The Biosynthesis and Metabolism of Betaines in Plants

2. THE BIOSYNTHESIS OF GLYCINEBETAIN (BETAIN) IN HIGHER PLANTS

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The synthesis of glycinebetaine may proceed by two possible routes: (a) by the stepwise methylation of glycine, with sarcosine and dimethylglycine as intermediates, or (b) by the stepwise methylation of ethanolamine to choline with subsequent oxidation of the latter base. In certain animal tissues there is abundant evidence of the formation of betaine by oxidation of choline, and this step would appear to be essential for the labilization of a methyl group of choline (Muntz, 1950). Since the work of Mann & Quastel (1937) and Bernheim & Bernheim (1938) who showed that rat-liver preparations are capable of oxidizing choline via betaine aldehyde to betaine, further investigation of the enzyme system has shown that the two components choline oxidase and betaine aldehyde oxidase are largely concentrated in the mitochondrial fractions of rat liver (Williams, 1952*a, b*). Although betaine is widely distributed in plants and occurs in high concentration in some families (Cromwell & Rennie, 1953*b*) little is known of the mode of synthesis and role of this compound in the metabolism of higher plants. Barrenscheen & Vályi-Nagy (1942) claim to have demonstrated a stimulation of betaine synthesis in homogenates of wheat seedlings to which glycine and methionine had been added, but repetition of these experiments in this laboratory failed to show any substantial conversion of glycine into betaine. In the present paper experiments are described in which possible precursors of betaine were infiltrated into leaves of *Beta vulgaris* L. and *Atriplex patula* L. with the object of determining the path of betaine synthesis. Preliminary experiments carried out by Cromwell & Rennie (1953*a*) on roots and leaves of seedling plants of *Beta vulgaris* from which homogenates were prepared seemed to show the presence of an enzyme which oxidized added choline to betaine. However, subsequent work showed that the activity of these homogenates was due to bacterial action.

EXPERIMENTAL

Materials and methods

Leaves of plants of *Beta vulgaris* L. and *Atriplex patula* L. in various stages of development were used in the following experiments. Two methods of introducing solutions of

possible precursors of betaine into the leaf tissues were adopted. In the first method the leaves were placed with their petioles in tubes containing the appropriate solution and allowed to take up the solution for periods of 24 or 48 hr. The solutions were adjusted to pH 7 where necessary, and to minimize bacterial growth were sterilized by boiling for 2 min. Immediately before the experiment one half of the leaf blade was removed as a control and kept in a moist atmosphere under a bell jar under the same light and temperature conditions as the experimental half. On completion of the experimental period the remaining half leaf was removed from the midrib and both halves dried at 80° for 2 hr., powdered and stored in a desiccator until required for analysis. In the second method, the solutions, neutralized where necessary, were introduced into the leaf tissues by vacuum infiltration. After infiltration and evaporation of excess of water, the leaves were kept in a moist atmosphere under a bell jar during the period of the experiment. In one series of experiments, half leaves were infiltrated with water as controls and the remaining half was infiltrated with the experimental solution. In a second series of experiments on *Atriplex patula*, entire opposite leaves were used, one member of the pair being infiltrated with water and the opposite member infiltrated with the experimental solution. Before infiltration, the leaves were surface sterilized by immersion in a 1% (v/v) solution of 'Dettol' (Reckitt and Coleman Ltd., Hull), and water and solutions used for infiltration were sterilized by boiling for 2 min. On completion of the experiment the leaves were dried at 80°, powdered and stored in a desiccator until required for analysis.

Choline chloride, glycine and DL-methionine used in these experiments were purified by recrystallization. Dimethylglycine was used as the Na salt and was prepared by the method of Michaelis & Schubert (1936). Betaine aldehyde, (formylmethyl)trimethylammonium hydroxide, was prepared by the method of Voet (1929). Betaine was estimated by the method of Cromwell & Rennie (1953*b*) after removal, where necessary, of dimethylglycine and other amino acids by oxidation with Ag_2O . As the determination of betaine by the above method depends on measurement of the concentration of reineckate ion, the purity of the reineckate precipitates was checked by determination of the melting point and by paper chromatography. For chromatography a solvent system consisting of 3 vol. *n*-propanol and 1 vol. *x* acetic acid was used with the ascending method (Whatman no. 1 filter paper). The spots were detected by use of a 0.25% (w/v) solution of potassium bismuth iodide in ethanol or acetone to which one drop of glacial acetic acid had been added. It was found that the use of acetone as solvent gave a better definition of the spots than ethanol.

A saturated solution of bismuth triiodide in ethanol slightly acidified with 10% (v/v) H_2SO_4 (1 drop 10% H_2SO_4 /10 ml. solution) also proved a satisfactory reagent for the detection of choline and the betaines. It is essential for good results that the papers should be thoroughly dried for at least 2 hr. at room temperature in a fume cupboard with forced draught before application of the above reagents. With the solvent system *n*-propanol-*N*-acetic acid, betaine has an R_f value of 0.39 and choline 0.54. Betaine reineckate dissolved in 75% (v/v) acetone can be placed directly on the paper but the acetone solution of choline reineckate must first be treated with dilute $AgNO_3$ solution to liberate the base before application to the paper.

RESULTS

The results of experiments on the uptake of solutions of choline through the petioles of leaves of *Beta vulgaris* are given in Table 1. In each experiment the uptake of choline has resulted in an increase in the betaine content of the leaves. The effect of infiltration of solutions of choline and other possible precursors of betaine into leaves of *Beta vulgaris* and *Atriplex patula* are shown in Tables 2 and 3. Infiltration of solutions of choline resulted in substantial increases in the betaine content of the leaves. The infiltration of betaine aldehyde did not stimulate the formation of betaine to the same extent as choline, but this may have been due to the lack of purity of the betaine aldehyde used in the experiments. Solutions of glycine and methionine in combination appeared to have little effect on the content of betaine, whereas solutions containing dimethylglycine in combination with methionine caused slight increases. The betaine content of leaves infiltrated with solutions of choline and kept during the experimental period in an atmosphere of nitrogen showed little variation from the controls.

DISCUSSION

The evidence presented by the foregoing experiments shows that of the possible precursors of betaine introduced into the leaf tissues of the experimental plants, choline is responsible for the greatest increase in the content of betaine and the inference is drawn that betaine is probably formed by the oxidation of choline. It will be convenient first to consider the effect of the infiltration of choline on the betaine content of the leaves of the annual plant *Atriplex patula*. The ability of the leaves to convert choline into betaine appears to depend to a considerable extent on the growth phase of the plants from which they are removed. Leaves from young plants and from old mature plants do not bring about conversion as readily as leaves from plants in the stage of active vegetative growth at mid-season. It is of interest to compare the production of betaine in the present experiments with the production of trimethylamine by the leaves of the closely related annual plant *Chenopodium vulvaria* L. The production of trimethylamine from choline by this plant has been shown to be greatest during the phase of active vegetative growth and as the plant reaches maturity the content of trimethylamine declines (Cromwell, 1950). The peak of activity for the transformation of choline into betaine or its breakdown to trimethylamine therefore coincides with the phase of active vegetative growth preceding maturity of the tissues, and it may be assumed that at this period the activity of the enzymes concerned is at its maximum. Alternatively, the synthesis of choline may reach a peak at this period and thus provide an optimum concentration of substrate for the enzymes responsible for the conversion of choline into betaine or breakdown to trimethylamine. In *Beta vulgaris*,

Table 1. *The effect of uptake of choline on the betaine content of leaves of Beta vulgaris by use of the half-leaf method*

Source of leaves	Duration of experiment (hr.)	Half-leaf sample	Composition of solution	Values in parenthesis refer to control experiments.	
				Betaine content (mg./g. dry wt.)	Δ (Betaine content) (mg./g. dry wt.)
Plants in vegetative phase	24	Experimental	Water	4.35 (4.00)	+0.35
		Experimental	Choline chloride (0.07M)	3.28 (2.16)	+1.12
Plants in vegetative phase	24	Experimental	Water	7.03 (5.82)	+1.21
		Experimental	Choline chloride (0.036M)	7.75 (5.70)	+2.05
Plants in vegetative phase	48	Experimental	Water	2.93 (2.89)	+0.04
		Experimental	Choline chloride (0.036M)	3.88 (2.61)	+1.27
		Experimental	Choline chloride (0.07M)	5.98 (3.31)	+2.67
Mature plants after flowering	48	Experimental	Choline chloride (0.036M)	5.68 (2.91)	+2.77

Table 2. *The betaine content of leaves of Beta vulgaris after infiltration with possible precursor substances*

The half-leaf method was used throughout these experiments and the half leaves were either placed in darkness or given a period of illumination (approx. 16 hr.) equivalent to a normal day during the experimental period. All infiltrated solutions 0.02M, except betaine aldehyde chloride, 0.05M. Values in parentheses refer to the control experiments in which water was infiltrated.

Age of plants (months)	Duration of experiment (hr.)	Illumination	Solution infiltrated	Betaine content (mg./g. dry wt.)	Δ (Betaine content) (mg./g. dry wt.)
3	72	{ Dark	Choline chloride	14.20 (11.80)	+2.4
		{ Light	Choline chloride	15.00 (9.28)	+5.72
3	72	{ Dark	Choline chloride	14.00 (10.20)	+3.80
		{ Light	Choline chloride	13.74 (7.17)	+6.57
3	48	Light	Choline chloride	6.87 (4.48)	+2.39
3	72	{ Dark	Glycine + methionine	10.10 (9.80)	+0.30
		{ Light	Glycine + methionine	8.75 (9.85)	-1.10
3	72	{ Dark	Dimethylglycine + methionine	9.56 (9.07)	+0.49
		{ Light	Dimethylglycine + methionine	12.00 (11.30)	+0.70
1-2	24	Light	Betaine aldehyde chloride	9.50 (8.50)	+1.0
1-2	24	{ Light	Choline chloride	16.20 (13.40)	+2.80
		{ Light	Betaine aldehyde chloride	15.70 (13.80)	+1.90
1-2	24	Light	Betaine aldehyde chloride	6.97 (5.92)	+1.05
1-2*	24	Light	Choline chloride	3.47 (3.03)	+0.44

* Leaves kept in atmosphere of nitrogen during experimental period.

Table 3. *The betaine content of leaves of Atriplex patula after infiltration with solutions of choline chloride*

Half leaves (Expts. 1-6) and entire leaves (Expts. 7, 8, 9) were infiltrated with 0.02M choline chloride and during the experimental period were either placed in darkness or given a period of illumination (approx. 16 hr.) equivalent to a normal day. Values in parentheses refer to the control experiments in which water was infiltrated.

Expt.	Source of leaves	Duration of experiment (hr.)	Illumination	Solution infiltrated	Betaine content (mg./g. dry wt.)	Δ (Betaine content) (mg./g. dry wt.)
1	Plants in early vegetative phase	48	{ Dark	Choline chloride	10.10 (9.00)	+1.10
			{ Light	Choline chloride	9.50 (7.65)	+1.85
2		48	{ Dark	Choline chloride	9.60 (6.90)	+2.70
			{ Light	Choline chloride	11.23 (7.07)	+4.16
3	Plants in late vegetative phase	24	{ Dark	Choline chloride	4.08 (3.13)	+0.95
			{ Light	Choline chloride	6.91 (4.70)	+2.21
4		48	{ Dark	Choline chloride	6.52 (4.94)	+1.58
			{ Light	Choline chloride	9.04 (5.88)	+3.16
5		6	Light	Choline chloride	7.86 (6.52)	+1.34
6		8	Light	Choline chloride	7.40 (6.52)	+0.88
7		24	{ Dark	Choline chloride	10.76 (8.46)	+2.30
			{ Light	Choline chloride	10.70 (6.74)	+3.96
8	Mature plants after flowering	24	{ Light	Choline chloride*	13.22	+0.78
			{ Light	Choline chloride†	12.94	+0.50
			{ Light	Choline chloride	13.82	+1.38
9†		24	Light	Choline chloride	3.86 (3.97)	-0.11

* 0.05M solution.

† 0.033M solution.

‡ Leaves kept in atmosphere of nitrogen during experimental period.

a biennial plant, the infiltration of choline into leaves of plants in the active vegetative phase of growth stimulates the formation of betaine to a considerable extent. Experiments on etiolated wheat seedlings to be described in a later paper have shown that no conversion of choline into betaine takes place when choline or its precursors are fed to the seedlings. Young seedling leaves of *B. vulgaris* have a comparatively low content of betaine and a progressive rise occurs with increasing age of the plants during the vegetative period. Similarly, young seedling leaves of *Chenopodium vulvaria* contain very little trimethylamine. The suggestion is put forward that the production of betaine in seedling plants does not take place to a great extent but as the seedlings grow to the mature plant, the capacity for production of betaine increases to a maximum. Kirkwood & Marion (1951) have shown that choline methyl groups are not utilized by sprouting barley to form the methyl group of hordenine, and Dubeck & Kirkwood (1952) found that the choline methyl group is not a precursor of the *N*-methyl group of ricinine in seedlings of *Ricinus communis* grown in darkness. Byerrum & Wing (1953), however, have shown that in young plants of *Nicotiana rustica* the methyl carbon of choline can be transferred to give the *N*-methyl carbon of nicotine, and these authors have suggested that, as in animal tissues, the compound which acts as the methyl donor in *Nicotiana* is betaine and that choline must first be oxidized to betaine before its methyl carbon becomes available for *N*-methylation. This suggestion receives support from the fact that betaine is known to be present in the leaves of *Nicotiana* spp. (Barger, 1914). Similarly Dubeck & Kirkwood (1952) have suggested that the inability of choline to contribute to the methyl groups of hordenine and ricinine in seedlings of *Hordeum* and *Ricinus* may be due to lack of the enzyme necessary to carry out the oxidation of choline to betaine. However, etiolated seedlings of *Hordeum* contain small amounts of betaine (Bregoff, Roberts & Delwiche, 1953) which (if betaine acts as a donor of methyl groups) should be available for transfer of methyl carbon to hordenine. If therefore the betaine of barley seedlings is derived from choline, the methyl carbon of choline would be expected to make some contribution to the *N*-methyl group of hordenine. It is noteworthy that in both sets of infiltration experiments, exposure of the leaves to light during the experimental period resulted in a higher betaine content than in leaves kept in darkness. On the assumption that the intracellular concentration of oxygen would be higher in leaves in which photosynthesis is taking place than in leaves kept in darkness, the higher conversion of choline into betaine in leaves exposed to light could be regarded as evidence in favour of

the view that betaine is formed by the oxidation of choline. Further evidence for oxidative conversion is given by the inhibition of betaine formation which took place when leaves infiltrated with choline were kept in an atmosphere of nitrogen during the experimental period and by the observation that infiltration of betaine aldehyde led to an increase in the betaine content of the leaves. Little or no evidence was obtained from the present experiments for the synthesis of betaine by the stepwise methylation of glycine.

Attempts were made to study the enzyme system involved in the conversion of choline into betaine in leaves by the preparation of tissue homogenates, but all preparations proved to be quite inactive even in the presence of added cytochrome *c* and various cofactors. It was concluded that breakdown of the cell structure resulted in the loss of essential components of the enzyme system or resulted in the production of inhibitors. It is hoped to carry out experiments on mitochondrial fractions of leaf tissues in an endeavour to make a further study of the enzyme system.

Preliminary experiments (Cromwell & Rennie, 1953*a*) which appeared to show that homogenates of roots of seedling plants of *Beta vulgaris* brought about the conversion of added choline into betaine were repeated and it was found that, despite the use of thymol as an antiseptic during the incubation period, bacterial activity was responsible for the conversion. When toluene was used in place of thymol no conversion of added choline took place either in homogenates of young roots of *Beta* or in homogenates of etiolated wheat seedlings. Homogenates sterilized by passage through a Seitz filter also failed to bring about the conversion of added choline into betaine. Accordingly, the suggestion made in an earlier paper (Cromwell & Rennie, 1953*b*) that betaine is synthesized in the root system of young seedlings of sugar beet and translocated to the shoot system is not supported by the results of experiments described in the present paper.

It has been stated that bacterial systems have provided no evidence for the formation of betaine from choline (Welch & Nicol, 1952) but work carried out in the present investigation has shown that certain Gram-negative bacteria, possibly belonging to the genera *Pseudomonas* and *Flavobacterium* isolated from non-sterile homogenates of wheat seedlings, are able to oxidize choline to betaine quite readily. Further work on the identification of these organisms is in progress.

SUMMARY

1. Experiments on the effects of infiltration of possible precursors of betaine into the leaves of *Beta vulgaris* and *Atriplex patula* have been described.

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*.