dioxide whereas, in its natural environment, the rumen of the sheep, the carbon dioxide tension is high, and this must be borne in mind in any assessment of the role it plays in the rumen.

SUMMARY

1. Washed suspensions of LC, a Gram-negative coccus isolated from the rumen of sheep, attack glucose, fructose, sorbitol, mannitol, lactate, pyruvate, DL-serine and formate anaerobically with the production of carbon dioxide and a gas which we assume to be hydrogen.

2. When pyruvate is fermented under an atmosphere of hydrogen there is an initial outburst of hydrogen followed by a consumption of the gas.

3. Addition of either acetate, propionate, or *n*-butyrate to pyruvate fermentations increases the uptake of hydrogen with increased formation of *n*-butyrate, *n*-pentanoate and *n*-hexanoate respectively.

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The Biosynthesis and Metabolism of Betaines in Plants

1. THE ESTIMATION AND DISTRIBUTION OF GLYCINEBETAINE (BETAINE) IN *BETA VULGARIS* L. AND OTHER PLANTS

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Betaine is found in a wide variety of plants (Guggenheim, 1940) and occurs in relatively high concentration in the tissues of members of the Chenopodiaceae and the Gramineae and also in *Lycium chinense* Mill. (Solanaceae), and *Vicia* species of the Leguminosae. Betaine occurs in all organs of these plants, the highest values usually being found in the leaves. In animal tissues betaine is formed by the enzymic oxidation of choline (Mann & Quastel, 1937) and plays an important part in the process of transmethylation (Muntz, 1950). It was therefore of interest to study the biosynthesis and metabolism of this compound and of other betaines in the plants in which they normally occur.

Since betaine is a very stable substance, methods available for its estimation have been restricted to the use of non-specific precipitants such as potassium triiodide (Staněk, 1904; Blood & Cranfield, 1936; Reifer, 1941), phosphotungstic acid (Davies & Dowden, 1936) and ammonium reineckate (Walker & Erlandsen, 1951). For the purposes of the present

investigation an accurate and reasonably rapid method for the estimation of betaine in plant tissues was desirable and since none of the above methods proved reliable when applied to young actively growing tissues, attention was directed to the modification and improvement of the reineckate method of Walker & Erlandsen (1951). This method is based upon the fact that betaine, being a very weak base, forms with strong acids salts which can be titrated with alkali. Walker & Erlandsen (1951), in the application of their method to beet molasses and diffusion juices, first precipitated betaine as the reineckate which was filtered off and dissolved in aqueous acetone. The reineckate ion was then removed with silver nitrate and the betaine nitrate thus formed was titrated with alkali to pH 6 with methyl red as indicator. The authors state that although choline and other basic nitrogenous substances are precipitated by ammonium reineckate, the salts formed on treatment of the precipitate with silver nitrate are not sufficiently acidic to be titrated with alkali to pH 6.

When this method was applied to purified extracts of tissues of young roots and seedlings it became apparent that other basic substances were present which were coprecipitated with betaine when the extracts were treated with ammonium reineckate at pH 1. The reineckates of some of these substances gave acidic salts when the reineckate ion was removed with silver nitrate and were thus included in the estimation as betaine. It was found that most of the interfering substances could be effectively removed by treatment of the plant extracts with silver oxide according to the procedure of Herbst & Clarke (1934), who showed that amino acids and partially methylated amino acids, but not betaine, undergo oxidative deamination when boiled with silver oxide.

EXPERIMENTAL AND RESULTS

The estimation of betaine in plant tissues

The plant material was dried at 80° in an oven with a forced draught, finely powdered, and 0·1–1·0 g. was weighed into the extraction thimble of a micro-Soxhlet apparatus and extracted with methanol for 1 hr. The extract was transferred to a boiling tube, small pieces of porous pot were added to prevent bumping and the methanol removed at 80° on a water bath. The residue was taken up with distilled water (5 ml.) and powdered activated charcoal (0·5–2 g.) was added according to the amount of plant material originally weighed out. The solution was brought almost to boiling and filtered through a sintered-glass crucible, the disk of which was covered with a thin layer of Gooch asbestos. The colourless filtrate was collected in a test tube and the boiling tube rinsed three times with 2 ml. portions of hot water which were run through the crucible and added to the filtrate which was then transferred quantitatively to a small evaporating dish and concentrated on a boiling-water bath to approx. 3 ml. The concentrated solution was returned to the test tube and the basin washed twice with 1 ml. portions of water giving a total volume of 5 ml. The pH of the solution was adjusted to 1 (approx.) by addition of 0·05 ml. of 10 N-HCl, and 10 ml. of a saturated solution of ammonium reineckate (previously adjusted to pH 1) were added and the tube kept at 4° for 1 hr.

The reineckate precipitate was filtered off in a chilled sintered-glass crucible of medium porosity and washed four times with 2 ml. portions of water-saturated ether. The underside of the crucible was dried and the precipitate dissolved in 75 % (v/v) acetone (5 ml.). The reineckate ion was next removed by the addition of 5 ml. of $AgNO_3$ reagent (a solution 0·1 N with respect to both $AgNO_3$ and $NaNO_3$), the precipitate of silver reineckate centrifuged off and the clear supernatant decanted into a boiling tube. The precipitate was washed twice with 5 ml. portions of $AgNO_3$ reagent and twice with 5 ml. portions of distilled water, and the washings added to the boiling tube together with small pieces of porous pot. The boiling tube was warmed gently to remove acetone and 4 ml. of 0·2 N-NaOH were slowly added and the solution boiled gently until the volume was reduced to 5 ml. (approx.). After cooling, 0·15 ml. 10 N-HCl was added and the precipitate of AgCl removed by filtration through a sintered-glass crucible, the disk of which was covered with Gooch asbestos. The AgCl precipitate was washed three times with 2 ml. portions of water and the combined filtrate and washings were concentrated to 3 ml. (approx.) in a small basin on the water bath and returned to the test tube. The basin was washed twice with 1 ml. portions of water and the washings added to the test tube to make a total volume of 5 ml. (approx.). The pH was adjusted to 1, 10 ml. of saturated ammonium reineckate solution were added and the tube kept at 4° for 1 hr. The precipitate was filtered off in a chilled sintered-glass crucible of medium porosity and washed with 3 ml. portions of water-saturated ether until the washings were acid-free. The betaine reineckate was dissolved in 75 % acetone (v/v) and the solution collected in a conical centrifuge tube of 10 ml. capacity. Next 5 ml. of $AgNO_3$ reagent were added and the silver reineckate removed by centrifugation. The supernatant was carefully decanted off into a 100 ml. conical flask and the silver reineckate was washed free of betaine nitrate with 5 ml. portions of distilled water to a total volume of 40 ml. (filtrate + washings). To the combined filtrate and washings were added 4 drops of 0·1 % methyl red dissolved in ethanol and the solution was titrated to pH 6 with 0·02 N-diphenylguanidine in 70 % ethanol: 1 m-equiv. of betaine nitrate = 1 m-equiv. of diphenylguanidine. (The stock solution of 0·05 N-diphenylguanidine in 70 % ethanol was prepared from diphenylguanidine twice recrystallized from boiling toluene (Carlton, 1922).)

It will be observed in the procedure for removal of interfering substances that Ag_2O is produced from the excess of $AgNO_3$ remaining after removal of the reineckate ion as silver reineckate. The amount of NaOH added is carefully adjusted so that a slight excess of unchanged $AgNO_3$ remains in the solution. The pH of the solution is thus maintained at a point near neutrality, a condition essential for complete oxidation of amino acids and partially methylated amino acids (Herbst & Clarke, 1934). Guanine and adenine, if present, are coprecipitated with betaine but are removed as insoluble silver compounds when the crude reineckate precipitate is dissolved in aqueous acetone and treated with $AgNO_3$. Other betaines such as trigonelline, stachydrine

and betonicine are unaffected by treatment with Ag_2O and if present, will give relatively insoluble precipitates with ammonium reineckate at pH 1. On removal of the reineckate ion with silver nitrate, the nitrates of these betaines will give acidic solutions and will be estimated as betaine. Caution is therefore necessary in the interpretation of results if the presence of other betaines has been established by preliminary examination of tissue extracts by means of paper chromatography: the melting point and chromium content of the purified betaine reineckate were therefore used as criteria of purity. The method gives the most satisfactory results when the amount of betaine in the sample of plant material taken for analysis lies within the range 1–10 mg. A recovery of 99–101 % was obtained when known amounts of betaine were added to tissue extracts and to mixtures of amino acids and purines.

Application of the method to the determination of betaine in Beta vulgaris *L., and other plants*

As soon as possible after harvesting, the plant material was dried and stored in a desiccator until required for analysis. Except where otherwise stated, all leaves (with petioles) of the shoot system of individual plants were included in the sample taken for analysis. Table 1 shows the values for betaine (mean values of two estimations) in plants of *Beta vulgaris* L., *Atriplex patula* L., and *A. hortensis* L., in progressive stages of development. In Table 2 the values for betaine in various other plants are given.

DISCUSSION

Staněk (1906, 1911), as the result of an investigation of the betaine content of a variety of plants, concluded that young actively growing tissues show a higher concentration than mature tissues. The results of the present investigation substantially support the findings of Staněk with the exception that leaves of young seedling plants showed a lower betaine content than the leaves of older plants. In fact, the betaine content of the whole leaf system of young plants increased throughout the vegetative

Table 1. *The betaine content of tissues of* Beta vulgaris *L.,* Atriplex patula *L. and* Atriplex hortensis *L. during the growth period*

Plant	Age (days)	Betaine content (g./100 g. dry wt.) Leaf	Petiole	Root
Beta vulgaris	25	0·25	—	—
	33	0·36	—	1·27
	44	0·48	—	—
	56	0·64	—	—
	62	0·84	—	1·15
	75	0·87	—	—
	81	1·02	—	—
	96	1·32	1·09	—
	132	1·86	1·65	0·79
	140	2·20	—	—
	167	2·55 (outer leaves) 3·34 (middle leaves) 4·85 (inner leaves)	1·08 (upper half) 0·99 (lower half)	0·18
Atriplex patula	30	1·32	—	—
	60	1·71	—	—
	80	2·64 (plants in bud)	—	—
	90	2·48 (plants in flower)	—	—
	120	1·84 (plants fruiting)	—	—
A. hortensis	39	0·78	—	—
	48	1·04	—	—
	107	0·99 (old leaves) 1·26 (young leaves)	—	—

Table 2. *The betaine content of miscellaneous plants*

Plant	Material	Betaine content (g./100 g. dry wt.)
Spinacea oleracea L.	Leaves (35 days)	0·27
Chenopodium vulvaria L.	Leaves (mature)	1·86
C. bonus-henricus L.	Leaves (mature)	2·23
Atriplex canescens James	Leaves (mature)	3·27
A. halimus L.	Leaves (mature)	4·90
Amaranthus caudatus L.	Leaves (mature)	0·54
Lycium chinense Mill.	Leaves (mature)	1·95
Vicia faba L. Seedlings germinated in dark	Cotyledons	0·22
	Shoots	1·47
	Roots	1·10
Wheat (*Triticum vulgare* Vill. var. Atle) Etiolated seedlings (8 days)	Coleoptiles	0·83
	Roots	0·42
Beta vulgaris L. Etiolated leaves from mature roots	—	5·00

period and reached a maximum at the time of flowering. Moreover, young seedling leaves of *Beta vulgaris* contained much less betaine than young actively growing leaves sprouting from the crown of a mature root. A concentration gradient of betaine is established during the vegetative phase of growth of the plants examined, the betaine content rising steadily from the root to the leaf. On the contrary, the young unfolding leaves at the centre of the crown of the mature root contain more betaine than the older leaves at the periphery of the crown. Leaves of the shrubby evergreen species of Atriplex (*A. halimus* and *A. canescens*) are noteworthy for their high content of betaine. Care is necessary in the interpretation of results of the analysis of young as compared with mature tissues when dry weight is taken as the basis of calculation.

However, an explanation of the above results is possible in the light of recent work which has been carried out on the formation of betaine in *Beta vulgaris* (Cromwell & Rennie, 1953). This work has shown that betaine is formed in the root tissues by the enzymic oxidation of choline. Since choline oxidase is not present in the leaf tissues it seems likely that betaine must have been translocated from the roots to the leaves. Failing the utilization of betaine in metabolic processes, it would be expected that, with continuous production in the root, a gradual accumulation of betaine would take place in the shoot system during the growing period. Similarly, the high concentration of betaine in the young leaves growing from the centre of the crown of a mature root could be explained as due to translocation from a relatively large mass of root tissue which in the resting condition contains a considerable amount of betaine. The high concentration of betaine in the leaves of the perennial evergreen species likewise could be explained as the result of a slow accumulation over a period of time.

SUMMARY

1. A method for the estimation of betaine in plant tissues is described.

2. This method incorporates an oxidation procedure for the removal of substances which are present in young tissues and which interfere in the estimation of betaine by the reineckate method.

3. The improved method has been employed for the estimation of betaine in organs of various plants in all stages of development, and the conclusion is reached that betaine formed in the roots is translocated to the shoot system and accumulates in the stems and leaves.

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