and the osmotically determined value of the molecular weight, together with an assumed value of 0·75 for the partial specific volume, allow the frictional ratio f/f_0 (Svedberg & Pedersen, 1940) to be calculated: the value obtained is 1·38. Comparison with the data for myoglobin (Theorell, 1932) shows a marked difference: myoglobin has a molecular weight of about 17,000, a sedimentation constant of $2·04 \times 10^{-13}$ and is more nearly spherical having $f/f_0 = 1·1$.

SUMMARY

1. Preliminary measurements of the osmotic pressure of methaemoglobin prepared from the tracheal cells of the larva of *Gastrophilus* (the bot fly) yield a mean value of 34,000 ± 3000 for the molecular weight.

2. The material was found to sediment homogeneously in the ultracentrifuge, with sedimentation constant $2·5 \times 10^{-13}$.

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The Estimation of Free Choline in Plants

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Choline, being a constituent of lecithin, is probably present in every living cell. Schulze & Trier (1912*b*) and Klein & Zeller (1930) have examined more than 100 plants of widely different systematic origin and, with the exception of the three lichens examined, were able to detect free choline in all cases.

In the animal choline has lipotropic activity; it promotes phospholipin turn-over, prevents the development of fatty liver, protects against haemorrhagic kidney degeneration, and is concerned in the conversion of homocystine to methionine. Its vitamin-like action may be related to its activity as a methyl donator (du Vigneaud, Chandler, Moyer & Keppel, 1939; du Vigneaud, Chandler, Cohn & Brown, 1940; Chandler & du Vigneaud, 1940). The significance of choline in the methylation processes of higher plants has not hitherto been investigated. Klein & Linser (1932, 1933) have opened up the subject of choline metabolism in higher plants by their study of variations of lecithin and choline contents during germination of various seeds.

The present paper describes a method suitable for routine estimations of the free choline content of plant tissues in metabolic studies. Preliminary data on the distribution of free choline in the potato plant are presented.

Quantitative estimation of choline

Estimations of choline in animal tissues have been based upon the formation of the sparingly soluble choline periodide, bismuth-iodide, platinichloride, aurichloride or reineckate; upon the decomposition of choline into trimethylamine or upon acetylation followed by biological assay.

Roman (1930) has made a careful study of the conditions necessary for the quantitative precipitation of choline as periodide (ennea-iodide) and found that, taking all precautions, it was possible to estimate 0·005–5 mg. choline in pure aqueous solution with an error of less than ±5%. The achievement of quantitative yields is, however, made a matter of considerable difficulty by variability in the composition of the precipitate, the extreme readiness with which it looses iodine and its ready solubility in potassium iodide. The application of the method to plant extracts encounters the additional difficulties that iodine will give precipitates with alkaloids and many other bases, a difficulty not satisfactorily prevented by addition of sodium bicarbonate (Schulze & Trier, 1912*a*; Vickery, 1925), and that the solubility of choline periodide is, in general, increased by the presence of salts. Methods involving potassium bismuth iodide (Jahns, 1885) as precipitant suffer from similar difficulties. Strack & Schwaneberg (1937) have noted that even pure bases can, under certain conditions, give an atypical gold salt and that from mixed solutions mixed salts are precipitated, so that gold chloride is unsuitable for the precipitation, identification or estimation of bases. Precipitation

of plant extracts by gold, platinum or mercuric chlorides would yield complex mixtures from which the choline derivative could only be separated by further reactions and extensive crystallizations.

Methods of estimation based upon precipitation of choline as reineckate have been described (Kapfhammer & Bischoff, 1930; Beattie, 1936; Johnston, Irvin & Walton, 1939; Jacobi, Baumann & Meck, 1941; Engel, 1942). Strack & Schwaneberg (1937) and Neuberger & Sanger (1942) have pointed out that whilst all basic substances give reineckates relatively insoluble at acid or sometimes even at neutral or mildly alkaline reactions, only quaternary bases without a carboxyl group such as choline and methylpyridinium chloride give reineckates insoluble at pH 12–13. In our experience, however, aqueous plant extracts treated with reineckate at pH 12 yield precipitates so intractable to filtration that decomposition occurs on the filter. Previously described methods (Beattie, 1936; Johnston *et al.* 1939; Miller & Lowe, 1940) for estimation of choline content of the reineckate precipitate are applicable only with pure choline reineckate, or choline reineckate contaminated with traces of non-basic material and are therefore not directly applicable to the precipitates obtained by treating plant extracts with ammonium reineckate at neutral reaction.

Gulewitsch (1898) found that when choline is treated with either conc. KOH solution or moist Ag_2O it underwent a hydramine fission with formation of trimethylamine. Klein & Zeller (1930) and Klein & Linser (1932) describe a method of estimating choline by decomposition with boiling concentrated alkali and distillation and titration of the trimethylamine formed. The method involves treatment of the plant extract for 3 hr. with 33% (w/v) NaOH at high temperatures (up to 180°). These conditions cause profound changes in plant extracts, and betaine, one of the most resistant bases likely to be encountered, is partially decomposed into trimethylamine. An estimate of the ammonia formed must be made and applied as a correction. Using a pure choline solution the error with 15 mg. choline was ±10%, and with 1 mg. ±30%. Klein & Linser (1932) attempted to avoid interference from alkaloids and other bases by choosing plants from which these substances were regarded as absent.

Lintzel & Fomin (1931*a*) have studied the oxidative decomposition of choline and of a number of allied substances using permanganate in acid and alkaline solution. Choline when treated with alkaline permanganate yielded trimethylamine and glycollic acid:

$$CH_2(OH).CH_2.N(CH_3)_3OH \xrightarrow{O_2} CH_2(OH).COOH + N(CH_3)_3.$$

Trimethylamine was similarly produced by alkaline oxidation of acetylcholine, neurin and carnitine but neither betaine nor γ-butyro-betaine were attacked. Neurin can, however, be distinguished from choline as it yields trimethylamine when treated with acid permanganate, whereas, under these conditions, choline is oxidized to betaine. Neither neurin nor carnitine have hitherto been reported as constituents of higher plants. Lintzel & Fomin (1931*b*) and Lintzel & Monasterio (1931) were therefore able to elaborate methods of estimation based upon the almost quantitative formation of trimethylamine resulting from treatment of choline solutions with dilute alkaline permanganate under carefully controlled conditions. The method of Lintzel & Fomin (1931*b*), however, involves evaporation of the acid distillate containing the trimethylamine to small bulk on a water-bath prior to its estimation. Poor yields were obtained in preliminary trials of this method, and it was shown that this was due to inevitable and variable loss of trimethylamine during the evaporation. The procedure of Lintzel & Monasterio (1931) has formed the basis of the method of estimation of choline in crude plant extracts described below.

EXPERIMENTAL

Procedure 1. *Estimation of choline in the form of its hydrochloride, reineckate or mercurichloride*

The apparatus used is shown in Fig. 1. The procedure is as follows: The choline derivative with 25 ml. water is contained in flask *A*. The ammonia

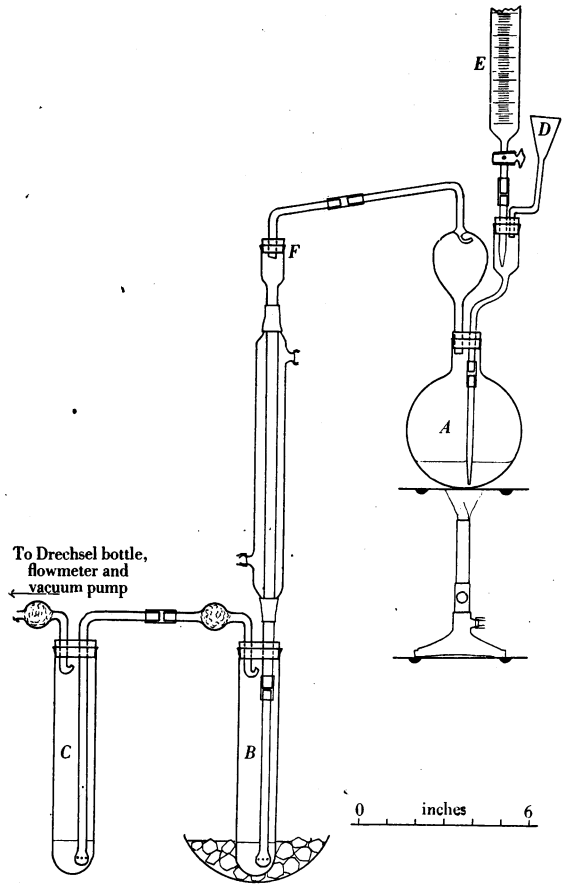

Fig. 1. Apparatus for estimation of choline. *A*, reaction flask; *B* and *C*, ammonia-traps charged with alkaline formaldehyde; *D*, alkali and air inlet; *E*, burette charged with $KMnO_4$ solution.

traps (*B* and *C*) are each charged with 10 ml. 40% formaldehyde, 5 ml. 40% (w/v) NaOH and a drop of caprylic alcohol. No liquid is allowed to splash on to the inner walls of the tubes. Both tubes are cooled on ice immediately after charging and the

lower part of the tube *B* is surrounded by ice during the distillation. The trimethylamine is trapped in a Drechsel bottle containing 5, 10 or 15 ml. 0·02 N-H_2SO_4. A gentle stream of air (20 l./hr.) is drawn through the apparatus, and 10 ml. 40 % (w/v) NaOH is pipetted into flask *A* via *D*, the contents of flask *A* heated to boiling, the derivative decomposed and then the addition of the 0·5 % (w/v) $KMnO_4$ commenced from the burette *E*. The $KMnO_4$ is added dropwise at a rate commencing at 0·5 ml./min. and increasing gradually to 2·0 ml./min. The addition of the $KMnO_4$ should be completed in 15–30 min. and the mixture in flask *A* is gently boiled during this period. Completion of the oxidation is marked by a green colour clearly visible at the surface of the boiling liquid and persisting for at least 1 min. after stopping the addition of $KMnO_4$. The top of the condenser is now disconnected at *F*, the inner tube washed down with 10 ml. 40 % (w/v) NaOH, the ice removed from around tube *B*, and the aeration rate raised to 45 l./hr. and maintained at that rate for a further 1 hr. The excess 0·02 N-H_2SO_4 in the Drechsel bottle is immediately titrated with Tashiro's indicator (see Steward & Street, 1946) against a standard approx. N/50 solution of trimethylamine delivered from a CO_2-free micro-burette. With yields of trimethylamine ranging from 0·14 to 3·2 mg. N duplicates agreed to 0·05 ml. 0·02 N-trimethylamine (0·014 mg. N).

The above procedure must be strictly observed. The concentration or rate of addition of $KMnO_4$ must not be increased. Formation of spray on the walls of the ammonia traps renders the aeration of the trimethylamine incomplete. The excess acid must be immediately titrated, since retained formaldehyde gradually oxidizes to formic acid. Lintzel & Monasterio (1931) used phenolphthalein as indicator because of its relative insensitivity to ammonia. The present procedure completely removes ammonia from the air stream even when the equivalent of 140 mg. NH_3-N is liberated in flask *A* and thereby makes possible the use of Tashiro's indicator giving a sharper end-point in the trimethylamine-H_2SO_4 titration.

Table 1. *Recovery of choline*-N *as trimethylamine*

Derivative of choline	Range of choline-N (mg.)	Recovery of N as trimethylamine (%)
	Procedure 1	
Mercurichloride	0·79–1·1	87·0, 86·1, 85·9, 87·2
Mercurichloride	3·00–3·3	96·4, 96·2, 95·1, 96·9
Reineckate	0·80–1·1	85·7, 84·6, 83·7, 85·1
Chloride	0·80–1·0	85·0, 84·1, 83·2, 85·2
Procedure 1 but with traps *B* and *C* charged with alkali only		
Mercurichloride	0·79–1·1	96·5, 98·8, 98·1, 97·4
Mercurichloride	3·0 –3·3	99·6, 100·4, 99·3, 98·9

Preliminary trials with this procedure gave the yields shown in Table 1.

Formation of ammonia during oxidation. The yields of trimethylamine shown in Table 1 fell short of the theoretical by some 14–15 % with quantities of about 1 mg. N and by some 3–5 % with quantities of about 3 mg. N. Substitution of water for formaldehyde in the ammonia traps raised the yields almost to theoretical. Examination of the alkaline formaldehyde in ammonia trap *B*, after an estimation, demonstrated the presence of hexamine. The hexamine-N content, over the range 0·79–3·25 mg. choline-N, was determined by acid hydrolysis, removal of formaldehyde and distillation of the ammonia into standard acid. The results are shown in Table 2. These results, together with those shown

Table 2. *Ammonia formed during oxidation of choline with alkaline permanganate*

Choline-N (mg.)	Ammonium-N (mg.)	Choline-N (mg.)	Ammonium-N (mg.)
0·79	0·12	1·82	0·14
0·92	0·13	3·0	0·17
1·12	0·14	3·25	0·15

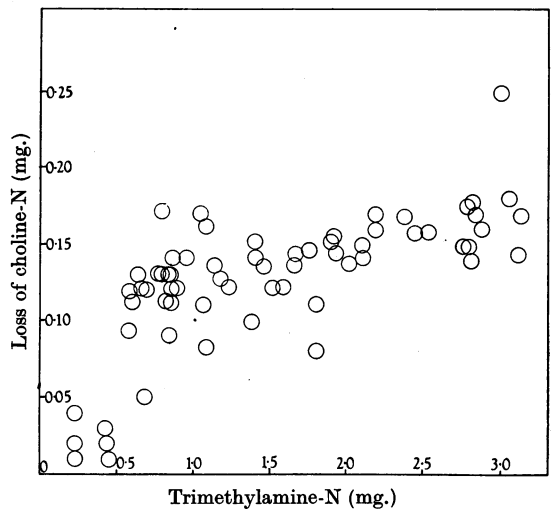

Fig. 2. Loss of choline-N plotted against yield of trimethylamine-N by Procedure 1, using quantities of choline-N up to 3·25 mg.

in Fig. 2, indicate that under the conditions of Procedure 1 and over the range 0·70–3·25 mg. choline-N, the amount of N liberated as ammonia falls within the range 0·11–0·18 mg. (mean value 0·14 mg.). With amounts of choline-N below 0·70 mg. the amount of ammonia liberated falls rapidly.

Table 3 summarizes the recoveries of choline-N from choline chloride, reineckate and mercurichloride by application of the equation:

$$\text{choline-N} = 0{\cdot}28f\,(T_1 - T_2) + 0{\cdot}14 \text{ mg.},$$

where $(T_1 - T_2)$ = ml. of 0·02 N-H_2SO_4 neutralized, in terms of the blank and test titration readings of 0·02 N-trimethylamine, and f the factor representing possible deviations from an exact 0·02 N solution.

added to such extracts could not be recovered as trimethylamine. Procedure 1 cannot, therefore, be directly applied for estimation of choline in crude plant extracts.

Table 3. *Recovery of choline*-N *by application of the equation* choline-N = 0·28 f $(T_1 - T_2)$ + 0·14 mg.

Choline derivative	Range covered (mg. N)	No. of determinations	Recovery as deviation from theoretical (%) Mean deviation	Maximum deviation
Chloride	0·68–2·94	25	1·3	4·4
Reineckate	0·8 –3·1	10	1·7	4·5
Mercurichloride	0·9 –3·25	15	1·6	3·5

Specificity. Lintzel & Fomin (1931*a*) found that alkaline permanganate produced trimethylamine with acetylcholine but not with betaine. We have confirmed these findings and also shown that no trimethylamine is evolved from trigonelline.

Procedure 2. *Estimation of free choline in plant extracts*

The procedure is as follows: The dried plant tissue is finely powdered and exhausted with cold toluene-saturated water or 80 % (v/v) ethanol. The extract is concentrated to small bulk *in vacuo* at a water-bath temperature of 40°. (Towards the end of the concentration a sample of the extract should be tested for volatile bases other than ammonia and monomethylamine, and if present such bases should be removed by continuing the vacuum distillation in presence of excess MgO. The concentrate, freed from excess MgO by filtration and brought to pH 7 with HCl, is then adjusted to a definite volume and heated to 80° as in the standard procedure.) The concentrate is heated to 80° for 10 min., cooled and filtered. The filtrate (10 ml.), which should contain not less than 0·7 mg. choline-N, is treated with 10 ml. of freshly prepared 4 % (w/v) aqueous solution of ammonium reineckate, followed by 0·4 g. of finely powdered solid ammonium reineckate, allowed to stand overnight, filtered on no. 50 Whatman paper by suction and the precipitate washed on the filter with 10 ml. cold water. The drained filter paper and precipitate are then transferred to flask *A*, 25 ml. water added and the trimethylamine-N estimated by Procedure 1.

Application of Procedure 1 *directly to plant extracts*

Concentrated plant extracts prepared, as described above, from dried roots and leaves of *Atropa* spp. and from dried King Edward potato tubers when treated by Procedure 1 yielded only traces of trimethylamine and excessive quantities of $KMnO_4$ solution were required to obtain even a transient green colour in flask *A*. Known amounts of choline

Precipitation of choline as reineckate. Choline reineckate is soluble to the extent of 0·02 % in water at 18°, and in a saturated solution of ammonium reineckate only to 0·0015 % (Strack & Schwaneberg, 1937). The precipitation adopted in Procedure 2 should therefore occasion a loss of choline-N within the limits 0·01–0·07 mg. Actual losses recorded by this procedure are shown in Table 4. The solubility

Table 4. *Loss of choline*-N *resulting from precipitation with ammonium reineckate in Procedure* 2

Choline-N (mg.) Contained in 10 ml. choline chloride solution	Loss due to precipitation
2·951	0·038
2·250	0·032
	0·058
	0·026
	0·060
1·512	0·038
	0·038
1·243	0·029
	0·070
	0·026
	0·040
0·821	0·055
	0·063
	0·039

Average loss = 0·044 mg.

of choline reineckate is but little affected by pH and it has been shown (Strack & Schwaneberg, 1937; Neuberger & Sanger, 1942) that ammonium reineckate is therefore a very specific precipitant for choline in strongly alkaline solution. The concentrated plant extracts used here gave with reineckate at pH 11–12 very fine precipitates difficult to filter. These precipitates were contaminated with material precipitated by the alkali used, and underwent partial decomposition during the protracted filtration. Yields obtained by precipitation at pH 11–12 were lower than by precipitation at pH 5–6. The

pH 11–12 precipitates obtained from the potato tuber extracts contained 69–72 % of choline reineckate. Precipitation of choline at pH 5–6 was therefore adopted since contamination with other reineckates does not interfere with the estimation of choline content by Procedure 1.

Table 5. *Recovery of added choline-N as trimethylamine-N from plant extracts by Procedure 2*

Extract	Amount of added choline-N (mg.)	Recovery of N as trimethylamine (%)
Indian belladonna leaf	0·1605	98·3, 96·9
	0·4815	100·3, 96·7
	0·6420	99·5, 96·6
	0·8020	99·7, 100·4
King Edward potato tuber	0·1605	95·0, 100·9
	0·3210	96·5, 98·0
	0·4815	95·7, 96·8
	0·6420	100·7, 98·4
	0·8020	101·8, 100·1

Recovery of choline added to plant extracts. The method of estimating choline in plant extracts involves two sources of loss, both of which are constant in magnitude. The constancy of this loss should make possible quantitative recovery of the whole of any added choline-N as trimethylamine-N, the whole of the loss being carried in the original value for the choline-N content of the plant extract. The results of recovery experiments using an extract of the dried leaves of Indian belladonna (*Atropa acuminata*) and an extract of dried King Edward potato tubers are shown in Table 5.

Free choline-N content of dried plant tissues

The free choline-N content of selected dried plant tissues has been determined by Procedure 2. The choline-N content of the extract sample has been calculated from the equation

$$\text{choline-N} = 0{\cdot}28\, f(T_1 - T_2) + 0{\cdot}18 \text{ mg.}$$

The results are shown in Table 6.

DISCUSSION

The method described is applicable to aqueous or aqueous ethanolic extracts of plant tissues. It is highly specific for choline, serving to distinguish it from other closely related bases with the exception of acetylcholine. Trimethylamine and other volatile bases (except ammonia and monomethylamine) if present in the extracts must be removed by a vacuum distillation in presence of excess mild alkali as they are likely to give reineckates sparingly soluble at neutral or slightly acid reaction.

Table 6. *Choline-N content of dried plant tissues*

Source	Organ of plant	Free choline-N (mg./100 g.) Dry tissue	Free choline-N (mg./100 g.) Fresh tissue
Atropa belladonna (English belladonna)	Leaves	16·0	—
	Roots	1·5	—
Atropa belladonna (Hungarian belladonna)	Roots	1·9	—
Atropa acuminata (Indian belladonna)	Leaves	13·1	—
	Roots	3·9	—
Hyoscyamus muticus (Egyptian henbane)	Inflorescences	1·1	—
	Leaves	1·5	—
	Stems	1·0	—
	Seeds	5·7	—
Solanum tuberosum (King Edward potato)	Sprouting tubers	8·9	1·78
Plant collected after 68 days' growth in sand culture. N supplied as $Ca(NO_3)_2$ (0·001 M)	Tops	2·6	0·30
	Stem bases	8·7	1·37
	Roots	7·1	0·56
	New tubers	1·6	0·26
	Depleted tubers	Nil	—
Plant as above but collected after 82 days' growth	Leaves	12·7	1·38
	Stems and petioles	8·4	0·79
	Roots	4·4	0·16
	New tubers	1·6	0·28
Plant collected after 68 days' growth in sand culture. N supplied as $(NH_4)_2SO_4$ (0·001 M)	Tops	15·4	1·83
	Stem bases	9·5	1·72
	Roots	6·0	0·45
	New tubers	5·6	0·90
	Depleted tubers	3·8	0·22
Plant as above but collected after 82 days' growth	Leaves	5·5	0·77
	Stems and petioles	11·2	1·12
	Roots	8·0	0·71
	New tubers	2·1	0·45

The method has been used to determine the amount of free choline in certain plant tissues. Total choline (free and combined) can be determined by the same procedure if the plant tissue is extracted with absolute methanol and the phospholipins hydrolyzed with barium hydroxide, as recommended by Engel (1942). The hydrolysate is then neutralized, filtered and treated with ammonium reineckate as described in Procedure 2.

Previous work (Street, Kenyon & Watson, 1946) on the nitrogenous constituents of the potato plant has focused attention upon the 'other nitrogen' ('residual nitrogen') as possibly containing the nitrogen compounds involved in translocation. This fraction includes the nitrogen of simple bases. The present results show that choline-N accounts for only a small percentage of the 'other nitrogen' (in the cases analyzed 2·8 and 1·2 % in leaves; 4·7 and 8·6 % in stems and petioles; 2·5, 1·6, 4·5 and 3·5 % in roots; 4·0 and 9·4 % in stem bases; 0·7, 2·2, 4·1, 1·9 % in new tubers and nil and 6·7 % in depleted tubers).

The present data confirms the existence of free choline in plant tissues. The content of free choline-N is small and variable. It will form a part of future work to examine how far concentration gradients of free choline-N are established in the growing plant.

SUMMARY

1. A modification of the Lintzel & Monasterio (1931) method is described for the determination of the choline-N content of choline derivatives. With amounts of choline-N ranging from 0·7 to 3·2 mg. this method gives quantitative recovery with a mean deviation from theoretical of not more than ± 2 %, and a maximum deviation of ± 5 %. The method is highly specific and, with the exception of acetylcholine, serves to distinguish choline from all closely related bases likely to be encountered in plant extracts.

2. The above method has been combined with a preliminary reinecke salt precipitation for the determination of the free choline-N content of crude extracts of dried plant tissues. Choline added to such extracts, in amounts equivalent to 0·16–0·8 mg. choline-N, could be quantitatively recovered with a mean deviation from theoretical of not more than ± 2 % and a maximum deviation of ± 5 %.

3. The method has been applied in the determination of the free choline-N content of the organs of King Edward potato plants and of several other important Solanaceous plants.

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