during the course of further work to be reported at a later date. It is certain that there are differences in the effect of diet on different tissues as Long (1953) has found that, although the hexokinase activity of intestinal mucosa is depressed by a low-carbohydrate diet, that of intestinal muscle is unaffected.

In so far as attempts to reproduce the anomalies of carbohydrate metabolism observed in the adult ruminant are concerned, these have been successful and it may be concluded from the similarities between the adult ruminant and the fat-fed non-ruminant that many of the apparent abnormalities in the former are due to the peculiar digestive processes of the ruminant rather than to specific hormonal or enzymic differences.

SUMMARY

1. After 17 hr. fasting, blood-sugar levels from rats and hamsters fed on a high-fat diet from weaning are consistently lower than those from similar animals on a high-carbohydrate diet.

2. The reduced tolerance to intravenously injected glucose induced by a high-fat diet has been confirmed using rats.

3. Restriction of the carbohydrate intake by feeding excess fat or protein depresses both glucose uptake and glycogen synthesis by isolated rat diaphragm.

4. The hexokinase activity of kidney homogenates from rats and hamsters is depressed by high-fat diets.

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Evidence for the Occurrence of γ -Methylene- α -oxoglutaric Acid in Groundnut Plants (*Arachis hypogaea*)

By L. FOWDEN AND J. A. WEBB

Department of Botany, University College, Gower Street, London, W.C. 1

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The isolation of two new amino acids, γ -methyleneglutamic acid and γ -methyleneglutamine, from groundnut plants (*Arachis hypogaea*) was described by Done & Fowden (1951, 1952). More recently these two compounds have been isolated from tulips (*Tulipa gesneriana*) by Zacharius, Pollard & Steward (1954), and their presence has been indicated in hops (*Humulus lupulus*) by Harris & Tatchell (1953). Although these compounds have a limited distribution within the plant kingdom, there are indications that they are of considerable importance in the metabolism of those species in

which they occur. γ -Methyleneglutamic acid can, under the influence of the transaminase systems present in groundnut plants, transfer its α -amino group to α -oxoglutaric, oxalacetic or pyruvic acids (Fowden & Done, 1953a), whilst γ -methyleneglutamine appears to be important in the transport of nitrogen within this species (Fowden, 1954).

α -Oxo acids have been the subject of several detailed investigations, and α -oxoglutaric, oxalacetic, pyruvic and glyoxylic acids have, by characterization as their 2:4-dinitrophenylhydrazine (DNPH) derivatives, been found to be widely

distributed in plant and animal tissues. Recently Steward and his co-workers, using a new method for the identification of DNPH derivatives, have obtained preliminary evidence for the presence of additional keto acids in plants, and detected the keto-acid analogues of valine and γ -methylene-glutamic or γ -methylglutamic acids (Towers, Thompson & Steward, 1954). Towers & Steward (1954) claim to have isolated the latter keto acid, as its DNPH derivative, from tulips. After completion of the transamination studies using groundnut extracts referred to above, we considered γ -methylene- α -oxoglutaric acid to be a possible constituent of groundnut plants, and commenced an investigation of the α -keto acids present in groundnut seedlings. We now report the isolation of this unsaturated keto acid as its DNPH derivative, together with more rigorous proof of its chemical identity than has so far been published. A brief description of some of the present experiments has appeared earlier (Fowden & Webb, 1954).

Preliminary experiments were performed to reveal the number and probable nature of the keto acids present in six-day-old seedlings. Aqueous deproteinized extracts of the seedlings were treated to form the DNPH derivatives of the keto acids, which were then separated on paper chromatograms. One strong spot, moving just in advance of that of the derivative of α -oxoglutaric acid, was not attributable to any known keto acid, but did occupy the same position on chromatograms as the derivative of the keto acid (presumably γ -methylene- α -oxoglutaric acid) arising from γ -methyleneglutamic acid by biological transamination.

Having established the probable nature of the new keto acid, larger quantities of seedlings were extracted, and about 12 mg. of the pure DNPH derivative of the acid finally obtained, after it had

been separated from the derivatives of other keto acids by cellulose-powder column and paper-chromatographic procedures. Since the keto acid has not been chemically synthesized, a biologically synthesized reference substance was prepared by transamination from γ -methyleneglutamic acid and α -oxoglutaric acid; the structure of this amino acid has now been confirmed by chemical synthesis (Wailes, Whiting & Fowden, 1954). The natural and synthetic DNPH derivatives so obtained behaved identically when subjected to the following treatments: (a) paper chromatography, (b) melting-point determinations, (c) hydrogenation in the presence of Adam's catalyst, (d) visible spectra determinations, (e) infrared spectra determinations on Nujol mulls, (f) X-ray spectra determinations of their ammonium salts and (g) oxidation by hydrogen peroxide.

Hydrogenation, in yielding an amino acid identical with γ -methylglutamic acid, alone established that the new keto acid was probably γ -methylene- (or γ -methyl-) α -oxoglutaric acid. To ensure that reduction of the $\text{C}=\text{C}$ bond had not accompanied transamination during the biological synthesis of γ -methylene- α -oxoglutaric acid from γ -methyleneglutamic acid (so yielding γ -methyl- α -oxoglutaric acid), the DNPH derivatives of the natural and synthetic acids were oxidized with hydrogen peroxide, and the products investigated by organic acid-paper chromatography. Malonic acid was the only oxidation product that could be so identified, and would be expected according to scheme A. The derivative of γ -methyl- α -oxoglutaric acid would be expected to yield methylsuccinic acid by similar treatment (see scheme B). A small quantity of this latter derivative, prepared by transamination from synthetic γ -methylglutamic acid, did in fact yield methylsuccinic acid as the main oxidation product.

Scheme A.

Scheme B.

EXPERIMENTAL

Plant material. Groundnut seeds were grown in moist sawdust at 25–30° for 6 days. Each seedling weighed about 2 g. fresh weight upon harvesting, and had developed a branched root system and the first two immature leaves.

Preparation of the DNPH derivatives of the keto acids. The seedlings, washed free from adhering sawdust, were macerated with an equal weight of distilled water in an 'Atomix' blender. The mush was strained through muslin, and the pH of the filtrate adjusted to approximately 4.2 with H_2SO_4 . The proteins were coagulated by warming to 50°, and removed by centrifuging. The keto acids were converted into their DNPH derivatives by treatment of 10 vol. of the supernatant with 1 vol. of a saturated solution of 2:4-dinitrophenylhydrazine in 2*N*-HCl for 20 min. at 40°. The derivatives were extracted into ether by four successive extractions (1 vol. ether to 4 vol. supernatant), and the combined ether extracts reduced to one-fifth of their original bulk. The concentrated ether extract was then shaken with successive small volumes of 10% (w/v) Na_2CO_3 soln. until no further appreciable amounts of acidic DNPH derivatives entered the Na_2CO_3 layers. Unchanged 2:4-dinitrophenylhydrazine remained in the ether layer at this stage. The combined Na_2CO_3 layers, maintained at 0°, were made slightly acid by the addition of conc. HCl and the DNPH derivatives of the keto acids again completely transferred to ether. These extracts were combined and evaporated to dryness, yielding a residue consisting mainly of the DNPH derivatives of the keto acids, together with smaller amounts of dark-coloured resinous substances. This extraction procedure was modified from those published by El Hawary & Thompson (1953) and Cavallini & Frontali (1954).

Preliminary identification experiments

In these experiments 100 g. fresh weight of seedlings were treated as above to obtain the mixed DNPH derivatives of their oxo-acid constituents. The residue was dissolved in the minimum volume of 0.5*N*- NH_3 soln., and a measured fraction applied as a short band to a sheet of Whatman no. 3 filter paper. A similar band was applied adjacent to the first, and contained the derivatives of α -oxoglutaric, glyoxylic and pyruvic acids prepared from 40 $\mu g.$ of each of the pure acids by a procedure similar to that above. The chromatogram was then developed for 20 hr. using *n*-butanol saturated with 6% (w/v) NH_3 soln. as the mobile phase (Fincham, 1953). The appearance of the chromatogram is reproduced in Fig. 1. The derivatives of pyruvic and glyoxylic acids each gave two spots on the developed chromatogram. The seedlings contained oxo acids co-chromatographing with α -oxoglutaric, pyruvic and glyoxylic acids, and four additional acids giving DNPH derivatives. One of these formed a strong band moving slightly in advance (R_F 0.14) of the α -oxoglutaric acid derivative, and was the subject of the further investigations to be described. The three other unknown derivatives formed fainter bands and moved at lower R_F values than α -oxoglutaric acid. Their nature has not been further investigated.

A small amount of the DNPH derivative of γ -methylene- α -oxoglutaric acid, admixed with a larger amount of the α -oxoglutaric acid derivative, was prepared by the procedure outlined above from the mixture of the two keto acids resulting from transamination between 2 mg. γ -methylene-glutamic acid (natural) and 2 mg. of α -oxoglutaric acid in

the presence of a dialysed groundnut extract (reaction conditions as Fowden & Done, 1953*a*). The derivative of γ -methylene- α -oxoglutaric acid also flowed slightly in advance of that of α -oxoglutaric acid, and was inseparable from the unknown (R_F 0.14) after the chromatogram had been developed for 3 days in the *n*-butanol- NH_3 solvent system.

Larger-scale preparations

Isolation of the unknown DNPH derivative from seedlings. Groundnut seedlings (2 kg., fresh wt.) were treated by the above extraction procedure and yielded about 1 g. of residue containing the mixed DNPH derivatives. A preliminary purification of the unknown derivative was accomplished on a cellulose-powder column. Dry Whatman cellulose powder (200 g.), previously extracted with *N*-HCl followed by *N*-NaOH, was mixed with *n*-butanol saturated with 6% (w/v) NH_3 soln. and poured as a slurry into a column of 50 mm. diameter. Frequent tamping ensured tight packing, and gave a column of 290 mm. length. The DNPH derivatives were dissolved in 20 ml. of the same solvent, and applied slowly to the column. The column was developed with more solvent, slight suction being used to obtain a percolation rate of about 50 ml./hr. After 5 hr. the development was stopped, the unknown material having formed a separate, and fairly well-defined band about 8 cm. down the column. The DNPH derivative of α -oxoglutaric acid had also formed a fairly discrete band, but the derivatives of glyoxylic and pyruvic acids, which moved down the column more rapidly, remained incompletely resolved. Excess solvent was allowed to drain from the column, and

Fig. 1. Representation of a chromatogram on Whatman no. 3 filter paper developed in the *n*-butanol- NH_3 solvent system containing the DNPH derivatives prepared from (a) a standard mixture (containing 40 $\mu g.$ each acid) of pure α -oxoglutaric acid (A), glyoxylic acid (B_1 and B_2) and pyruvic acid (C_1 and C_2), and (b) the keto acids occurring in extracts of six-day-old groundnut seedlings. X is the unknown keto-acid derivative investigated in this paper; Y represents three further unknown compounds which have not been investigated.

then the cellulose packing was carefully extruded to obtain the portion containing the unknown derivative. The remaining solvent was removed from this by drying *in vacuo* at laboratory temperature.

The derivative was extracted from the dried cellulose powder using four lots of 25 ml. of 0.5N-NH₃ soln. The combined extracts were made slightly acid by addition of conc. HCl, and the derivative was then quantitatively transferred to ether. A light-yellow residue remained on removal of the ether. Paper chromatograms revealed that it consisted of the unknown, contaminated by small amounts of the derivative of α -oxoglutaric acid. The contaminant was removed by applying the residue in a 25 cm. band near to the top edge of a sheet of Whatman no. 3 filter paper and developing the chromatogram for 40 hr. in the *n*-butanol-NH₃ solvent system. Complete resolution of the two substances occurred, and the unknown derivative was eluted from the stronger band by 0.5N-NH₃ soln. After acidifying the extract with conc. HCl, the derivative was again transferred to ether, and obtained as a yellow residue upon evaporation. This residue was re-extracted with dry ether (any NH₄Cl carried over into the former wet ether layers would remain undissolved), and the extract again evaporated to yield 12 mg. of the unknown.

Preparation of the DNPH derivative of γ -methylene- α -oxoglutaric acid by transamination. An extract of four-day-old groundnut seedlings was prepared by grinding them with washed sand and a small amount of 0.025M potassium phosphate buffer, pH 7.4. The centrifuged extract was dialysed for 20 hr. against a stream of 0.05M potassium phosphate buffer, pH 7.4 at 2°. No amino or keto acids could be detected in this dialysed extract, which was used as a source of transaminases. A 10 ml. portion of the extract was added to a mixture of 25 mg. of α -oxoglutaric acid and 25 mg. of γ -methyleneglutamic acid (natural), previously adjusted to pH 7.4 with NaOH and made up to 10 ml. with water. This reaction mixture was incubated at 37° for 4 hr.; at the end of this time determinations on measured fractions showed that glutamic and γ -methyleneglutamic acids were the only amino acids, and α -oxoglutaric and γ -methylene- α -oxoglutaric acids the only keto acids present in the mixture. The keto acids were converted into their DNPH derivatives and isolated as previously; 40 mg. of mixed derivatives resulted, about 70% consisted of the α -oxoglutaric acid derivative. The two derivatives were first separated on a 40 x 220 mm. cellulose column, followed by final purification of the γ -methylene- α -oxoglutaric acid derivative on paper chromatograms as described above for the unknown. The yield of the pure derivative was 10 mg.

Identity of the unknown derivative

The unknown derivative was shown to be identical with the biologically synthesized γ -methylene- α -oxoglutaric acid derivative by the following criteria.

Chromatographic behaviour. The two derivatives were inseparable on paper chromatograms developed in the *n*-butanol-NH₃ solvent for 5 days, during which time they had moved about 35 cm. down the paper.

Melting-point determinations. The unknown had m.p. 228° (decomp.), whilst the DNPH derivative of γ -methylene- α -oxoglutaric acid had m.p. 224° (decomp.). The mixed m.p. was 226° (decomp.). All m.p.'s were corrected.

Hydrogenation. Towers *et al.* (1954) showed that when DNPH derivatives of keto acids were hydrogenated in the

presence of a suitable catalyst, the analogous amino acids were produced, and could be identified by paper-chromatographic methods. Solutions of about 1 mg. of the unknown and of the synthetic derivative in 0.5N acetic acid, to which a trace of Adam's platinum oxide had been added, were hydrogenated using a hydrogen pressure of 50 lb./sq.in. in a stainless-steel bomb with constant shaking. No derivative remained after 15 min. as judged by the colourless nature of the solutions. The acid conditions used greatly reduced the time stated as necessary for complete reaction by Towers *et al.* (1954), who worked in neutral solution. The solutions were evaporated to dryness, and the residues redissolved in 100 μ l. portions of distilled water. Each solution (20 μ l.) was applied as spots to each of four chromatograms, together with marker spots containing suitable mixtures of known amino acids including γ -methylglutamic acid. The chromatograms were developed in water-saturated phenol, the *n*-butanol-NH₃ solvent above, collidine-lutidine mixture (Dent, 1947), and the *n*-butanol-acetic acid mixture of Partridge (1948), and the amino acids detected after ninhydrin spraying. Both the unknown and synthetic derivatives yielded a well marked spot of an amino acid inseparable from γ -methylglutamic acid in all four solvent systems, together with a trace of another amino acid identified as γ -amino- α -methylbutyric acid by chromatographic comparison with a sample of this amino acid prepared by hydrogenation of γ -amino- α -methylglutamic acid, isolated by Fowden & Done (1953b). This non- α -amino acid presumably arose by slight decarboxylation during the reduction of the DNPH derivatives, and this may be compared with a similar behaviour observed for the derivatives of other dicarboxylic keto acids (Towers *et al.* 1954). We have observed that there is a tendency for decarboxylation to occur more rapidly, with the production of increased amounts of ω -amino acids, as the pH of the solution subjected to reduction is raised.

Visible absorption spectra. The absorption spectra of equimolar solutions of the two derivatives in N-NaOH, and of a similar solution of the DNPH derivative of α -oxoglutaric acid, were determined over the wavelength range 380–700 m μ . using a Unicam SP. 500 spectrophotometer (Fig. 2).

Fig. 2. Reproduction of the visible absorption spectra of the DNPH derivatives of keto acids; 0.005% (w/v) in N-NaOH. —, derivative of α -oxoglutaric acid; + - - +, derivative of the biologically synthesized γ -methylene- α -oxoglutaric acid; • - - •, derivative of the unknown keto acid investigated.

Those of the unknown and the derivative of γ -methylene- α -oxoglutaric acid are seen to be virtually identical, but they differ from that of the α -oxoglutaric acid derivative.

Infrared spectra. The infrared absorption spectra of Nujol mulls of the two derivatives were compared (Fig. 3). It will be seen that the two spectra possess all the same main absorption frequencies, and only differ very slightly in the relative intensities of these absorption peaks.

X-ray-diffraction spectra. The two derivatives in the form of their ammonium salts were subjected to comparative X-ray-diffraction analysis. The diffraction lines obtained from the unknown were more diffuse than those obtained from the synthetic derivative, but the two spectra appeared to be essentially identical. When the unknown and synthetic derivatives were compared as free acids, their diffraction patterns differed, presumably due to differences in the crystalline forms of the two derivatives.

Oxidative degradation. Trial experiments showed that the DNPH derivative of α -oxoglutaric acid could be oxidized to succinic acid (the expected product) by H_2O_2 . The α -oxoglutaric acid derivative (1 mg.) was treated with 100 μl . of 30% (w/v) H_2O_2 soln. at 100° for 20 min., and the remaining solution chromatographed on a sheet of Whatman no. 2 filter-paper strip against marker spots of α -oxoglutaric and succinic acids containing 100 μg . of each acid. The lower organic layer of the two-phase mixture produced after equilibration of 80 ml. CHCl_3 , 80 ml. *tert*-amyl alcohol, 80 ml. water and 30 ml. 98–100% (w/v) formic acid was used as the developing solvent (L. E. Bentley, unpublished), and after complete removal of the solvent, the chromatogram was evenly sprayed with a 60% (v/v) ethanolic solution of bromocresol green adjusted to pH 5.5. The positions of the organic acids were revealed as yellow areas on a greenish blue background. The only detectable organic

acid produced during the oxidation flowed to a position identical with that of succinic acid (R_F 0.63); the spot of α -oxoglutaric acid had an R_F value of 0.46.

The same conditions were used to oxidize the unknown and the derivative of γ -methylene- α -oxoglutaric acid, which, if identical, should both yield malonic acid. The latter acid was added as an additional marker to the chromatograms developed in the same solvent. The only oxidation product detected in either oxidized solution was an organic acid moving at the same R_F value (0.54) as that determined for malonic acid.

In order to establish that the DNPH derivative of γ -methyl- α -oxoglutaric acid did not give malonic acid as the main oxidation product under these conditions, a small amount of the derivative was prepared and isolated in a similar manner to that already described for γ -methylene- α -oxoglutaric acid, by using 5 mg. of γ -methylglutamic acid as the starting material in the transamination reaction. On oxidation of the derivative of γ -methyl- α -oxoglutaric acid with H_2O_2 , an organic acid chromatographically identical with methylsuccinic acid (R_F 0.77) appeared as the main oxidation product.

DISCUSSION

From the literature it appears that γ -methylene- α -oxoglutaric acid is a new keto acid that has not been chemically synthesized. In order to obtain the DNPH derivative of the keto acid, we had therefore to resort to a biological synthetic method, using γ -methyleneglutamic acid as the starting material. The recent chemical synthesis of this amino acid (Wailes *et al.* 1954) has established its structure,

Fig. 3. Reproduction of the infrared absorption spectra of Nujol mulls of (a) the DNPH derivative of γ -methylene- α -oxoglutaric acid, and (b) the derivative of the unknown keto acid investigated.

whilst many detailed investigations of the transamination reaction have established its mechanism as firmly as those of most chemical syntheses. There can therefore be little doubt concerning the structure of the biologically synthesized keto acid, unless enzymic reduction of the —C=C— bond had also occurred.

The evidence obtained by hydrogenation and oxidative degradation, coupled with our general experience with groundnut plants, in which we have never obtained any evidence for the natural occurrence of the saturated γ -methyl derivative of glutamic acid or of enzymes capable of reducing the γ -methylene derivative, proved that the biological synthesis did in fact yield the unsaturated γ -methylene- α -oxoglutaric acid. The identity of the DNPH derivative of this keto acid with the material isolated from the groundnut seedlings is firmly established by the variety of the comparative methods used.

Although Towers & Steward (1954) consider their substance *B* to be the 2:4-dinitrophenylhydrazone of γ -methylene- α -oxoglutaric acid, they record a much higher m.p. ($268\text{--}270^\circ$ decomp.) for their material than we have observed for either the derivative of the natural keto acid (228°) or the biologically synthesized acid (224°). The reason for this discrepancy is not immediately obvious, for although geometrical isomerism is possible in DNPH derivatives, it seems unlikely that such isomers would differ widely in their m.p.'s. We consider that we have presented the stronger evidence in support of the exact nature of our isolated derivative, and note that our recorded m.p.'s ($224\text{--}228^\circ$) are of the order that might be predicted from those recorded for other DNPH derivatives of α -keto dicarboxylic acids (oxalacetic acid, 211° ; α -oxoglutaric acid, 219° ; from El Hawary & Thompson, 1953).

Seedlings, rather than mature plants, were extracted because they could be grown and handled more easily. Earlier analyses (Fowden, 1954) had also demonstrated that a very rapid production of γ -methyleneglutamine and γ -methyleneglutamic acid accompanied the process of germination, and a similarly rapid accumulation of γ -methylene- α -oxoglutaric acid seemed likely. Further experiments are in progress to determine the rates of production of this acid and other keto acids during seedling growth, and their distribution in the different organs of more mature plants.

Provided the process used for the extraction of the DNPH derivative of γ -methylene- α -oxoglutaric acid from the seedlings was reasonably efficient, the yield (12 mg. from 2 kg. fresh weight) suggests that the difficulties likely to be encountered in the isolation of useful amounts of the free acid from seedlings would be very great. These facts empha-

size the need for a suitable chemical method of synthesis of the keto acid, giving amounts that would be of use in studying its metabolism in the plant. The low concentrations of this and other oxo acids found in seedlings are probably indicative of their rapid rates of turnover, and it seems plausible that γ -methylene- α -oxoglutaric acid is an intermediate stage in the synthesis of γ -methylene-glutamic acid from carbohydrate or lipid storage materials, being converted into the amino acid either by transamination or directly by reductive amination with ammonia.

SUMMARY

1. The keto acids present in extracts of groundnut seedlings (*Arachis hypogaea*) were investigated and characterized by paper chromatography of their 2:4-dinitrophenylhydrazine (DNPH) derivatives. α -Oxoglutaric, glyoxylic and pyruvic acids were identified, whilst comparable amounts of a new keto acid were also present in the seedlings.

2. Using cellulose-powder column and paper-chromatographic procedures, 12 mg. of the DNPH derivative of the new keto acid were isolated from 2 kg. of seedlings.

3. Using a variety of physical and chemical methods, the DNPH derivative of the new keto acid was shown to be identical with the corresponding derivative of γ -methylene- α -oxoglutaric acid

prepared by biological transamination from the analogous amino acid.

4. The implications of the presence of the new keto acid in the seedlings are discussed.

We wish to thank Professor Kathleen Lonsdale, F.R.S., and Dr L. J. Bellamy for arranging the X-ray and infrared spectra comparisons respectively, Dr L. E. Bentley for supplying unpublished information regarding the solvent used for organic acid chromatography, and Dr J. A. Elvidge for a gift of methylsuccinic acid. We are also indebted to the Royal Society for a grant in aid of apparatus, whilst one of us (J. A. W.) expresses gratitude to the Agricultural Research Council for a personal maintenance grant.

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The Estimation of Thiols and Disulphides by Potentiometric Titration with Silver Nitrate

BY R. CECIL AND J. R. MCPHEE

Department of Biochemistry, University of Oxford

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This paper describes further developments in the estimation of thiols by potentiometric titration with silver nitrate. A previous paper (Cecil, 1950) described a method in which the thiols were allowed to react with a known excess of silver nitrate followed by back-titration with potassium bromide. Although the results then obtained agreed well with the theoretical figures, it has now been shown that the agreement was due to compensation of errors.

It has been found that these errors can be avoided by titrating the thiols directly with the silver nitrate in buffered solutions. The silver mercaptides formed tend, in most cases, to form complexes with additional silver ion which can interfere with the determination of the thiol end point. However, the stability of these complexes varies greatly with pH, and a range of pH has been found for every compound in which the thiol group can be titrated satisfactorily.

As the pH is raised, so the concentration of silver ion in equilibrium with free thiol falls and becomes so low that reproducible potentials cannot be obtained with a silver electrode. This difficulty has been overcome by the development of the silver thiol electrode (Cecil, 1954) which is reversible to the thiol as well as to the silver ion.

As in the previous work, the disulphides are first allowed to react with sodium sulphite and the thiol formed estimated in the same way.

THEORETICAL

Reaction of thiols with silver nitrate. The reaction of thiols with silver nitrate is described by the equations

In the presence of solid silver mercaptide

where S is defined as the solubility product. The equilibrium equation for (2) is

$$K = \frac{[\text{RS}^-] \times [\text{H}^+]}{[\text{RSH}]}, \quad (4)$$

K being the dissociation constant of the thiol. This may be expressed in terms of total thiol concentration, c , where $c = [\text{RSH}] + [\text{RS}^-]$ so that (4) now becomes

$$[\text{RS}^-] = c \cdot K / (K + [\text{H}^+]), \quad (5)$$

and substituting in (3)

$$S = [\text{Ag}^+] c \cdot K / (K + [\text{H}^+]). \quad (6)$$

The end-point titre (c_{ep}) is reached when the total silver added equals the total thiol originally present:

$$[\text{Ag}^+_{\text{ep}}] + [\text{RSAg}] = c_{\text{ep}} + [\text{RSAg}],$$

or

$$[\text{Ag}^+_{\text{ep}}] = c_{\text{ep}}. \quad (7)$$

From (6) the silver ion concentration at the end point is

$$[\text{Ag}^+_{\text{ep}}] = \left(S \frac{K + [\text{H}^+]}{K} \right)^{\frac{1}{2}} = \sqrt{S'}, \quad (8)$$

where S' is the apparent solubility product under any one set of conditions.

This equation is of limited application at present because of the difficulties of defining the various quantities. K is known only for thiols that do not contain amino groups. Different ionic species of the same compound are likely to have different solubility products and these must be taken into account when using eqn. 6. Lastly, species other than thiol, for example sulphite and silver mercaptides, bind silver ion sufficiently to depress the end-point concentration below the predicted value. To this extent therefore the method is empirical, and sufficient of each titration curve must be plotted to determine the end-point potential.

If there is to be a simple relation between $[\text{Ag}^+]$ and c during a titration, then the term $K/(K + [\text{H}^+])$ must remain constant. Adequate buffering is therefore essential. Because of the low silver ion concentration prevailing in the presence of thiol, the usual buffer salts acetate, phosphate, borate, as well as nitric or sulphuric acid, may be used, provided their concentrations are kept low.