2. The infiltration of solutions of choline and betaine aldehyde resulted in a significant increase in the betaine content of the tissues.

3. Little variation from controls was observed when solutions of glycine in combination with methionine were infiltrated into the leaves.

4. When leaves infiltrated with solutions of choline were kept in an atmosphere of nitrogen during the experimental period significant increases in the content of betaine were not observed.

5. Leaves infiltrated with solutions of choline and exposed to light during the experimental period showed a greater increase in betaine content than leaves kept in darkness during the experimental period.

6. The conclusion is reached that the synthesis of betaine in the tissues of higher plants takes place largely as the result of the oxidation of choline.

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The Biosynthesis and Metabolism of Betaines in Plants

3. STUDIES ON THE BIOSYNTHESIS OF PRECURSORS OF GLYCINEBETAINE IN SEEDLINGS OF WHEAT (*TRITICUM VULGARE* VILL.)

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Evidence has been obtained that infiltration of choline into leaves of *Beta vulgaris* L. and *Atriplex patula* L. leads to an increase in the betaine content of the leaves and the inference has been drawn that betaine is formed in the tissues of plants by oxidation of choline (Cromwell & Rennie, 1954). It was therefore of interest to investigate the mode of synthesis of choline on the assumption that it is a direct precursor of betaine. The extensive literature on the biosynthesis of choline in animal tissues has been reviewed by Jukes (1947), and it is now well established that choline is formed by the stepwise methylation of ethanolamine in the tissues of the rat. In plants, however, the biosynthesis of choline has received little attention, and the object of the present work was to determine whether or not the synthesis of choline follows the same path in plants as in animals, the method being to feed wheat seedlings with possible precursors. Kirkwood & Marion (1951) have shown that when potassium formate labelled with ^{14}C is fed to sprouting barley, the labelled carbon appears in the methyl groups of choline and hordenine. These authors suggest that the formylation of amines followed by reduction is a general route for the synthesis of *N*-methyl groups in plants and animals. Recent work by Ahmad & Karim (1953) on the biosynthesis of choline in germinating seedlings of the chick-pea has shown that methionine, acetone and methanol, substances which give rise to choline methyl groups in the animal, also stimulate choline synthesis in the plant. Steensholt (1946) found that the methyl group of methionine was not utilized by etiolated wheat seedlings for the *N*-methylation of ethanolamine to choline. It is clear, therefore, that the problem of choline synthesis in plants as in animals involves a study not only of transmethylation reactions in which intact methyl groups are transferred, but also of the synthesis of 'biologically labile' methyl groups *de novo*.

EXPERIMENTAL

Materials and methods

Seeds of *Triticum vulgare* (vars. Atle and Fylgia) were used throughout these experiments. To hasten germination the seeds were treated with conc. H_2SO_4 for 1 min. and rapidly washed free from acid in a stream of tap water. Sterilization of the seeds was carried out by immersion for 5 min. in a solution containing 0·1% (w/v) $HgCl_2$ and 0·5% (v/v) 'Teepol' detergent (Shell Chemicals Ltd., London), followed by washing in sterile distilled water. Roux bottles (1 l. capacity) containing 80 g. of coarse quartz sand were used for germination and were fitted with cotton-wool air filters of the conventional pattern to enable the cultures to be aerated under sterile conditions. The appropriate culture solution (40 ml.) was placed in each of the bottles, the entire apparatus sterilized by autoclaving and allowed to cool in the aseptic cabinet. Each bottle was sown with 7·5 g. (dry wt.) of seed which was evenly distributed over the surface of the sand by holding the bottle in the horizontal position and imparting a vigorous swirling motion to the slurry of sand. The bottles were placed in the incubator at 19° and set at an angle of 10° from the horizontal to allow the culture solution to drain to the bottom of the bottle. After germination of the seed had taken place (1–2 days) the bottles were lowered to the horizontal position for the remainder of the growth period, thus allowing the culture solution to bathe the root system of the seedlings. The cultures were aerated daily for a period sufficient to allow of the complete removal of accumulated CO_2. For this purpose, the outgoing air stream was allowed to bubble through a solution of methyl red indicator and aeration continued until the indicator changed to orange-yellow. At the end of the growth period (6–7 days) the seedlings were removed from the bottles, freed from adhering sand grains by means of a coarse sieve, well washed in running water and finally dried in an oven with forced draught at 80°. After drying, the coleoptiles and roots were easily removed from the seeds by rubbing gently over a coarse-meshed sieve. The dried material was finely powdered in a mortar and stored in a desiccator until required for analysis. The culture solutions contained 0·0125% (w/v) of the substances used as possible precursors of choline and of methylating agents, either singly or in combination, and where necessary the pH of the solutions was adjusted to 7. Controls using distilled water were set up and each experiment was carried out in triplicate.

The sodium salt of dimethylglycine was prepared by the method of Michaelis & Schubert (1936). All other compounds used were commercial samples purified either by redistillation or by recrystallization. DL-Methionine was used as the methyl donor and formate was supplied as the potassium salt.

Estimation of choline

The dried plant material (0·1–1 g.) was extracted with methanol, following the method for the estimation of betaine (Cromwell & Rennie, 1953) to the stage of clarification with charcoal. At this stage the solution (5 ml.) was adjusted to pH 11–12 with 5% (w/v) NaOH, 5 ml. of a saturated aqueous solution of ammonium reineckate added and the tube placed in ice water for 1 hr. The precipitate of choline reineckate was filtered off on a chilled sintered glass crucible of medium porosity, the disk of which was covered with a thin layer of Gooch asbestos. The precipitate was washed with 5 ml. of cold ammonium reineckate solution and the filtrate and washings set aside for the estimation of betaine by the method of Cromwell & Rennie (1953). The precipitate of choline reineckate was washed with three portions of 2 ml. of *n*-propanol and finally with ether. A small glass rod was used to facilitate the complete removal of ammonium reineckate during the washing of the precipitate. The underside of the crucible was dried and the precipitate dissolved in 75% (v/v) acetone (4 ml.) and the solution collected in a test tube. The quantitative estimation of the choline reineckate was then carried out by the method of Marenzi & Cardini (1943). This method was slightly modified by adding the H_2SO_4 solution to the ethanolic solution of *sym.*-diphenylcarbazide before development of the colour.

RESULTS

The results of feeding etiolated wheat seedlings with possible precursors of choline and with compounds which might yield either intact methyl groups or which might contribute to the synthesis of labile methyl groups are given in Table 1. The possible precursor substances were either fed alone to the plants or in combination with the methylating compounds. Adequate precautions were taken to ensure that co-precipitation of unchanged methylated amines with choline did not take place as these substances were readily taken up by the seedlings. It was found that ammonium reineckate at pH 11–12 precipitates choline with no interference from methylethanolamine or dimethylethanolamine if these bases are present in admixture with choline. Analysis and melting point determinations were used to check the purity of the choline precipitates. Dimethylglycine is co-precipitated with betaine in acid solution and therefore was removed by oxidation with silver oxide prior to the estimation of betaine (Cromwell & Rennie, 1953).

The uptake of ethanolamine did not result in a significant increase in the content of either choline or betaine, but this base when fed in combination with formate caused a slight increase in the content of choline and a decrease in the content of betaine. The combination of ethanolamine with methionine led to a decrease in both choline and betaine values. When seedlings were allowed to take up methylethanolamine or dimethylethanolamine a remarkable stimulation of choline synthesis took place, and although the additional effect of the presence of methylating compounds varied somewhat, the combination of dimethylethanolamine with formate gave an increase of 172% in the choline value, but had comparatively little effect on the betaine content of the tissues. The combination of methylethanolamine with formate and/or methionine resulted in a loss of betaine. The uptake of glycine alone and in combination with methionine brought about a slight increase in choline values and a slight decrease in betaine. On the contrary, feeding with dimethylglycine alone or in combination with

Table 1. *The effect of possible precursor substances and substances yielding methyl groups on the biosynthesis of choline and betaine in etiolated wheat seedlings*

Experiments were carried out in triplicate and all analyses in duplicate. Choline and betaine values are expressed as mg./g. dry wt. Differences between experimental and control values are given on a percentage basis.

Possible precursor substance		Source of methyl groups: Choline	Source of methyl groups: Betaine	Methionine: Choline	Methionine: Betaine	Formate: Choline	Formate: Betaine	Methionine + Formate: Choline	Methionine + Formate: Betaine
No addition	Control	—	—	0·58	4·55	0·63	6·13	—	—
	(Mean variation)			(0·09) +15	(0·05) +15	(0·02) +8	(0·16) +2		
	Experimental			0·67	5·25	0·68	6·28		
	(Mean variation)			(0·07)	(0·22)	(0·05)	(0·28)		
Ethanolamine	Control	0·71	5·24	0·71	5·24	0·71	5·24	0·71	5·24
	(Mean variation)	(0·06) +15	(0·24) +8	(0·06) −11	(0·24) −15	(0·06) +20	(0·24) −8	(0·06) +14	(0·24) +10
	Experimental	0·82	5·66	0·63	4·47	0·85	4·80	0·81	5·78
	(Mean variation)	(0·07)	(0·15)	(0·08)	(0·25)	(0·09)	(0·13)	(0·04)	(0·30)
N-Methylethanolamine	Control	0·70	6·49	0·70	6·49	0·70	6·49	0·70	6·49
	(Mean variation)	(0·07) +112	(0·11) −1	(0·07) +81	(0·11) −27	(0·07) +102	(0·11) −27	(0·07) +123	(0·11) −29
	Experimental	1·48	6·41	1·27	4·74	1·41	4·76	1·56	4·63
	(Mean variation)	(0·13)	(0·13)	(0·25)	(0·11)	(0·09)	(0·20)	(0·09)	(0·25)
Dimethylethanolamine	Control	0·61	5·34	0·61	5·34	0·61	5·34	—	—
	(Mean variation)	(0·04) +93	(0·28) +6	(0·04) +80	(0·28) +18	(0·04) +172	(0·28) +3		
	Experimental	1·18	5·68	1·10	6·28	1·66	5·48		
	(Mean variation)	(0·15)	(0·55)	(0·14)	(0·27)	(0·19)	(0·21)		
Glycine	Control	0·69	6·23	0·69	6·23	—	—	—	—
	(Mean variation)	(0·06) +22	(0·19) −4	(0·06) +16	(0·19) −16				
	Experimental	0·84	5·97	0·80	5·24				
	(Mean variation)	(0·08)	(0·38)	(0·09)	(0·24)				
Sarcosine	Control	—	—	—	6·15	—	—	—	—
	(Mean variation)				(0·25) +9				
	Experimental				6·68				
	(Mean variation)				(0·44)				
Dimethylglycine (Na salt)	Control	0·69	5·73	0·69	5·73	0·69	5·73	0·69	5·73
	(Mean variation)	(0·06) −12	(0·13) +12	(0·06) −26	(0·13) +14	(0·06) +4	(0·13) +15	(0·06) +7	(0·13) −4
	Experimental	0·61	6·42	0·51	6·57	0·72	6·62	0·74	5·52
	(Mean variation)	(0·04)	(0·16)	(0·04)	(0·24)	(0·07)	(0·17)	(0·06)	(0·10)

methionine resulted in slight losses of choline but slight increases in the betaine values. The uptake of methionine and formate separately and in combination did not bring about a significant increase of either choline or betaine and sarcosine was ineffective as a stimulant of betaine synthesis.

DISCUSSION

Bearing in mind the difficulties attending the interpretation of results of feeding experiments on intact, actively growing seedlings, the work described in this paper may be regarded as giving support to the conclusion that choline is synthesized in the plant by the stepwise methylation of *N*-methylethanolamine and possibly by the stepwise methylation of ethanolamine. If the free choline in the tissues were to undergo immediate conversion into betaine or to take part in the synthesis of phospholipids, the difficulties of interpretation would be further increased. However, there appears to be little or no conversion of choline into betaine in the seedlings under the conditions of the experiments and phospholipids are not synthesized to any extent in the tissues of young, actively growing plants (Jordan & Chibnall, 1933; Byerrum & Wing, 1953). The experiments of Ahmad & Karim (1953) on the seedlings of the chick-pea have shown that feeding with DL-methonine stimulates the synthesis of choline to a considerable extent. In the present series of experiments, feeding of etiolated wheat seedlings with methionine or formate without the ethanolamines did not lead to a significant increase in the choline content of the tissues and it may be assumed that the effective concentration of endogenous methionine or 'formate' was not the limiting factor in choline synthesis. The uptake of ethanolamine alone and in combination with methionine and formate did not stimulate the synthesis of choline and in these experiments the apparent inability of etiolated wheat seedlings to use methionine for the methylation of ethanolamine is in agreement with the findings of Steensholt (1946). Similarly, mutant 34486 of *Neurospora crassa* is unable to utilize methionine for the methylation of ethanolamine (Horowitz & Beadle, 1943; Jukes, Oleson & Dornbush, 1945), and Jukes (1941) found that the chick is unable to utilize ethanolamine in the presence of methionine as a substitute for choline in a purified diet. However, when formate labelled with ^{14}C is fed to sprouting barley, the radioactive carbon appears in the methyl groups of choline (Kirkwood & Marion, 1951) and an incorporation of formate carbon into the methyl group of nicotine was reported by Brown & Byerrum (1952). More recently, Byerrum & Wing (1953) have shown that the carbon of the methyl groups of choline can be transferred to the *N*-methyl group of nicotine. It would therefore appear that in the tissues of *Nicotiana rustica* L. formate makes a contribution to the methyl groups of choline. From the experimental evidence available, the tentative deduction can be made that ethanolamine is not methylated to *N*-methylethanolamine by the transfer of an intact methyl group from methionine but requires for this reaction a one-carbon methyl group precursor, the carbon of which may be derived from formate, formaldehyde, methanol or acetone. The work of Arnstein (1951) on the biosynthesis of choline in the tissues of the rat has shown that L-serine is decarboxylated to ethanolamine and that methanol, formate, the β-carbon atom of serine and the α-carbon atom of glycine are precursors of choline methyl groups. Furthermore, Arnstein (1951) concluded that the rate of synthesis of choline methyl groups from the above precursors is slow compared with the rate of synthesis of the ethanolamine moiety and suggests that the apparently slow rate of methyl group synthesis is due mainly to an adequate supply of labile methyl groups in the diet. Similarly, if the rate of synthesis of labile methyl groups in etiolated wheat seedlings is slow, the lack of a significant stimulation of the formation of choline when ethanolamine is fed could be explained. Alternatively, ethanolamine when fed to etiolated wheat seedlings may undergo breakdown and therefore may be available to a limited extent only, for methylation to *N*-methylethanolamine. Weissbach & Sprinson (1953) have shown that ethanolamine is deaminated in the tissues of the rat with the formation of glycolaldehyde, which is oxidized through glycollic acid to glyoxylic acid which then becomes aminated to glycine. The synthesis of serine from glycine takes place through the incorporation of 'formate' and decarboxylation of serine results in the formation of ethanolamine. In the tissues of the rat, therefore, a cycle of reactions involving ethanolamine may take place. In the present series of experiments the stimulation of choline synthesis observed when *N*-methylethanolamine or dimethylethanolamine were fed to the seedlings may be ascribed to the ability of these compounds to accept an intact methyl group from methionine or other methyl donor. Moreover, the synthesis of the methyl group of methionine may be stimulated by formate, but on the other hand a one-carbon compound derived from formate may have been directly responsible for the methylation of these amines in the experiments in which formate was fed. When *N*-methylethanolamine and dimethylethanolamine were fed alone, the tissue content of choline showed a significant rise, thus lending support to the previous assumption that a substantially adequate endogenous supply of methyl donors was present in the tissues of the seedlings. In contradistinction to ethanolamine, it is unlikely that either *N*-methyl-

ethanolamine or dimethylethanolamine would be deaminated to any great extent and these substances would therefore be freely available for choline synthesis. The slight increase in choline synthesis observed when glycine was fed may be due to the ability of the α-carbon atom of glycine to act as a precursor of ethanolamine and the methyl groups of choline. The loss of betaine in some of these experiments is difficult to explain. The seed of wheat contains approx. 0·092 % of betaine (Guggenheim, 1940) and as the young seedling does not appear to synthesize betaine from choline or other possible precursors it is assumed that the betaine present in the seedling is translocated from the seed. As the fate of betaine was not determined in these experiments it is impossible to do more than suggest that on the analogy of the behaviour of betaine in animal tissues, this substance can also act as a methyl donor in plants. In experiments in which a loss of betaine was recorded, a methyl group derived from betaine might have made some contribution to the methylation of *N*-methylethanolamine and dimethylethanolamine to choline.

SUMMARY

1. Experiments on the effects of feeding possible precursors of choline to etiolated wheat seedlings grown under sterile conditions have been described.

2. The biosynthesis of choline was stimulated to a marked degree when *N*-methylethanolamine or dimethylethanolamine were fed alone and in combination with methionine and/or formate. *N*-Methylethanolamine or dimethylethanolamine used in combination with formate stimulated choline synthesis to a greater extent than the combination of these precursors with methionine.

3. The feeding of ethanolamine either alone or in combination with methionine and/or formate did not result in a significant increase in the choline content of the tissues.

4. The feeding of glycine alone and in combination with methionine brought about a slight increase in the choline content of the tissues but a slight decrease in the content of betaine. The feeding of dimethylglycine alone or in combination with methionine resulted in slight losses of choline but a slight increase in the content of betaine. A slight increase in the content of betaine was observed when dimethylglycine was fed in combination with formate.

5. Conversion of choline into betaine did not appear to take place in etiolated wheat seedlings under the conditions of the experiments. The possible significance of losses of betaine which occurred in some experiments is discussed.

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The Biosynthesis of Proteins

2. SYNTHESIS OF MILK PROTEINS BY THE GOAT

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In the first paper of this series (Campbell & Work, 1952) it was shown that the lactating rabbit synthesized milk protein mainly from the free amino acids of the blood. The distribution of radioactivity between casein and whey proteins showed, however, that if casein is synthesized entirely from free amino acids then a portion of the whey nitrogen must be supplied as bound amino acid, probably in the form of whole protein or peptide.

It has been shown recently (Askonas, Campbell, Humphrey & Work, 1954) that the immune globulin of rabbit milk is not synthesized in the mammary

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