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THE ORIGIN OF THE O - METHYL AND N - METHYL GROUPS IN THE ALKALOID RICININE

by

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ABSTRACT.

The origin of the O - methyl and the N - methyl group in the alkaloid ricinine has been determined. Carbon 14 labelled sodium bicarbonate, sodium formate and C^{14} methyl labelled L - methionine and choline were administered to growing seedlings of Ricinus communis which elaborate the alkaloid ricinine. Methionine was the only metabolite that produced C^{14} activity in the alkaloid. The activity was located entirely in the methyl groups and the specific activity of the O - methyl and N - methyl group was found, within experimental error to be identical.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Dr. S. Kirkwood for his aid and encouragement throughout the whole of this work.

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INTRODUCTION

Little is known about the metabolic processes in the higher plants. The methylation process, however, is gaining widespread attention. The origin of the nitrogen-methyl group in the alkaloids hordenine and nicotine has been determined and shown to arise from formate and the methyl group of methionine.

The origin of the oxygen-methyl group has never been determined. The alkaloid ricinine, which is elaborated by the growing seedlings of Ricinus communis, contains an oxygen-methyl group and therefore gives a means for investigating the possible origins of this group. In addition to the O - methyl group ricinine also contains an N - methyl group; the presence of both these groups in one molecule allows a determination of any similarity or difference that may exist in the origins of the O - methyl and N - methyl groups.

In determining the origin of the methyl groups in ricinine it was decided to administer in separate experiments carbon-14 methyl labelled choline, L - methionine and carbon-14 labelled sodium formate and sodium bicarbonate to growing seedlings of Ricinus communis. Choline and methionine are known transmethylating agents and formate has been shown to be a precursor of the labile methyl; bicarbonate was included to illustrate the role, if any, played by carbon dioxide in methylation. If appreciable carbon-14 activity could be detected in the methyl groups of ricinine, which was extracted from seedlings receiving one of the above metabolites, it could be concluded that the particular metabolite is a source of the methyl groups of ricinine.

HISTORICAL INTRODUCTION

Methylation in Biological Systems

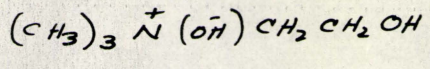
Biological methylation plays a key role in the metabolic processes of plants, animals and micro-organisms. Since the methyl containing compounds, choline (I) and methionine (II) are known to be present in all living cells, it can be concluded that the processes giving rise to methyl groups are essential to life. Despite the widespread occurence of the methylation process, its presence was not conclusively established until comparatively recent times. There was speculation on the presence of biological methylation or alkylation however, at an early period in the history of biochemical investigation.

Methylation as a result of bacterial action was first proposed by Basedow in 1846 (1). This hypothesis was proposed in connection with fatalities that were occuring as a result of mold action on arsenic containing wallpapers. It was thought that the toxic substance was cacodyl oxide (III); no experimental evidence was presented in support of this view.

The first experimental evidence in support of biological alkylation (methylation) was presented by Gosio (2) in 1891; he exposed potato mash containing arsenious oxide to the air and observed that a garlic like odour was emitted, which, Gosio concluded, was an alkyl arsine. The organism responsible for this process was the mold, Scopulariopsis brevicaulis.

An attempt was made by Biginelli (3) to characterize this gas (Gosio gas), by aspirating it through a solution of mercuric chloride

and hydrochloric acid. The resulting precipitate was assigned a composition that indicated that the gas was diethyl arsine (IV).



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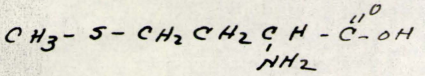

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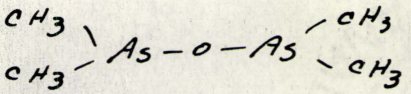

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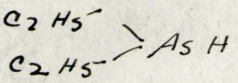

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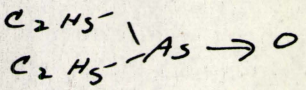

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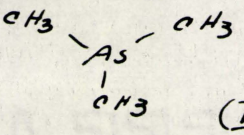


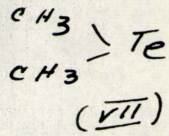
(VI)

Klason (4), from a reconsideration of Biginelli's work, concluded that it was diethylarsine oxide (V). Wigren (5) synthesized both these compounds and showed that their behaviour towards acidic mercuric chloride solution was different from that of Gosio gas. As a result of the uncertainty regarding Gosio gas, an investigation was started by Challenger (6) who showed that the gas was trimethyl arsine (VI). This was the first experimental evidence proving the presence of a methylating process in a biological system.

Challenger subsequently illustrated the biological methylation of inorganic tellurium and selenium compounds to dimethyl telluride (VII) and dimethyl selenide (VIII) respectively (7,8).

Investigations were later extended into the field of the sulphur compounds. Attempts were made to obtain dimethyl sulphide by the use of two different strains of Scopulariopsis brevicaulis. Negative results were obtained with inorganic sulphur, sodium sulphite

and sodium tetrathionate. The occurrence in nature of compounds, such as methionine (II), suggested the possibility of a biological methylation of sulphur.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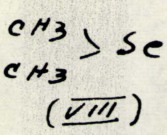
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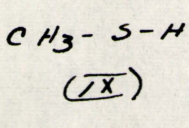
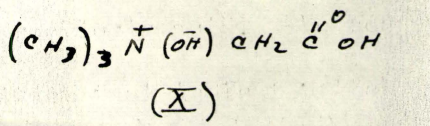

(IX)

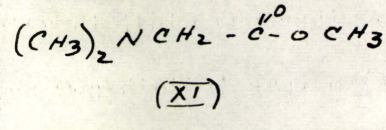
Subsequent work by Birkenshaw et al. (9) showed that the wood destroying fungus Schizophyllum commune Fr., when grown on an aqueous medium containing glucose, inorganic salts, and a trace of Marmite converts inorganic sulphate to methyl mercaptan (IX). This observation illustrated the biological methylation of sulphur.

In considering the origin of the methyl groups produced during biological methylation, it was originally considered that formaldehyde might play a role as a precursor in the formation of this group (10). This hypothesis probably arose because of the ease with which the Eschweiler reaction proceeds in vitro (11, 12). No direct biochemical evidence has been presented in support of the formaldehyde hypothesis.

The suggestion that certain biological methylations in animals might be conditioned by methyl containing compounds present in the body was presented by Hofmeister (13). Riesser, (14) subsequently suggested that the methyl groups of choline (I) and betaine (X) might play a role in the biological methylation process. This hypothesis was probably advanced because of Willstatter's work, (15) which showed that, on heating, betaine forms methyl dimethylaminoacetate (XI), a reaction clearly involving the migration of a methyl group.



(X)



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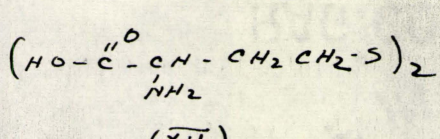

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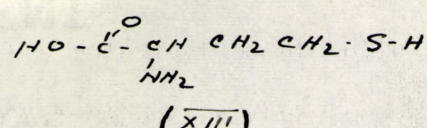


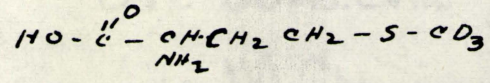
(XIII)

Biochemical evidence in support of the methyl transfer hypothesis was presented by du Vigneaud (16, 17, 18); he showed in his work with rats that homocystine (XII) after its conversion to homocysteine (XIII) can replace methionine (II) in the diet only if choline or betaine are present. It was suggested that the methyl group is transferred from the nitrogen of choline or betaine to the sulphur of homocysteine, thus giving rise to the essential amino acid methionine. It was also suggested by the above authors that the reaction may be reversible, methionine acting as a methyl donor to a choline precursor.

The transmethylation hypothesis gained additional support from du Vigneaud's work with specimens of deuteriomethyl labelled methionine (XIV) (19). This substance was fed to rats maintained on a choline methionine free diet, and it was subsequently shown that the deuterium content present in the methyl groups of isolated choline and creatine (XV) was eighty-five percent of the theoretical possible amount. This result substantiated the conclusion that these reactions are true transmethylations. The results also disprove the formaldehyde theory of methylation. If deuterium labelled formaldehyde was eliminated from methionine at some stage of the reaction and combined with a choline precursor with subsequent reduction to a methyl group, the deuterium

content of the resulting group could not rise above two-thirds of that present in the methyl group of the methionine administered.

The transmethylation hypothesis has gained further support by recent work of Keller et. al. (20) in which the technique of double labelling was employed. The methyl of methionine was labelled with both deuterium and carbon-14; upon administration of the compound to a rat and subsequent isolation of the choline, the ratio of D to C^{14} in the choline methyls was identical to that of the administered methionine methyl.



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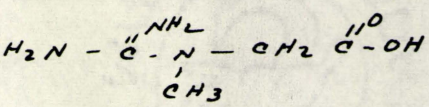

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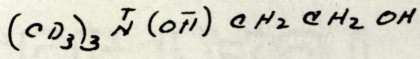
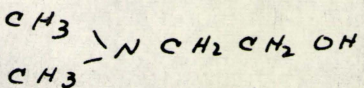


(XVI)

The role of choline in transmethylation was illustrated by du Vigneaud et. al. (21) by administering trideuteriocholine (XVI) to rats maintained on a choline methionine free diet containing homocysteine. The deuteriomethyl group was detected in tissue methionine.

The possibility that mono and dimethylaminoethanol play a role as methyl donors has been investigated by du Vigneaud (22). When dimethylaminoethanol (XVII) was fed to young rats maintained on a methyl free basal diet containing adequate amounts of homocystine, growth

was not as good as when choline was fed; this suggested that methionine was not readily formed from dimethylaminoethanol. Deuteriodimethyl-aminoethanol under similar conditions was converted into deuteriocholine, which in turn gave rise to deuterium containing methyl in creatine by transmethylation. The ratio of deuterium in body choline to deuterium in body creatine was large in the above experiment whereas when deuteriomethionine was fed the ratio was almost unity. These results indicate that dimethylaminoethanol does not take part directly in transmethylation, but it must accept a methyl group from methionine, or some other methyl donor, thus giving rise to choline and accounting for the limited growth producing power. These facts indicate that choline when engaged in transmethylation releases only one methyl group giving dimethylaminoethanol with no further loss of a methyl group. Experiments with deuteriomethyl-aminoethanol led to similar conclusions; monomethylamino-ethanol (XVI) does not play a direct role in transmethylation. The inability of the aminoethanols to transfer their methyl groups has been attributed to the absence of a quaternary nitrogen atom which is present in choline and betaine.



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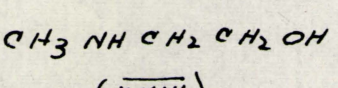


(XVIII)

Additional evidence in support of the view that the amino-ethanols are direct precursors of choline was presented by Horowitz et.al. (23), who used mutant strains of Neurospora crassa. One mutant cannot synthesize methylaminoethanol but can methylate it to choline if an exogenous supply is available. The other mutant strain is capable

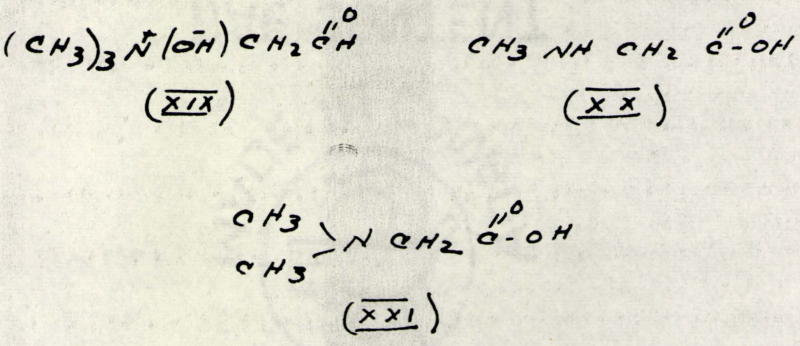
of producing methylaminoethanol, but is unable to convert it to choline at a normal rate, consequently it accumulates and is regarded as a normal intermediate in choline synthesis.

Stetten (24) with the aid of N^{15} labelled aminoethanols, has presented evidence in support of the view that these substances are choline precursors in animals. Nitrogen-15 labelled ethanolamine, when administered to rats, was converted to tissue choline.

Betaine (X) an oxidized form of choline, has been shown to take part in transmethylation. Du Vigneaud et.al. (19) fed betaine with deuterium labelled methyl groups and N^{15} in the amino group to growing rats. Isotopic analysis of the choline and creatine isolated from the rat tissues showed that betaine was an effective methyl donor. The low N^{15} content found in the tissue choline proved that the administered betaine was not reduced to choline.

Dubnoff (25) has recently described some work on the relation of choline and betaine in enzyme systems. He states that the methyl groups of choline are not themselves labile, but become available for methionine formation from homocystine only if the animal can oxidize choline to betaine. In vitro enzyme studies show that methionine formation is rapid from betaine both aerobically and anaerobically in suitable organs of all animals tested, but choline is effective only for those organs derived from animals which possess choline oxidase. Some recent in vitro work by Muntz (26) with rat liver homogenates strongly support these conclusions. Williams (27) has shown with the aid of rat liver homogenates that choline, betaine aldehyde (XIX) and betaine are equivalent methyl donors under aerobic conditions. However,

anaerobically only betaine and betaine aldehyde are capable of transmethylation and choline is almost inactive in furnishing methyl groups to homocysteine; these results indicate the necessity of choline oxidase for the utilization of choline methyl groupsin transmethylation. With the aid of isotopic indicators du Vigneaud has shown that the other glycine derivatives sarcosine (XX) and dimethylglycine (XXI) are ineffective in transmethylation (28, 29).



(XIX) (XX) (XXI)

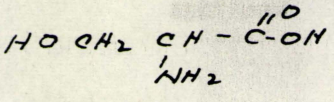
Synthesis of Labile Methyl in the Animal

From the work thus far reviewed the hypothesis arose that the animal organism is incapable of generating methyl groups. The first piece of experimental evidence in opposition to this hypothesis was reported by du Vigneaud et. al. (30). The authors maintained the concentration of deuterium in the body water of two rats at about 3 atom percent. The deuterium content of the choline isolated from the tissues showed that between 7.7 to 8.5 percent of the choline methyl hydrogen was derived from the body water. The authors state that it is very unlikely that isotopic exchange could cause the appearance of deuterium in the methyl groups under these conditions. The possibility of methyl synthesis by intestinal bacteria was considered as a source

of these methyls and this view was held by du Vigneaud up to the time of Sakami's work (31).

This bacterial hypothesis was subsequently disproved by some recent work of du Vigneaud et. al. (32). Germ free rats maintained on water containing deterium were shown to contain deuterium labelled choline methyls to an extent of 3.3 and 6.4 percent of that in the body water. This work clearly indicates that the synthesis of the labile methyl group is possible in animals, but it does not reveal the primal origin of the group.

Formate has been shown to take part in the metabolism of one carbon fragments. Buchanan and Sonne (33) showed that formate is a precursor of the 2 and 8 carbons of the uric acid nucleus. Competitive inhibition studies on biological systems reveal that the biosynthesis of purines occur through a utilization of this one carbon fragment (34, 35). Additional evidence for the utilization of formate has been shown in connection with the biosynthesis of the amino acid serine (XXII). Sakami (36) reported that rats receiving C^{14} labelled formate and glycine labelled with C^{13} in the carboxyl group were capable of synthesizing serine labelled with C^{14} in the β position and C^{13} in the carboxyl group. Siekevitz and Greenberg (37) were able to show the reverse effect, that formate can be produced in rat liver slices from carbon atom 3 of serine.



(XXII)

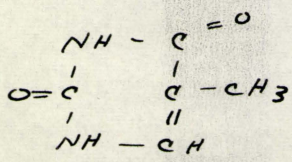
Sakami (38) has shown that the methyl group of acetone is a precursor of the β carbon of serine and the labile methyls of choline and methionine. Since the β carbon of serine was shown to be derived from formate, the methyl group of acetone must be metabolized via formate. That the labile methyl groups of choline and methionine are derived from the methyl of acetone suggested that formate might be a direct precursor of these groups. Subsequent in vivo and in vitro work by Sakami (39) revealed that C^{14} labelled formate is a precursor of labile methyls.

Additional evidence in support of the formate labile methyl relationship was reported by Arnstein (40) D L - 3 - C^{14} serine, L - 3 - C^{14} - serine, C^{14} labelled methanol and 2 - C^{14} glycine all known sources of formate have been shown to give rise to the methyl group of choline.

The above results provide conclusive evidence in support of the hypothesis that labile methyls are capable of being synthesized by animals and that formate plays a key role in this metabolic process. Occasionally du Vigneaud (30) found animals capable of showing some growth on a methyl free diet containing homocystine; this fact can be readily interpreted in view of Sakami's work.

There is no conclusive evidence in support of a mechanism for the conversion of formate to the completely reduced form of a labile methyl. The hypothesis that serine, which is known to arise from glycine and formate, is a direct precursor of labile methyls is gaining widespread acceptance. Sprinson has shown that the β carbon of serine appears to be a precursor of labile methyls without passing through

a higher level of oxidation. (41) Double labelled serine containing deuterium and C^{14} in the β position was prepared by the reduction of ethyl formyl hippurate in the presence of deuterated water. This substance when fed to rats was shown to give rise to the methyl groups of choline and thymine (XXIII). The deuterium C^{14} ratio in these methyls was approximately the same as that present in the original serine This fact suggested that the β carbon of serine is transferred together with its hydrogens and that it does not pass through an intermediate of a higher level of oxidation.



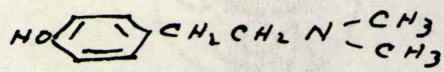
(XXIII)

As pointed out by Sprinson, this conclusion was open to criticism since the method employed for the production of the deuterium labelled serine could easily give rise to a preferentially attached deuterium. If this was the case and the Ogston concept (42) applies to the enzyme systems involved, a preferential extraction of hydrogen on the β carbon could occur without altering the deuterium carbon 14 ratio in any subsequent one carbon fragment. Elwyn (43) however, has recently reported that the method employed for the synthesis of serine labelled in the position with deuterium does not give rise to a preferentially attached deuterium. L-serine was prepared by the reduction of ethyl (D, C^{14}-formyl) hippurate with Al-Hg and normal water. This results in a labelling only in the β position and if any assymetric synthesis was achieved, the deuterium is attached in a configuration opposite to that of the first serine.

Administration of this amino acid to rats also resulted in an equal dilution of C^{14} and D in the synthesis of the methyl groups of choline and thiamine, in agreement with previous findings.

Methylation in Plants

As a result of the large accumulation of data regarding transmethylation, and the synthesis of labile methyl groups in animals, investigations are now being extended into the methylation processes of plants. Kirkwood and Marion (44) have shown with the aid of different barley strains that the methylation of the alkaloid hordenine (XXIV) proceeds via tyramine (XXV) in a stepwise manner similar to the methylation of ethanolamine to choline. With the aid of C^{14} labelled formate, Kirkwood and Marion (45) subsequently showed that this metabolite was a precursor of the N - methyl groups of hordenine, again analogous to the formation of the methyl groups of choline in animals.



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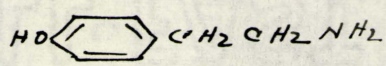
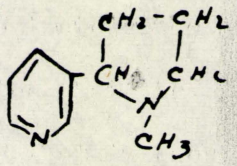


(XXV)

Unpublished work from this laboratory indicates that methionine is also a source of the methyl groups of hordenine (46). Choline was found to play no part in the transmethylating scheme in barley. This result has been explained on the basis that betaine as pointed out by previous investigators, is the active transmethylator; if the choline oxidase system for the conversion of choline to betaine is absent in the

barley plant, transmethylation would not occur.

Brown and Byerrum (47) have recently reported that the N - methyl group of the alkaloid nicotine (XXVI), present in the tabacco plant, arises from formate and methionine; choline was not investigated by these authors.



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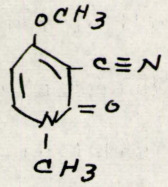


(XXVII)

In the experimental part of this thesis the transmethylation scheme in plants has been extended to the alkaloid ricinine (XXVII), which is found in the castor bean Ricinus communis. The particular feature of this alkaloid is the presence of both an O - methyl and an N - methyl group. The biogenisis of the O - methyl group had never been established and ricinine offers a means to establish any similarity or difference that may exist in the biogenesis of the N - methyl and O - methyl group.

During the course of the present work with ricinine, evidence for the origin of the O - methyl group in nature was presented by Byerrum and Flokstra (48). These authors administered C^{14} methyl labelled D L - methionine and C^{14} labelled formate to growing barley plants; isolation of the barley lignin followed by subsequent degradation revealed that the O - methyl groups in lignin were labelled with C^{14}. It was therefore concluded that the administered metabolites were precursors of the O - methyl group.

EXPERIMENTAL AND RESULTS

Measurement of Carbon 14 Activity

All C^{14} activity was measured using the Nuclear Instruments "Q" gas counter. Samples for counting were prepared by evaporating an infinitely thin layer of the material being counted on to the bottom of an aluminum counting tray. In measuring the activities of ricinine and its degradation products a piece of thin lens paper (thickness $<$ 1 mgm. $cm.^{-2}$) was placed on the bottom of the aluminum counting tray. The presence of the lens paper was found to be advantageous in comparing the relative activities of these compounds. Different solvents had to be used to bring these compounds into solution; in preparing a sample of ricinine for counting methanol was used; with tetramethyl ammonium reineckate aqueous acetone was employed. Since each of these solvents possess different wetting properties towards aluminum uniform infinitely thin layers of identical geometry could not be produced on the bottom of the counting tray upon evaporation of the solvent. Acetone in particular tended to pile the tetramethyl ammonium reineckate onto the edges of the tray, this altered the geometry of the sample as compared to a sample of ricinine prepared with the aid of methanol. With the use of lens paper a very uniform distribution of material was obtained throughout the lens paper, thus giving an identical geometry with all samples regardless of the solvent employed. Some β ray absorption due to the lens paper was present but this was inconsequential in view of the relative results that were being sought.

Synthesis of C^{14} Labelled Compounds

Synthesis of C^{14} labelled sodium bicarbonate:

The C^{14} labelled sodium bicarbonate was prepared by adjusting an aqueous solution of C^{14} labelled sodium carbonate as supplied by Atomic Energy of Canada Ltd., to a pH of 8.3 with 0.1N hydrochloric acid solution.

Synthesis of C^{14} labelled sodium formate:

Carbon^{14} labelled sodium formate was prepared by a method devised by the author which has been submitted for publication. The procedure outlined is an improvement of the method described by Melville et. al. (49) which is an adaptation of the method of Bredig and Carter (50). This involved the reduction of bicarbonate with hydrogen over palladium catalyst at 70 degrees C. and 100 atmospheres pressure. Bredig and Carter specify that it is necessary to use the potassium salt to obtain good yields. Anker (51) failed to observe any difference between the sodium and potassium salts and this has been confirmed in this laboratory. It appears that sodium formate is strongly adsorbed on the palladium catalyst and the failure of Bredig and Carter to get good yields was probably due to inadequate washing of the catalyst. Anker reports considerably lower yields than Melville et. al. and he ascribes these to the use of a commercial catalyst.

The method described here is adapted to the use of sodium carbonate as the starting material rather than the barium carbonate as used by the American workers. Sodium carbonate containing excess sodium hydroxide is the standard from in which C^{14} is supplied by Atomic Energy

of Canada Limited. The method described has several advantages over that of Melville et. al. The whole operation is carried out in a single glass vessel which eliminates Melville's apparatus train. The catalyst is generated in situ and this results in a palladium black of uniformly high activity and does away with preparation of catalyst by the method of Willstatter and Waldschmidt - Leitz (52). The method appears to lend itself to any scale of operation from the microgram to the gram scale and the yields of successive runs compare with those reported by Melville et. al.

Anhydrous sodium carbonate (80.0 mgm.) was added to 2 ml. of water in the glass liner of a Parr high pressure hydrogenation apparatus, and sufficient of a solution of labelled sodium carbonate added to bring the activity up to the desired level. The solution was then adjusted to a pH of 8.3 (phenolphthalien indicator by adding o.1 N hydrochloric acid. An accurately weighed sample of about 140 mgm. of palladous chloride was then added to the reaction vessel, followed by sufficient, standardized, sodium hydroxide solution to combine with the hydrogen ion released when the palladous ion was reduced to metalic palladium. The glass liner was then sealed into the hydrogenation apparatus and the reduction carried out for 24 hours at 70 degrees C.and 100 atmospheres of hydrogen. The reaction mixture was then filtered using vacuum and the palladium black thoroughly washed with water at 80 degrees C. to remove the strongly adsorbed formate. Yields were invariably between 90 and 95 percent of the theoretical.

The unexposed palladium black prepared during the course of the hydrogenation is very active as compared to the palladium black produced by the classical Willstatter method (52). The author believes that the

production of palladium black catalyst by the reduction of palladous chloride in situ during the hydrogenation could be used to advantage in many hydrogenations that require this catalyst.

Synthesis of C^{14} methyl labelled L - methionine:

The S - benzyl - L - homocysteine used for the preparation of C^{14} methyl labelled L - methionine was prepared using the method described by du Vigneaud and Patterson (53) DL - homocysteine was converted to S - benzyl DL - homocysteine which in turn was formylated to N - formyl - S - benzyl DL - homocysteine. This formyl derivative was resolved with the aid of brucine into the D and L isomers which were subsequently converted to S benzyl - D homocysteine and S - benzyl - L - homocysteine.

Carbon-14 methyl labelled L - methionine was prepared from C^{14} labelled methyl iodide and S - benzyl - L - homocysteine using the procedure outlined by Melville (49). The apparatus used for this preparation is shown in Fig. 1. 0.37 ml. of carrier methyl iodide was distilled through tip "a" into trap B which was cooled in liquid air. Vessel A was removed, tip "a" was sealed, the apparatus was evacuated through F and seal E was closed thus isolating the whole apparatus under a vacuum. Break seal "b" on sample tube C containing one tenth of a millicurrie of C^{14} labelled methyl iodide was broken with the magnetic breaker, thus allowing the contents of sample tube C to condense into trap B containing the carrier methyl iodide. The mixed methyl iodide samples were alternately condensed from trap B into sample tube C to promote good mixing of the carrier methyl iodide with the labelled methyl iodide sample. The entire methyl iodide sample was finally frozen into

trap B, kept at liquid air temperatures and seal "c" was broken while covered with rubber tubing containing nitrogen slightly above atmospheric pressure. Tip "a" was blown out to an aperture of about 2 mm. and vessel A containing about 30 ml. of distilled liquid ammonia and 1.45 gm. of S benzyl - L - homocysteine was fitted into the ground glass joint, clamped and surrounded by a dry ice acetone bath at -100°C. With trap D at liquid air temperatures to remove water and carbon dioxide, a slow stream of nitrogen was bubbled from tip "c" through the apparatus until the S benzyl - L - homocysteine had completely dissolved. Small pieces of freshly cut sodium were added through the side arm of Flask A until a blue colour persisted in the liquid ammonia for several minutes indicating that a stoichiometric quantity had been added. The temperature of vessel A was raised to -50°C. with a dry ice acetone bath, and the liquid air bath surrounding trap B was removed. With a continuous flow of nitrogen passing through the apparatus the methyl iodide in trap B was slowly raised to room temperature, vaporized and swept into reaction vessel A, trap B was finally heated to about 40°C. until all of the methyl iodide had vaporized, this took about thirty minutes. Two 0.025 ml. samples of unlabelled methyl iodide were added to the reaction mixture before it gave a negative nitroprusside test, indicating the absence of a free sulfhydryl group. The liquid ammonia was now allowed to evaporate in the current of nitrogen, allowed to escape through the side arm and led into a water drain.

The white solid residue was dissolved in 15 to 20 ml. of water taken to a pH of 6 with hydroiodic acid and filtered. The filtrate was concentrated to about 5 ml. under vacuum and the mixture was heated

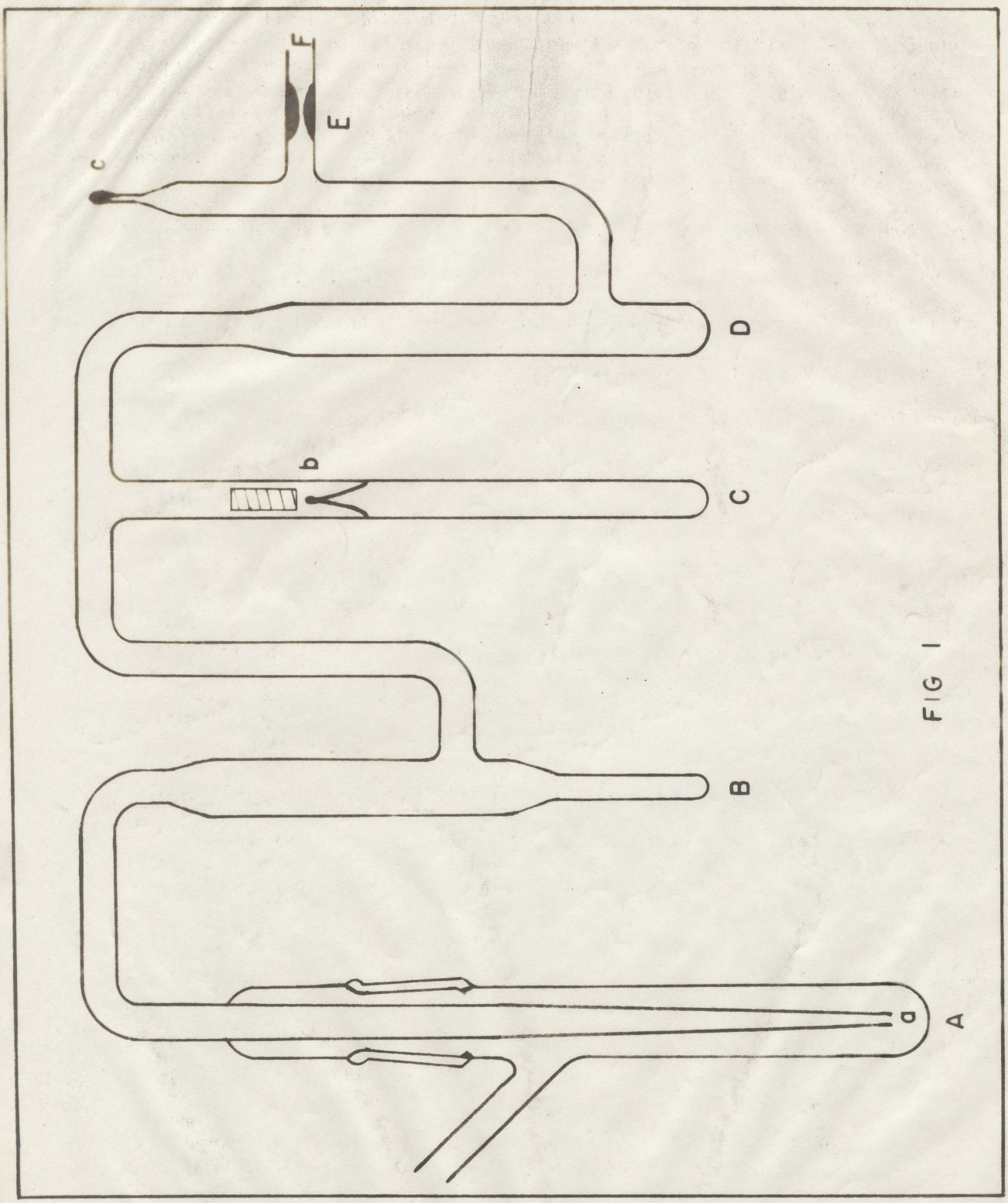


FIG I

slightly on a steam bath until the crystalline methionine had gone into solution. About 100 ml. of boiling ethanol was added to this solution and crystallization was allowed to proceed at room temperature, the mixture was finally allowed to crystallize at 0°C. overnight. The crystals of L - methionine were separated from the mother liquor by filtration washed with cold ethanol and dried.

The yield was 600 mgm.; 60 percent based on the total amount of S - benzyl - L - homocysteine used. The total activity in the sample was 8.0 x 10 counts per minute.

Synthesis of C^{14} methyl labelled choline:

Carbon-14 methyl labelled choline was prepared by employing the method described by Feiger and du Vigneaud (54). Carbon-14 labelled methyl iodide was allowed to react with an excess of ethanolamine and the produced choline was isolated as the reineckate salt. The choline was purified by employing the method described by Kapfhammer and Bischoff (55).

Growth of Ricinus communis (Castor bean)

The castor beans used in this investigation were supplied by Drs. C. K. Whitehair and D. C. Van Horn of the Agricultural Experimental Station at Stillwater Oklahoma.

The germination and growth of the castor beans was carried out in the complete absence of light. Under these conditions it has been shown that the etiolated castor seedling has the greatest alkaloid producing power (56). Growth in the absence of light also eliminates photosynthesis which would cause appreciable random labelling of the

ricinine molecule. A temperature of 25°C. was maintained throughout the germination and growth of the castor seedlings.

About 360 gm. of castor beans were evenly distributed on the bottoms of six 12" x 12" pyrex trays and kept well wetted until germination was widespread as evidenced by the splitting of the castor beans. The germinating beans were removed from the trays and thoroughly soaked and washed with distilled water, then placed in clean glass trays and covered with a uniform layer of medium vermiculite (a form of mica which is a non nourishing inert plant support medium); about 100 gm. of this material was added to each tray. The layer of vermiculite was kept well dampened by daily watering with distilled water. Under the above conditions the growth of the young castor seedlings was rapid and uniform.

The radioactive compounds sodium bicarbonate, sodium formate, choline, and L - methionine were administered to the young seedlings by including them in the daily water ration, following the addition of the vermiculite medium. These compounds appeared to have no toxic effects on the young seedlings since they maintained a healthy appearance during the whole period of growth. These compounds were found to be readily absorbed by the young seedlings, since in separate experiments it was shown that when they were administered to the roots C^{14} activity could be detected in the cotyledons and hypocotyls.

The young seedlings were harvested when the majority of them had reached a height of from 15 to 20 cm. This height was usually attained about eighteen days after the initial planting of the beans. The seedlings were prepared for the extraction of the alkaloid ricinine

by removing the roots, which have been shown to contain little or no ricinine (56) and drying the cotyledons and hypocotyls at 100°C. The weight of the dry cotyledons and hypocotyls was in the vicinity of 75 gm.

Extraction and Purification of the Alkaloid Ricinine

The alkaloid ricinine was extracted from the dried cotyledons and hypocotyls using a modification of the method outlined by Weevers (56).

The dried cotyledons and hypocotyls were thoroughly crushed, placed in an extraction bag and thoroughly extracted for forty-eight hours with methanol employing a soxhlet extractor. The methanol solution containing the alkaloid was taken down to about 500 ml. on a steam bath and the residue was washed into a two litre beaker with about 1,200 ml. of boiling distilled water. The aqueous solution was allowed to cool to about 40°C. and a saturated solution of lead acetate was added in excess to precipitate the amino acids in the form of lead salts. This precipitate was filtered off and thoroughly washed with about 700 ml. of hot water. The washings were combined with the filtrate and the whole was treated with hydrogen sulfide to remove the excess lead ion by precipitation as the sulfide. The precipitate was filtered off and washed with about 700 ml. of hot distilled water; the washings were combined with the filtrate. The clear bright orange filtrate was evaporated under vacuum to a volume of about 70 ml. This aqueous residue together with the precipitated ricinine was transferred quantitatively into a continuous chloroform extractor. Extraction with chloroform was carried out for about twenty-four hours and the chloroform extract

containing the alkaloid ricinine was evaporated to dryness under vacuum. The residue was taken up with hot methanol and the ricinine was allowed to crystallize from this solvent at 0°C., for about fifteen hours. The crystals were filtered and washed with cold methanol, the washings and the filtrate were combined and a second fraction of crystals was obtained from the liquors by evaporation of excess solvent, followed by crystallization at 0°C. A third fraction of ricinine was obtained by precipitation of the alkaloid from the evaporated liquors of the second recrystallization with diethyl ether.

All the fractions were combined, placed into a Spath bulb and were sublimed at 195°C. and 0.05 cm. of Hg. Sublimation under these conditions was repeated followed by recrystallization from methanol and finally from an ethanol diethyl ether medium until the specific activity of the alkaloid had reached a constant value. The melting point of the purified ricinine was 200 - 201°C. (corrected). The reported melting point of ricinine is 201°C. (57).

Degradation of Ricinine

Description of the apparatus:

The labelled ricinine was degraded employing the apparatus shown in Fig. 2. The apparatus contains ground glass joints throughout. Trap D contains a 5 percent solution of sodium thiosulfite and cadmium sulfate which removes any hydrogen iodide or iodine that escapes from the reaction vessel A. Traps E and F contain a 5 percent ethanolic solution of trimethyl amine and traps G and H contain a solution of dilute sulphuric acid which prevents the escape of the volatile amine into the laboratory.

Procedure:

The procedure employed for the fission of the O - methyl and N - methyl groups from the alkaloid ricinine was a slight modification of the method outlined by Pregl (58), for the determination of the methoxy and methyl-imino groups. About 50 mgm. of ricinine were added to reaction vessel A followed by 15 ml. of constant boiling hydroiodic acid (Sp. Gr. 1.7). With traps E and F containing the 5 percent ethanolic solution of trimethylamine at dry ice acetone temperature and stopcocks T_1 and T_2 opened a slow stream of nitrogen of about two bubbles per second was passed through the apparatus. To split the O - methyl group flask A was slowly heated with a "glasscol" heater until the hydroiodic acid began to boil, water was now passed through condenser B. Slow refluxing was continued for two hours with a constant flow of nitrogen passing through the apparatus which swept the liberated methyl iodide into traps E and F. Heating was stopped, and the apparatus was thoroughly flushed with nitrogen for an additional hour. The dry ice acetone bath was removed from traps E and F, and the flow of nitrogen was continued for an additional hour until the traps had reached room temperature. The contents of traps E and F were quantitatively washed into a round bottom flask with distilled water, about 0.5 gm. of potassium hydroxide was added to the solution to remove any possible contamination due to trimethylammonium iodide and the solution was taken to complete dryness under vacuum. The residue was taken up with water and quantitatively rinsed into a 250 ml. beaker. The solution was taken to a pH of about 4 with dilute hydrochloric acid and the tetramethylammonium iodide was precipitated as the reineckate -

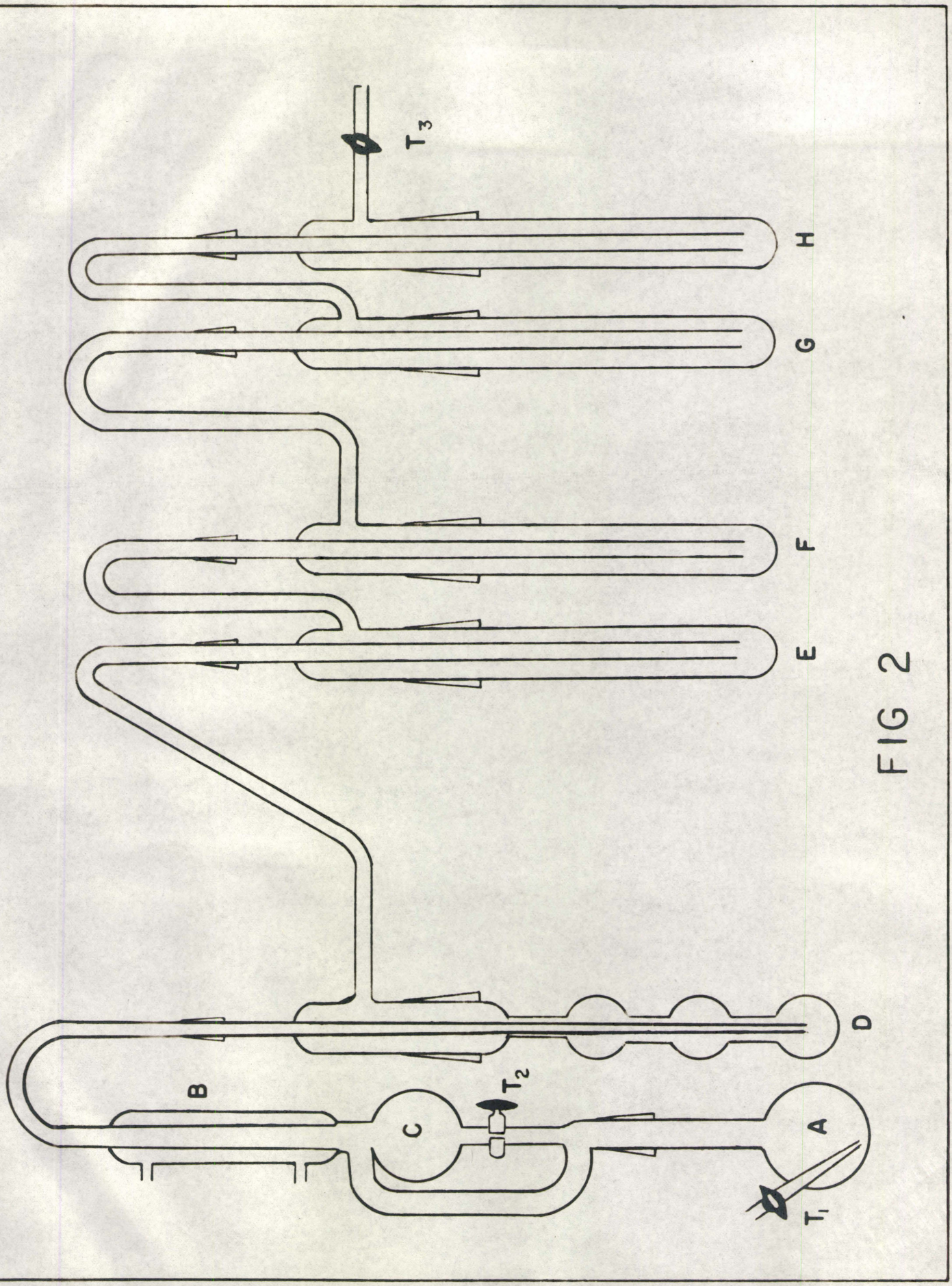


FIG 2

The yield of the O - methyl group in terms of the reineckate was 95% of the theroetical.

Subsequent fission of the N - methyl group in the above reaction mixture was accomplished by using Pregl's (58) methyl imino procedure. Traps D, E and F were recharged with the appropriate solutions and about 60 mgm. of ammonium iodide and two drops of a 10 percent solution of gold chloride were added to flask A containing the remains from the O - methyl split. With a slow stream of nitrogen flowing through the apparatus and traps E and F at dry ice acetone temperatures, stop cock T_2 was closed and the hydroiodic acid was distilled from Flask A into Flask C. The residue remaining in flask A was thermally decomposed at 320°C. for about thirty minutes, flask A was now allowed to cool and the hydroiodic acid readmitted to A by opening stopcock T_2. Distillation of the hydroiodic acid followed by decomposition of the residue was repeated. It was found that two such thermal decompositions gave the maximum amount of N - methyl group. The apparatus was again thoroughly flushed as outlined in the O - methyl procedure and the contents of traps E and F were also treated in an identical manner. The yield of N - methyl in terms of the reineckate varied from 60 to 70 percent.

Preliminary work on the degradation of ricinine showed that a very sharp separation could be obtained between the O - methyl and N - methyl groups. No methyl iodide could be detected with prolonged refluxing in the period between the two hour reflux with hydroiodic acid to obtain the O - methyl group and the first thermal decomposition to obtain the N - methyl group. In view of this result there was no possibility of contaminating one group with the other.

The samples of tetramethylammonium reineckate were purified to constant specific activity by repeated decomposition and reprecipitation of the reineckate in water according to Kapfhammer and Bischoff (55), alternating with precipitation from ethanol as the chloroplatinate, followed by decomposition with hydrogen sulphide. The O - methyl and N - methyl groups were finally counted in the form of the reineckate salt.

The results of the determination of the specific activities of the alkaloid ricinine and the degradation products are shown in Table 1.

TABLE I

Metabolite Administered	Weight mgm.	Activity counts/minute	Specific Activity (counts/minute/millimole)			
			Ricinine	O-methyl as tetramethyl ammonium reineckate	N-methyl as tetramethyl ammonium reineckate	Sum of the activities of the O- and N-methyl groups
L-Methionine	230	3.0×10^7	$6.48 \pm 0.18 \times 10^4$	$3.06 \pm 0.14 \times 10^4$	$3.23 \pm 0.15 \times 10^4$	$6.29 \pm 0.21 \times 10^4$
Choline Chloride	60	3.0×10^7	$0.0176 \pm 0.0013 \times 10^4$	-	-	-
Potassium Bicarbonate	60	7.0×10^7	$0.0741 \pm 0.0017 \times 10^4$	-	-	-
Sodium Formate	17	7.0×10^7	$0.0867 \pm 0.0019 \times 10^4$	$0.0170 \pm 0.0026 \times 10^4$	$0.153 \pm 0.0026 \times 10^4$	$0.0323 \pm 0.0037 \times 10^4$

DISCUSSION

The results in Table I indicate that in the alkaloid ricinine the biogenesis of the O - methyl group follows a pattern identical to that of the N - methyl group. The fact that the specific activities of both groups are approximately the same indicates that the enzyme system or systems responsible for their appearance in ricinine function at an equal rate.

Methionine is the only metabolite which gives rise to the O - methyl and N - methyl groups; this fact coupled with the work of Byerrum (47) and work now being carried out with hordenine indicates that this substance must play a key role in the transmethylation scheme.

In view of the work of Kirkwood and Marion with hordenine (45), Byerrum's work with nicotine (47), and the widespread utilization of formate in metabolic processes concerned with one carbon fragments, the absence of appreciable activity in the ricinine extracted from the beans that were administered C^{14} labelled sodium formate is rather surprising. As shown in Table I what little activity that is present, appears to be randomly distributed throughout the molecule. This suggests that formate has been oxidized to carbon dioxide with subsequent fixation of this substance and its utilization in the general metabolic processes of the castor bean. The anomalous results obtained with formate cannot be explained, although it could be suggested that an exogenous supply of formate is not readily utilized because of the large fat and protein supply that the growing seedlings have at their disposal; this is evident by the possession of a relatively large

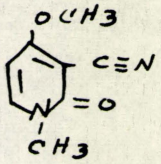
endosperm. The growing seedlings probably depend on the endosperm for their entire labile methyl supply, and do not develop the synthesis of labile methyls until the endosperm has been completely exhausted and photosynthetic processes have been put into full operation.

The absence of appreciable activity in the ricinine extracted from the castor beans that were administered C^{14} methyl labelled choline suggests that this substance is not utilized in the transmethylation scheme. This is not surprising since similar results were obtained with the alkaloid hordenine (45). The result can be explained by assuming that the choline oxidase system necessary for the conversion of choline to betaine which has been shown to be the active transmethylator in animal tissues, is absent in the plants investigated. This property may be common to all the higher plants but further investigation must be carried out before a valid conclusion can be made on this point. The small activity that is present in the ricinine is probably due to the oxidation of the choline methyls to carbon dioxide with subsequent fixation and random labelling of the molecule, as illustrated in the case of formate.

The absence of an appreciable activity in the ricinine which was extracted from the castor beans that were administered C^{14} labelled sodium bicarbonate is not surprising since carbon dioxide has never been found to play a role in the methylation process. The activity present is probably due to fixation of carbon dioxide followed by random labelling of the ricinine molecule.

The biosynthetic scheme responsible for the elaboration of the alkaloid ricinine in the growing castor seedling undoubtedly a

stepwise process. One possibility for an immediate precursor of ricinine in such a process is ricininic acid. (XXVIII)



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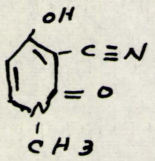


(XXVIII)

A comparison of the structures of ricininic acid and ricinine indicates that the final step in the biosynthesis of ricinine could be the formation of the O - methyl group. Such a step could easily be detected with the aid of C^{14} N - methyl labelled ricininic acid.

Carbon 14 N - methyl labelled ricininic acid can be prepared by the alkaline hydrolysis of ricinine (56) labelled with C^{14} in both the O - methyl and N - methyl groups by the castor seedlings which were administered C^{14} S - methyl labelled L - methionine. If C^{14} N - methyl labelled ricininic acid was administered to growing castor seedlings and the isolated ricinine was found to be labelled with C^{14} in the N - methyl group it could be concluded that ricininic acid is a direct precursor of ricinine.

The inability of formate to play an active role in the transmethylation scheme of Ricinus communis may prove to be a valuable aid in the study of the utilization of the β carbon of serine in biological methylation. Sprinson (41) has indicated that the β carbon of serine is utilized in the methylation process with retention of its hydrogen atoms. This would indicate that the β carbon of serine is not utilized as a result of an oxidative process such as conversion

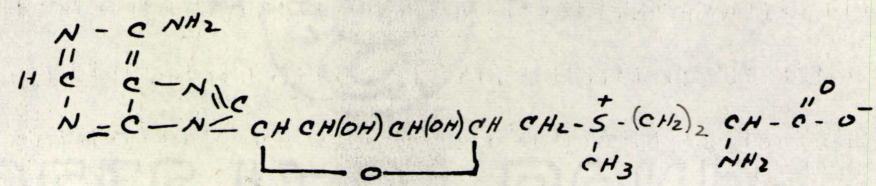
to formate, and subsequent reduction to labile methyl.

Additional experimental evidence on the utilization of serine in methylation could be obtained by administering 3 - C^{14} - L - serine to growing castor seedlings. Since formate is not utilized as a precursor of the methyls of ricinine via methionine the metabolism of serine via glycine and formate should not give rise to C^{14} labelled methyls of ricinine. If serine is utilized for the formation of labile methyls as indicated by Sprinson, the C^{14} labelled β carbon atom of serine might appear in the methyl groups of ricinine. If such a utilization could be demonstrated we would possess valuable evidence supporting the hypothesis that the β carbon atom of serine is a direct precursor of labile methyls without passing through a different level of oxidation such as formate.

An exact mechanism for the transmethylation process has not been experimentally established. However, the compounds involved in the process appear to have a common feature that is chemically favourable for the elimination of a methyl group. A comparison of the structures of the transmethylators betaine and choline with the non transmethylators dimethylglycine, sarcosine and the aminoethanols, indicates that a positively charged nitrogen is a requirement of the transmethylation process. The bond strengths of the methyl groups attached to the positively charged nitrogen are lower and more favourable to enzymatic elimination of a methyl group than those attached to an uncharged nitrogen atom. The Hoffman type reaction (elimination) in which organic fragments are liberated from sulphonium and ammonium compounds illustrates the energetically favourable conditions for

the breaking carbon bonds from the onium grouping. An observation by Willstatter (15) offers a striking example of the labile nature of a methyl group attached to a positively charged nitrogen atom. Betaine when heated is readily transformed to methyl dimethylamino-acetate; in this case an actual intramolecular transmethylation has occurred.

In view of the apparent necessity of a positively charged onium group before methyl transfer can occur, it is conceivable that methionine a known transmethylating agent might be converted to a sulfonium derivative prior to its liberation of a methyl group. This has recently been established by Cantoni (59) who showed that the active form of methionine is S - adenosyl methionine (XXIX) which arises by reaction with adenosine triphosphate.



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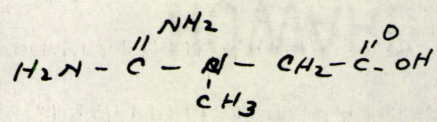

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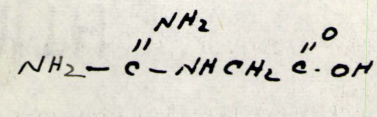

(XXX)

This active form of methionine has been shown to methylate quanidoacetic acid (XXX) to creatine (XV) in the presence of an enzyme known as a methylpherase. This work definitely confirms the view that the transfer of a methyl group is dependent on its attachment to a positively charged onium group.

REFERENCES

(1) Basedow, Schmidt's Jahrbuch 52, 89 (1846)

(2) Gosio, B., Arch. ital. biol., 18, 253, 298 (1893)

(3) Biginelli, P., Gass. chim. ital., 31, (1), 58 (1901)

(4) Klason, P., Ber., 47, 2634 (1914)

(5) Wigren, N., Ann., 437, 285 (1924)

(6) Challenger F., Advances in Enzymology 12, 431 (1951)

(7) Challenger, F., and North, H.E., J. Chem. Soc. 1934, 68

(8) Bird, M.L., and Challenger F., J. Chem. Soc., 1939, 163

(9) Birkenshaw, J.H., Findlay, W. P. K., and Webb, R.A., Biochem. J., 36, 526 (1942)

(10) Robinson, R., J. Roy. Soc. Arts 96, 795 (1948)

(11) Eschweiler, W. Ber. 38: 880 (1905)

(12) Raoul, Y., Compt. Rend., 204, 74 (1937)

(13) Hofmeister, F. Arch. exptl. Path. Pharmacol. 33, 198 (1894)

(14) Riesser, O., Z. physiol. chem., 86, 440 (1913)

(15) Willstatter, R., Ber., 35, 584 (1902)

(16) Chandler, J. P., and du Vigneaud, V., J. Biol. Chem., 135, 223 (1940)

(17) du Vigneaud, V. Chandler, J. P., Moyer, A.W., and Keppel, D.M., J. Biol. Chem., 131, 57 (1939)

(18) du Vigneaud, V. Chandler, J.P., Moyer, A.W., J. Biol. Chem., 130, 917 (1941)

(19) du Vigneaud, V. Cohn, M., Chandler, J. P., Schenk, J.R., and Simmonds, S., J. Biol. Chem., 140, 625 (1941)

(20) Keller, E. B., Rachelle, J. R., and du Vigneaud, V., J. Biol. Chem. 177, 733, (1949)

(21) du Vigneaud, V., Simmonds, S., Cohn, M., Chandler, J.P., J. Biol. Chem., 149, 519 (1943)

(22) du Vigneaud, V., Chandler, J.P., Simmonds, S., Moyer, A.W., and Cohn, M., J. Biol. Chem., 164, 603 (1946)

(23) Horowitz, N. H., Bonner, D., and Houlahan, M.B., J. Biol. Chem., 159, 145 (1945)

(24) Stetten, D., J. Biol. Chem., 140, 143 (1941)

(25) Dubnoff, J.W., Federation Proc., 8, 195 (1949)

(26) Muntz, J.A., J. Biol. Chem. 182, 489 (1950)

(27) Williams, J. N., Prog. Soc. Exptl. Biol. and Med., 78, 202 (1951)

(28) du Vigneaud, V., Simmonds, S., and Cohn, M., J. Biol. Chem. 166, 47 (1946)

(29) du Vigneaud, V., Simmonds, S., Chandler, J.P., and Cohn, M., J. Biol. Chem. 165, 639 (1946)

(30) du Vigneaud, V., Simmonds, S., Chandler, J.P., and Cohn M., J. Biol. Chem. 159, 755 (1945)

(31) Sakami, W., and Welch, A.D., J. Biol. Chem., 187, 379 (1950)

(32) du Vigneaud, V., Ressler, C., and Rachelle, J. R., Science, 112, 267 (1950)

(33) Buchanan, J.M., and Sonne, J.C., J. Biol. Chem. 166, 781 (1946)

(34) Shive, W., and Roberts E. C., J. Am. Chem. Soc., 69, 725 (1947)

(35) Stetten, M.R., and Fox, C.L., Jr., J. Biol. Chem. 161, 333 (1945)

(36) Sakami, W., J. Biol. Chem., 176, 995 (1948)

(37) Siekevitz, P., and Greenberg, D.M., J. Biol. Chem. 180, 845 (1949)

(38) Sakami, W., J. Biol. Chem., 179, 495 (1949)

(39) Sakami, W., J. Biol. Chem., 187, 369 (1950

(40) Arnstein, H.R.V., Biochem. J., 47 XVIII (1950)

(41) Elwyn, D., Weissbach, A., and Sprinson, D.B., J. Am. Chem. Soc., 73, 5509 (1951)

(42) Ogston, A.G., Nature, 162, 963 (1948)

(43) Elwyn, D., Fed. Proc. 11, 208 (1952)

(44) Kirkwood, S., Marion L., J. Am. Chem. Soc., 72, 2522 (1950)

(45) Kirkwood, S. Marion L., Can. J. Chem., 29: 30-36 (1951)

(46) Matchett, T. J., Marion L., and Kirkwood S., (unpublished data).

(47) Brown, S. A., and Byerrum, R.V., J. Am. Chem. Soc., 74, 1523 (1952)

(48) Byerrum, R.V., Flokstra, F.H. Fed., Proc. 11, 193 (1952)

(49) Melville, D.B., Rachele, J.R., and Keller, E.B., J. Biol. Chem., 169, 419 (1947)

(50) Bredig, G., and Carter, S.R., Ber. 47, 541 (1914)

(51) Anker, R. Nucleonics 7, 54 (1950)

(52) Willstatter, R. and Waldschmidt - Leitz, E., Ber. 54, 123, (1921)

(53) du Vigneaud, V., and Patterson, W.I., J. Biol. Chem., 109, 97 (1935)

(54) Feiger, M.F., and du Vigneaud J. Biol. Chem. 185, 53 (1950)

(55) Kapfhammer, J., and Bischoff, C.Z. Physiol. Chem. 191: 179 (1949)

(56) Weevers, Th., Rec. Trav. Botaniques Neerlandaise, 30, 336 (1933)

(57) Winterstein, E., Keller, J. and Weinhagen, A.B. Arch. Pharm., 255, 513, (1917)

(58) Pregl, F. Quantitative Organic Microanalysis 4th Eng. Ed'n., J.A.Churchill Ltd., London, 156 - 60 (1945)

(59) Cantoni, G.L., J. Am. Chem. Soc. 74, 2942 (1952)