soon as it is formed. Combination of glutathione with lens protein as suggested by Hermann & Moses (1945) would also explain the maintenance of glutathione concentration at the expense of the protein —SH. A drop in thiol content, or, more accurately, a decrease in the intensity of the nitroprusside reaction has been reported many times, both in clinical and experimental cataract. This change has been attributed to a change in the glutathione concentration, but in view of the results given here it is suggested that this is secondary to a change in the protein of the lens.

This change in reducing sulphur is not, in itself, responsible for the formation of cataract as it occurred in animals that did not show lens changes. All the rabbits that developed cataract had a high blood-sugar level, but out of ten with high blood-sugar levels only five developed cataract. At present no known change either in the chemistry of the lens or in the general metabolism of alloxan-treated rabbits can be shown to lead in all cases to cataract formation. The results reported here showing that an

immediate change occurs in the lens proteins after injection of alloxan make it probable that other early changes also take place. Study of these may be expected to increase our knowledge of cataract formation more than studies on the cataractous lens itself.

SUMMARY

1. Clinical and biochemical changes occurring in the eyes of rabbits during alloxan diabetes are described.

2. An attempt is made to explain the reduction in the amount of protein —SH on the basis of the suggested sulphur-containing oxidation-reduction systems of the lens.

I wish to acknowledge with gratitude my indebtedness to Miss Ida Mann for her advice and encouragement during the early part of this work and for performing the iridectomy operations, and to Dr W. E. Gye for examining the histological specimens. I also wish to thank Dr A. Pirie for her continued guidance and helpful criticism throughout the whole of these investigations.

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The Micro-estimation and Origin of Trimethylamine in *Chenopodium vulvaria* L.

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The presence of trimethylamine in the tissues of *Chenopodium vulvaria* L. was first established by Dessaignes (1852). Further observations on the occurrence of this base in *Ch. vulvaria* were made by Wicke (1862) and Klein & Steiner (1928). Trimethylamine is present in small amounts in the flowers of many plants and occurs with methylamine in the rhizome of *Acorus Calamus* and in the young shoots

of *Mercurialis annua* (Guggenheim, 1940). Challinor (1913) has reported the occurrence of trimethylamine in the tissues of *Rhagodia hastata*, and Kapeller-Adler & Vering (1931) showed that the concentration of trimethylamine may reach the value of 500 mg./100 g. dry weight in the tissues of the red algae *Ceramium rubrum* and *Rhodomela subfusca*. Trimethylamine diffuses from innumerable glands

on the surface of the stems, leaves and perianth of *Ch. vulvaria*, thus distinguishing this species from most, if not all others, of the genus. The immediate precursor of trimethylamine in *Ch. vulvaria* may be either choline or betaine as both these substances are present in the free state in the tissues. Steele (1949) states that in *Ch. vulvaria* trimethylamine is a decomposition product of betaine, but experimental evidence in support of this statement appears to be lacking. The object of the present work was to determine the mode of formation of trimethylamine in this plant and to study the enzyme system involved. For the micro-estimation of trimethylamine in fish muscle, Dyer (1945) used a modification of the picrate method used by Richter, Lee & Hill (1941) for the determination of amines in blood. However, neither this method nor the titrimetric method gave entirely satisfactory results when applied to the determination of trimethylamine in the tissues of *Ch. vulvaria*, and it became necessary to develop a more specific micromethod for the estimation of this amine. Palumbo (1948) observed that tertiary amines in the presence of *cis*-aconitic anhydride dissolved in acetic anhydride give reddish purple colours, while ammonia and primary and secondary amines do not react. This reaction was studied and adapted for the colorimetric estimation of trimethylamine in *Ch. vulvaria*.

EXPERIMENTAL AND RESULTS

Reagents

Cis-aconitic anhydride. Prepared from aconitic acid by the method of Malachowski & Maslowski (1928). The product was purified by recrystallizing four times from hot benzene. A 2.5 % solution in glacial acetic acid was freshly prepared before use.

Glacial acetic acid, A.R.; acetic anhydride, A.R.; toluene, sulphur free; choline chloride; betaine hydrochloride; Na₂SO₄ (anhydrous), A.R.; K₂CO₃, A.R.

The micro-estimation of trimethylamine

Procedure. A solution (4 or 5 ml.) containing not more than 100 µg. of trimethylamine was run into a 25 ml. cylindrical separating funnel followed by 10 ml. of toluene. A saturated solution of K₂CO₃ (3 ml.) was added, the funnel shaken vigorously for 1 min. and allowed to stand for 5 min. The K₂CO₃ solution was run off and the toluene solution of trimethylamine poured into a test tube containing 0.5 g. anhydrous Na₂SO₄. The tube was corked and agitated for 10 sec. Into a second tube 0.1 ml. of the *cis*-aconitic anhydride reagent, 2 ml. of acetic anhydride and 5 ml. of the toluene solution were pipetted in this order. The solutions were thoroughly mixed and the tube, lightly corked, was placed in a constant-temperature water bath at 65° for exactly 10 min. The contents of the tube were cooled to room temperature and after an interval of 15 min. the colour was read in the Spekker absorptiometer using the Ilford spectrum yellow-green filter. A standard solution of pure trimethylamine hydrochloride was used for the calibration curve. Fig. 1 shows absorptiometer readings (extinctions, *E*) plotted against concentrations in µg. of trimethylamine.

Comments on the method

A solution of trimethylamine in toluene gives, with the *cis*-aconitic anhydride reagent and acetic anhydride at 65°, a claret colour the intensity of which is proportional to the concentration of trimethylamine between the limits of 5–50 µg./5 ml. of toluene solution. The colour is stable for at least 30 min. after development, but fades gradually on long

Fig. 1. Calibration curve obtained with a standard solution of trimethylamine hydrochloride. (Drum of absorptiometer set to 1 with toluene blank. 0.5 cm. cells.)

standing. Other tertiary amines give red or purple colours with the reagent and, if not removed, interfere in the determination of trimethylamine. It has been found, however, that distillation at low temperature under reduced pressure removes trimethylamine from most of the tertiary amines likely to interfere. Toluene of the grade specified gives a faint pink colour with the reagent and it is necessary, therefore, to run a control blank with each set of estimations. It is important to observe the details of time and temperature in the development of the colour and for each fresh batch of reagents a calibration curve is essential. The solution of trimethylamine in toluene should be added as soon as possible to the mixture of the *cis*-aconitic anhydride reagent and acetic anhydride, otherwise a red colour gradually develops in the mixture on long standing at room temperature. Immediate addition of the toluene solution prevents the development of the colour.

Application of the method to the determination of trimethylamine in *Chenopodium vulvaria* L.

Accurate estimation of trimethylamine in *Ch. vulvaria* is rendered difficult by the fact that this volatile base diffuses freely from the epidermal glands. These glands are very delicate and are easily ruptured on handling. The factors controlling the liberation of trimethylamine from the glands have not yet been fully determined, but it has been observed that atmospheric humidity and temperature are of importance in this connexion. Furthermore, the pH value of the epidermal cells and leaf tissues was found to be 6.5, and that of the glands 8.2. Variation in the pH value of the glands may occur, however, and thus influence the rate of diffusion of trimethylamine from the glands.

Drying of the tissue results in considerable loss of trimethylamine, therefore it is essential to use fresh tissues for analysis. A preliminary examination of the trimethylamine content of whole leaves, and leaves from which the glands had been carefully removed with a soft brush, was made as follows. Leaves (0.5 g.) were detached with forceps from individual plants (raised from seed) at two stages of growth, i.e. young plants showing active vegetative growth and fully mature plants in flower. The leaves were ground to a paste in a mortar with 1 ml. *N*-HCl, 10 ml. distilled water were added and the extract transferred to the vacuum distillation apparatus. Antifoam solution (10 g. paraffin, m.p. 54°, in 100 ml. toluene) (1 ml.) and excess $Mg(OH)_2$ were added and the mixture distilled under reduced pressure at 40° into standard acid (5 ml.) until the total volume of the distillate reached 20 ml. The rate of distillation was controlled to give 15 ml. of distillate in 30 min. The distillate was made up to 25 or 50 ml. and a sample of 4 ml. taken for analysis.

conical flasks (*a-f*) and 3 ml. 0.1 M-phosphate buffer of the required pH were added. Flasks *a-d* each received 1 ml. of a 0.01 M-solution of choline chloride or betaine hydrochloride (previously neutralized to pH 7). Before the addition of buffer and substrate, the enzyme solution in flasks *c* and *d* was heated to 100° and cooled. Flasks *e* and *f* were used as controls. A crystal of thymol was added to each flask and the solutions allowed to stand at 24° for 48 hr. After this period the contents of each flask were transferred to the distillation apparatus, 1 ml. antifoam solution and excess of $Mg(OH)_2$ added, and distillation carried out for 20 min. under reduced pressure at 40° into 5 ml. standard acid. The distillate was made up to 25 ml. and 4 ml. were taken for the determination of trimethylamine.

The results of this experiment, which are set out in Table 2, show that trimethylamine is produced by the enzyme preparation at pH 7.8, but not at pH 6 from choline. Apparently no trimethylamine is

Table 1. *Trimethylamine content of leaves of Chenopodium vulvaria L.*

Plant no.	Trimethylamine ($\mu g./0.5$ g. wet wt.)					
	Young plants			Plants in flower		
	Intact leaves	Leaves without glands	Glands	Intact leaves	Leaves without glands	Glands
1	850	265	585	425	125	300
2	818	221	597	470	137	333
3	740	218	522	580	145	435
4	890	282	608	326	102	224
5	730	248	482	410	116	294
6	805	260	545	370	110	260
Mean	805	249	556	430	122	308

Table 1 shows the values for trimethylamine in intact leaves, leaves from which the glands had been removed, and (by difference) the glands.

The origin of trimethylamine in Chenopodium vulvaria L.

Enzymic breakdown of choline by certain members of the Enterobacteriaceae has been reported by Dyer & Wood (1947). These workers showed that trimethylamine is produced at pH 7.2 from choline and acetylcholine, but not from betaine. Cohen, Nisman & Raynaud (1947) found that certain species of *Clostridium* break down choline with the production of trimethylamine, and ethanolamine with the production of ammonia and ethylene glycol.

Preparation of an active enzyme extract from leaves of Chenopodium vulvaria L. The leaves (50–100 g.) were frozen with solid CO_2 , allowed to thaw and pressed in a powerful hydraulic press. The press cake was allowed to imbibe 100 ml. of 5% (v/v) glycerol solution and again pressed out. The press juice and glycerol extract were combined (130 ml.) and dialysed against distilled water at 2° for 18 hr. At the completion of dialysis the enzyme extract contained small amounts only of choline, betaine and trimethylamine. The enzyme preparation (2 ml.) was placed in each of six 25 ml.

formed from betaine. The specificity of action of the enzyme was examined by substitution of ethanolamine, *N*-methylethanolamine and dimethylethanolamine for choline in the above experiment. The

Table 2. *Production of trimethylamine from choline and betaine by an enzyme preparation from leaves of Chenopodium vulvaria L.*

(Flasks *a-d* contained either choline or betaine, enzyme solution and buffer; flasks *e* and *f* contained enzyme solution and buffer only; flasks *c* and *d* were heated to 100° and cooled.)

Flask	...	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>
pH	...	7.8	6.0	7.8	6.0	7.8	6.0
Trimethylamine ($\mu g./2$ ml. distillate mean value of 3 experiments)							
Choline		53	9	12	9	6	6
Betaine		8	7	8	8	6	6

enzyme was without action on these amines. An extract prepared in the same manner from leaves of *Ch. album* L. was used in a parallel experiment. Trimethylamine did not appear as a breakdown

product of either choline or betaine. Glycollic acid could not be detected as a product of the action of the enzyme on choline, but the presence of ethylene glycol was established by periodate oxidation to formaldehyde which was detected by the Schiff reaction (Feigl, 1946). It was not possible to isolate a derivative of this alcohol. The detection of formaldehyde after periodate oxidation indicates that the enzyme is a choline aminase similar to that found by Cohen *et al.* (1947) in bacteria. Attempts to purify the enzyme by precipitation with acetone and ammonium sulphate resulted in complete inactivation.

DISCUSSION

In a previous publication (Cromwell, 1949), a scheme showing the possible reactions leading to the formation of methylated amines in plants was formulated, and it was suggested that trimethylamine is derived from choline. The results of the present work show that in *Ch. vulvaria* enzymic breakdown of choline takes place with the production of trimethylamine. Betaine, which has also been considered to undergo breakdown with the formation of trimethylamine, is not attacked. Preliminary examination of the leaves of *Ch. vulvaria* at different stages of development of the plant shows that production of trimethylamine is greatest during the phase of active vegetative growth. As the plant reaches maturity the production of trimethylamine declines. Ducet (1948) has shown that during vegetative growth of the soya bean there is a tendency for synthesis of phospholipins to take place at the expense of soluble choline (free choline plus glycerophosphoryl choline), whereas during maturation of the seed, degradation of phospholipins takes place with the liberation of choline. If, as in Soya, the content of free choline in *Ch. vulvaria* is diminished during vegetative growth by synthesis of phospholipin it would be expected that as the plant reaches maturity the amount of choline available for the production of trimethylamine would show a progressive decline. The limited scope of the present experiments, however, makes it impossible to decide whether free choline is attacked by the enzyme before synthesis of phospholipins takes place, or whether degradation of phospholipins yields the choline, which is subsequently broken down to trimethylamine. Hanahan & Chaikoff (1947, 1948) have shown that an enzyme capable of degrading phospholipins with the production of choline is present in the leaves of the carrot and cabbage. If choline acts as a methyl donor in plants

its capacity to do so in *Ch. vulvaria* must be restricted by its tendency to lose all three methyl groups in combination with nitrogen as trimethylamine. On the other hand, betaine, which does not undergo breakdown to trimethylamine, may act as the donor of one methyl group in the process of trimethylation. In the other species of *Chenopodium* examined, trimethylamine does not occur as a breakdown product of choline. However, it is interesting to note that glands similar to those of *Ch. vulvaria* are present on the leaves of *Ch. album* and *Ch. glaucum*. Dyer & Wood (1947) found that, in the absence of available carbohydrate, organic acid was produced in the bacterial metabolism of choline. These workers suggest that the alcoholic side chain of choline is oxidized serving as a source of energy and trimethylamine is a by-product of the reaction. Ehrlich & Lange (1913) have shown that the yeast *Willia anomala* Hansen (= *Hansenula anomala* Hansen Syd.) is capable of growth when supplied with betaine as the sole source of nitrogen. Glycollic acid was produced in the culture medium, but trimethylamine was not detected. It is presumed that either trimethylamine was produced and immediately metabolized with the production of ammonia, or degradation of betaine took place by way of dimethylglycine with ultimate production of ammonia. In the experiments of Ehrlich & Lange (1913) and of Dyer & Wood (1947) the organisms were cultivated under abnormal conditions, the media lacking carbohydrate or a normal source of nitrogen. In *Ch. vulvaria* trimethylamine production, with consequent loss to the plant of nitrogen, is a feature of normal metabolism. Further work is in progress which, it is hoped, will throw more light on the nitrogen metabolism of this remarkable plant.

SUMMARY

1. A sensitive method for the micro-estimation of trimethylamine has been developed and employed for the estimation of trimethylamine in the leaves of *Chenopodium vulvaria* L.
2. An enzyme preparation from the leaves of *Ch. vulvaria* L. has been shown to cause breakdown, at pH 7.8, of added choline with the liberation of trimethylamine.
3. Betaine does not yield trimethylamine when added to this enzyme preparation.
4. The enzyme is inactivated by precipitation with ammonium sulphate and acetone and is sensitive to heat and changes of pH.

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The Synthesis of Peptides Related to Gramicidin S and the Significance of Optical Configuration in Antibiotic Peptides

2. PENTAPEPTIDES

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In Part 1 (Harris & Work, 1950) we reported that esters of both the tripeptides L-leucyl-L-phenylalanyl-L-proline and L-leucyl-D-phenylalanyl-L-proline synthesized by us showed some antibacterial action, but that they were very much less active than the natural antibiotic, gramicidin S, upon which they were modelled. Since there was no clear-cut distinction between the activities of the two optically isomeric peptides, it was decided to extend the peptide chain and to synthesize a pentapeptide containing all the amino-acid constituents of gramicidin S in their proper sequence and optical form, L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-(II). For comparison with this peptide we synthesized the optically isomeric pentapeptide containing L-phenylalanine in place of D-phenylalanine. By comparison of the antibacterial activity of these two peptides we hoped to reveal the part played by D-phenylalanine in the production of antibiotic action.

Our two isomeric open-chain pentapeptides, although comparable one with the other, are not quite comparable with gramicidin S since they possess, in addition to the free δ -amino group of the ornithine residue, a free carboxyl and a free α -amino group, both of which are absent from the natural antibiotic. The absence of reactive end groups in gramicidin S is most satisfactorily explained by the

assumption of a cyclic structure. We scarcely hoped to achieve controlled cyclization of so complex a molecule as our synthetic 'D' pentapeptide and therefore approached the problem by synthesis of an acylpentapeptide amide, α -(p-toluenesulphonyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline-amide). This compound (I) was comparable to gramicidin S (II) in that it possessed the correct amino-acid sequence, the correct optical form and the desired absence of reactive end groups.

For comparison with the acylpentapeptide possessing a free δ -NH₂ on the ornithine residue, we intended at first to prepare the corresponding pentapeptide with the α -NH₂ of valine free and the δ -NH₂ of ornithine acylated, but, as explained below, this last project was abandoned in view of unexpected experimental difficulties and because of the limited biological activity of our first acylpentapeptide amide.

The choice of the sequence valylornithylleucyl-phenylalanylprolyl was dictated by experimental rather than theoretical considerations. Since gramicidin S is believed to be cyclic, any one of the five constituent amino-acids can be terminal in an open-chain analogue. Preliminary experiments indicated that some of the possible intermediates would be easier to synthesize and purify than others, and the sequence finally chosen was that which seemed to