formate was a more direct precursor of the methylenedioxy groups than methionine methyl, it would be expected that a much higher level of activity would be obtained in the formate run, since the level of activity fed in the formate experiment was four times that in the methionine experiment. It is reasonable to postulate, therefore, that the above scheme takes place rather than formylation followed by reduction and ring closure.

The fact that choline methyl does not contribute activity is not surprising. It has been shown previously in the case of barley and R. communis that choline methyl contributes neither to the "labile methyl" nor formate pools of these plants (3,4). The Dicentra plants seem to follow the same pattern.

An inspection of Table II shows that if the activity in the

methylenedioxy groups and the N-methyl group is substracted from the total activity in the molecule, a residual activity approximately twice that in the N-methyl group is left. An inspection of the structure of protopine XXXVI (page 18) shows that it could have originated from two dihydroxyphenylalanine residues plus the carbon atom in the dotted circle. It is reasonable to postulate that the residual activity is in this carbon atom. It has been shown that the "formate pool" is concerned with the biosynthesis involving single carbon atoms (85). Therefore, this single carbon atom may have had its biogenesis in the "formate pool" of the plant.

SUMMARY AND CONCLUSIONS

Carbon-14 methyl labelled betaine was synthesized and fed to young barley and castor bean plants. It was found that betaine took part in the transmethylation scheme in the barley plant but not in the castor bean plant.

The methylenedioxy group and the N-methyl group of the alkaloid protopine were found to arise from methionine. It was found that formate was also a precursor, but not as direct a precursor as methionine. Since the activity of the methylenedioxy groups and the N-methyl group did not account for the total activity found in the protopine molecule, the methionine must contribute activity to some other carbon atom or atoms in this molecule. The position of this residual activity is speculated as appearing in the carbon atom which is encircled in XXXVI (page18).

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