SUMMARY

1. Condensation products of reductone have been prepared with *p*-aminobenzoic acid, 2-*p*-aminobenzenesulphonamidothiazole (sulphathiazole), 2-*p*-aminobenzenesulphonamidopyridine (sulphapyridine), 4:6-dimethyl-2-(*p*-aminobenzenesulphonamido)pyrimidine (sulphamezathine), *p*-aminomethylbenzenesulphonamide (maphenide, marfanil), 4-methyl-2-(*p*-aminobenzenesulphonamido)thiazole (sulphamethylthiazole), *p*-aminobenzenesulphonylguanidine, 4:4'-diaminodiphenylsulphone, *p*-(disodium- γ -phenyl- α -disulphopropyl)aminobenzenesulphonamide (soluseptasine)

ducts of reductone with the three last compounds were identical.

2. It has been shown that the condensation product of reductone and sulphathiazole is decomposed by *p*-aminobenzoic acid. The resultant product is identical with that obtained from reductone and *p*-aminobenzoic acid (*p*-2':3'-dihydroxy-prop-2'-enylideneaminobenzoic acid), but the reverse process is apparently not possible under similar conditions. Maphenide, however, displaces *p*-aminobenzoic acid from *p*-2':3'-dihydroxyprop-2'-enylideneaminobenzoic acid.

3. A theory of the mode of action of sulphonamide derivatives is discussed.

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$C_6H_5CH(SO_3Na)CH_2CH(SO_3Na)NHC_6H_4SO_2NH_2$,
p-(sodium- α -sulphoethyl)aminobenzenesulphonamide (Me. $CH(SO_3Na)NHC_6H_4SO_2NH_2$) and *p*-aminobenzenesulphonamide. The condensation pro-

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The Micro-estimation of Citric Acid

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The estimation of citric acid by its conversion into pentabromoacetone (Stahre, 1895) has been greatly improved, notably by Pucher, Sherman & Vickery (1936), who introduced a sensitive colorimetric procedure. Recent modifications (Perlman, Lardy & Johnson, 1944; Krebs & Eggleston, 1944; Goldberg & Bernheim, 1944; Hunter & Leloir, 1945; Speck, Moulder & Evans, 1946; Taussky & Shorr, 1947;

Natelson, Lugovoy & Pincus, 1947, 1948; Wolcott & Boyer, 1948) have further increased the reliability and sensitivity of the method. However, the fact that modifications of the method continue to appear indicates that satisfaction is not general. Most authors agree that the addition of permanganate is a critical step which must be performed slowly and cautiously.

We became aware of the defects of the method in the course of an investigation of the effect of various mono-, di- and tri-carboxylic acids on the activity of aconitase. This involved the estimation of citric acid in the presence of a large excess of one or more other organic acids. Under these conditions the recovery of citric acid was apt to be low by up to 30%, a result which was but little affected by the various preliminary treatments recommended. It appears from the published data that, although interference with the specificity of the method has been thoroughly investigated, interference with recovery has received less attention.

In the modification here proposed, permanganate is replaced by vanadic acid as oxidizing agent. In our experience this modification has the advantage of greater simplicity, as the oxidizing agent may be added in bulk, of greater elasticity, as the amount of oxidizing agent need not be adapted to concentrations of citric acid varying within a wide range, and of greater freedom from interference by other substrates. In addition, the method is even more specific than the older one; for example, β -hydroxybutyric acid gives no colour.

The use of vanadic acid was suggested by the observation of Pozzi-Escot (1946), who found that the oxidation of citric acid to acetonedicarboxylic acid by chlorate was catalysed by a trace of vanadic acid. The function of chlorate in this reaction is probably that of reoxidizing the reduced vanadyl ion, and it might be expected that in the presence of a sufficient quantity of vanadate the oxidation would proceed in the absence of chlorate. This is indeed the case.

METHOD

Reagents

Sulphuric acid, 27N- H_2SO_4 .

Bromine water, saturated.

Ammonium vanadate, 2% solution, dissolve by warming. The substance tends to crystallize on standing, and before use should be redissolved by warming.

Sodium thiosulphate, 5% solution.

Sodium sulphide (A.R.), 2% solution, freshly prepared.

Sodium sulphate (anhydrous).

Light petroleum, b.p. 80–100°. Purification: extract repeatedly with conc. H_2SO_4 , then with a saturated solution of KMnO_4 in 0.5N- H_2SO_4 and finally with a 5% solution of FeSO_4 in 0.5N- H_2SO_4 ; wash with water, dry and rectify.

Citric acid standard, 1 mg. of the crystalline acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$)/ml. (in N- H_2SO_4).

Procedure

(a) *For quantities of 0.1–1.0 mg. citric acid.* The solution (5 ml., containing not more than about 1 mg. citric acid) is mixed with 27N- H_2SO_4 (5 ml.) in a 30 ml. test tube. The mixture is cooled to room temperature, and bromine water (2 ml.) and ammonium vanadate solution (3 ml.) are added. The contents are well mixed and the stoppered tube is left in a water bath at 50° for 20 min. A slowly increasing turbidity indicates the formation of pentabromoacetone. The tube is again cooled to room temperature and excess Br_2 is removed

by adding 10–12 drops of $\text{Na}_2\text{S}_2\text{O}_3$ solution. The solution, which is now blue or bluish green, is extracted with 6 ml. light petroleum by mechanical shaking of the stoppered tube for 2–3 min. The aqueous layer is blown out with the aid of a wash bottle head, i.e. a rubber stopper fitted with two glass tubes, of which the longer one should be smoothly adjustable so as to reach to the bottom of the test tube. This glass tube is tapered at both ends and the lower tip is ground smooth to ensure an even contact. During its introduction through the petroleum layer the delivery tip is closed with a finger. When most of the aqueous layer has been blown out, the meniscus is allowed to rise slowly in the tube to the delivery tip. Sharp separation is thus achieved. After the addition of a pinch of anhydrous Na_2SO_4 the light petroleum extract is decanted into a clean test tube. Aqueous drops, if any should remain, must be prevented from running out. A 5 ml. portion of the extract is now transferred to a third test tube and shaken for 1 min. with 10 ml. of Na_2S solution. The coloured aqueous layer is blown on to a funnel fitted with a dry filter paper, and the absorption of the clear filtrate is measured in the Spekker absorptiometer or a similar instrument, using 1 cm. cells and Ilford no. 601 (spectrum violet) filters. Na_2S solution is used as a solvent blank. A calibration curve is determined in the usual way.

(b) *For quantities of 0.02–0.2 mg. citric acid.* The simplest way of increasing the sensitivity of the method is to reduce the volume of the Na_2S extract. The extent to which this is possible depends mainly on the minimum volume required in the colorimetric instrument available. For quantities of 20–200 μg . citric acid, with a photoelectric test-tube colorimeter (Hilger 'Biochem' absorptiometer), about 3 ml. are needed. The preparation of the pentabromoacetone extract is as described for the 0.1–1.0 mg. range. A 5 ml. sample of the extract is measured into a colorimeter tube and shaken with 3 ml. Na_2S solution. The tube is centrifuged lightly for 1 min. Readings are taken without removing the petroleum layer.

Comments

Precautions. The use of test tubes permits the simultaneous analysis of a large number of samples. The use of a measured portion of the light petroleum extract makes it possible to dispense with repetition of extractions and with washing operations (cf. Natelson *et al.* 1947). Unsatisfactory results can usually be traced to one of two sources of error: incomplete removal of free Br_2 or contamination of the petroleum extract with acid. The former is recognized by the coloration of the petroleum extract; unless this is completely colourless it is better to discard the sample than to complete the reduction of Br_2 by a further addition of thiosulphate. The use of thiosulphate is preferable to that of hydrazine sulphate, as its action is instantaneous and not accompanied by gas evolution which may be troublesome during the subsequent extraction. Contamination with acid may be due to faulty separation or to insufficient cleaning of wash bottle heads or stoppers. It is advisable to keep separate sets of these for the two extractions.

Colour stability. The addition of a stabilizing solvent to the Na_2S extract is unnecessary; likewise no advantage was found in using ice-cold Na_2S solutions, since lower readings were obtained than with solutions at room temperature. For all practical purposes the colour is stable for at least 30 min. at about 18°, especially if protected from strong light. Data on colour fading are contained in Table 1. The fading is slower in the more concentrated solutions.

Table 1. *Colour fading of sodium sulphide extracts after shaking with light petroleum solutions of pentabromoacetone*

Tem- perature (°)	Exposure to daylight	Time of measurement (hr.)							
		0	1	2	3	4	5	21	46
		Extinction coefficients							
17-20	+	0.100	0.087	0.081	0.075	0.072	0.071	0.043	—
	—	0.100	0.102	0.099	0.093	0.093	0.086	0.050	—
	+	0.201	0.178	0.155	0.153	0.150	0.147	0.087	0.063
	—	0.201	0.201	0.191	0.188	0.186	0.171	0.103	0.077
	+	1.00	0.99	0.92	0.89	0.87	0.83	0.58	0.390
	—	1.00	1.00	0.96	0.94	0.92	0.90	0.65	0.460
3	—	0.965	0.980	—	—	—	—	—	0.585

The light sensitivity of the colour is a little troublesome when the absorption is measured in tubes with the Hilger 'Biochem' absorptiometer. Here the incident light beam is concentrated on the solution in a sharp focus by a condensing lens. This causes a slow, but completely reversible, decrease of light absorption. Reliable results can be obtained by taking quick readings and by twisting the tube slowly during exposure, thus continuously mixing irradiated and unirradiated parts of the solution. No such colour instability was noticed when flat-sided cuvettes were used.

Accuracy. The calibration curve is a straight line going through the origin. The standard deviation of an estimation is well below 2% within the range of 0.2–1.0 mg. citric acid (Table 2). Standardization of the temperature (18°) and duration (1 min.) of the Na₂S extraction is important for obtaining reproducible results. If the extraction is prolonged for more than 1 min., a progressive reduction of colour intensity occurs.

Table 2. *Analyses of pure citric acid solutions*

(Extraction by 10 ml. Na₂S solution; Ilford filter no. 601.)

Cryst. citric acid (mg.)	No. of estima- tions	Extinction coefficient (mean)	Standard deviation	Yield of penta- bromo- acetone (%)
0.10	10	0.1034	0.00414	103
0.20	10	0.2029	0.00314	101
0.30	10	0.3075	0.00425	102
0.50	10	0.5025	0.00403	100
1.00	10	0.9820	0.00350	100

The colour intensity corresponds to a yield of pure pentabromoacetone of slightly over 100%. This is probably connected with the fact that, besides pentabromoacetone, small quantities of other bromoacetones, notably hexabromoacetone, are formed (Goldberg & Bernheim, 1944).

Specificity. The following compounds, in amounts of 0.1 mmol., gave no colour in the test: glucose, fructose, L-tyrosine, L-phenylalanine; and the following acids: glyoxylic, L-lactic, pyruvic, malonic, succinic, fumaric, maleic, tartronic, DL-malic, L-tartaric, α -ketoglutaric, L- α -hydroxyglutaric, crotonic, ($\pm$)- β -hydroxybutric, tricarballic, citraconic, *cis*- and *trans*-aconitic, ($\pm$)-isocitric, DL-threo- and DL-erythro-2:3-dihydroxybutyric, L-glutamic, L-aspartic and isoacetic.

Solutions of acetoacetic acid containing more than 0.01 mmol./ml. give a yellow colour, but not after a preliminary boiling with acid. A specimen of oxaloacetic acid also gave a positive reaction, 1 ml. of a 0.1M-solution simulating 0.225 mg. citric acid. This reaction was unaffected by preliminary boiling with acid and it may have been due to contamination of the specimen with citric acid or a related substance. Itaconic acid, in contrast to citraconic acid, produces a yellow colour, 1 ml. of a 0.1M-solution being equal in this respect to 0.80 mg. citric acid. This value was not changed by repeated recrystallization; it is, however, much smaller than that reported by Breusch & Tulus (1947).

In the presence of certain aromatic compounds, especially salicylic acid, it is necessary to carry out a preliminary bromination and to remove the bromination product by filtration or by extraction with light petroleum.

Recovery. The following substances, in amounts of 0.1 mmol., did not interfere with the recovery of 1 mg. citric acid by the usual procedure: glucose, pyruvic, succinic, fumaric, maleic, *cis*- and *trans*-aconitic, glutamic and β -hydroxybutyric acids. In the presence of some other substances, notably hydroxy acids, but also fructose, malonic and α -ketoglutaric acids, recoveries were only quantitative if an excess of Br₂ and vanadate was maintained. Undue reduction of Br₂ is recognized by the colour of the solution veering towards green during incubation at 50°. In this case more Br₂ is added in the form of a saturated solution of Br₂ in 9N-H₂SO₄, in portions of 1–2 ml., until the orange colour of the solution, or brown vapours above it, persist. Additional Br₂ (1–7 ml. of the solution) was required in presence of 0.1 mmol. of the following substances: fructose, malonic, lactic, malic, α -hydroxyglutaric, α -ketoglutaric, oxaloacetic, isocitric, tartaric, tartronic and 2:3-dihydroxybutyric acids. Provided the addition of bromine was early and sufficient, recoveries of citric acid were quantitative. In these experiments a 3% solution of ammonium vanadate was used.

The presence of any of the substances mentioned in high concentration is a complication which may require some adjustment of the standard procedure as far as the amounts of Br₂ and vanadate solutions are concerned. The vanadic acid method is more adaptable in this respect and gives better results than the permanganate method.

Pre-treatment with periodic acid. Some interfering substances may be removed by a preliminary treatment with periodic acid. These include not only α -glycols, such as tartaric acid, or polyhydroxy compounds, such as mono- or di-saccharides, etc., but also glyoxylic, malonic and tartronic

acids (Sprinson & Chargaff, 1946). The following procedure gave quantitative recovery of citric acid (1 mg.) in the presence of 0.1 mmol. of fructose or tartaric acid: 0.5 ml. of a saturated solution of sodium periodate is added to 3–5 ml. of the neutral solution to be analysed. After standing at room temperature for 15 min. the solution is acidified with 0.3 ml. 10N- H_2SO_4 and a 2M-solution of KI is added dropwise, until the solution is faintly yellow. Iodate formed in the reaction is thereby reduced to I_2 . After addition of 0.1 ml. 6% H_2O_2 (to reduce excess periodic acid to I_2) the solution is measured, filtered from separated I_2 and extracted twice with 3 ml. light petroleum. A sample of the filtrate (3–4 ml.) is mixed with 0.2 ml. 30% KBr (to remove excess H_2O_2), made up to 5 ml. and analysed by the usual procedure.

Modifications of the estimation of pentabromoacetone. Some recent modifications of citric acid analysis concern the estimation of pentabromoacetone. This substance is estimated either by its colour reaction with Na_2S or related compounds, or by procedures in which inorganic bromide is liberated and measured by titration or by optical methods. The latter procedures are less specific than the former, as the extract may contain other bromination products which liberate bromide, but do not give the colour reaction with sulphide (cf. Breusch & Tulus, 1947). In the method of Tausky & Shorr (1947) the solution of pentabromoacetone is shaken with ethanolic NaI solution and the liberated I_2 is measured optically. The colour intensity produced, and therefore the sensitivity of the method, is not superior to that of the Na_2S method. The method has further the great disadvantage that the colour intensity slowly increases for several hours (Table 3).

solutions changes to the yellow colour (absorption maximum 460 $\text{m}\mu$.) at alkaline pH and vice versa. However, with a 4% thiourea-2% borax solution containing 0.06% Na_2S the same pink colour is produced as with a neutral thiourea solution.

At present we are not sufficiently impressed by the advantages of the thiourea method to substitute it for the well-tried sulphide method.

Estimation of citric acid in biological fluids

Serum or plasma. The sample (3 ml.) is added slowly, with shaking, to 10% trichloroacetic acid (25 ml.). A portion of the filtrate (20–22 ml.) is transferred to a small conical flask containing one or two glass beads and the neck is lightly plugged with glass wool to prevent splashing. The solution is boiled down to 2–3 ml. on a hot plate and the residue is poured into a test tube and made up to 5 ml. with the rinsings of the flask and glass wool. The further analysis is carried out according to the procedure for the 0.02–0.2 mg. range. Recovery of added citric acid was good (Table 4). A number of analyses on human sera gave results between 15 and 35 $\mu\text{g./ml.}$

Recently, microcells for use with the Spekker absorptiometer became available to us. They permit estimations on 0.5 ml. solution and proved very useful for the estimation of citric acid in serum. The light petroleum extract was shaken with 1 ml. 2%

Table 3. *Extinctions of various extracts (10 ml.) after shaking with light petroleum solutions of pentabromoacetone (0.897 mg. in 5 ml.)*

Extraction by	Ilford spectrum filter no.	Time of measurement (hr.)			
		0	1	2	3
		Extinction coefficients			
Na ₂ S (2%)	601	0.500	0.490	0.475	0.460
Ethanolic NaI (10%)	601	0.445	0.500	0.530	0.540
Thiourea (4%) in borax (2%)	601	0.575	0.420	0.380	0.365
Thiourea (4%) + Na ₂ S (0.06%)	603	0.540	0.540	0.536	0.533

Natelson *et al.* (1948) have advocated the use of thiourea at pH 9.2, instead of Na_2S . They claim that the colour is stable at room temperature, and that the extinction coefficient is approximately twice that of the Na_2S colour. Using the Spekker absorptiometer with Ilford filter no. 601 (maximum transmission about 440 $\text{m}\mu$.) we obtained, with the method of Natelson *et al.*, an extinction coefficient only 10–15% higher than with Na_2S , but the stability of the colour was considerably less (Table 3).

Natelson *et al.* observed a different colour (absorption maximum at 510 $\text{m}\mu$.) after prolonged shaking of a neutral thiourea solution with a light petroleum solution of pentabromoacetone. We found that this colour develops rapidly, in about 0.5 min., if the thiourea solution contains 0.06% Na_2S . Under these conditions fading was indeed reduced to a minimum. The extinction coefficient, read with Ilford filter no. 603 (maximum transmission at about 500 $\text{m}\mu$.), was, however, substantially the same as that obtained by other methods (Table 3). According to Natelson *et al.* the pink colour (absorption maximum 510 $\text{m}\mu$.) of neutral thiourea

sodium sulphide solution and about 0.5 ml. of the coloured aqueous layer was withdrawn with a test pipette and placed in the microcell. When using this

Table 4. *Recovery of citric acid from horse serum*

Citric acid ($\mu\text{g.}$)			Recovery (%)
Added	Found	Recovered	
0	29.2	—	—
20	48.2	19	95
40	69.0	39.8	99.8
60	88.8	59.6	99.3
80	109.0	79.8	99.8

modification we had difficulties with trichloroacetic acid as deproteinizing agent. Metaphosphoric acid, too, was unsatisfactory, but tungstic acid filtrates gave consistent results. A disadvantage of tungstic acid filtrates is the appearance of a precipitate of

excess tungstic acid in the strongly acid solution in which the oxidation and bromination of citric acid is carried out. This does not interfere with the reaction, but it makes the removal of the aqueous layer by blowing out impossible. Instead, the petroleum layer is sucked off.

Cerebrospinal fluid. The c.s.f. (1 vol.) is mixed with 30 % trichloroacetic acid (0.1 vol.), and 1–3 ml. of the filtrate are directly analysed without further pre-treatment. Range: 40–80 $\mu\text{g./ml.}$ (cf. Benni, Larsson & Thunberg, 1943).

Urine. Protein, if present, is removed by precipitation with trichloroacetic acid, turbidities by filtration, acetoacetic acid by boiling with dilute acid, and aromatic compounds, such as salicylates, by preliminary bromination. No preliminary treatment whatever is required for normal urines which

are analysed directly after suitable dilution. Samples of 1–5 ml. of urine diluted fivefold usually give satisfactory readings. Recovery of added citric acid was quantitative.

SUMMARY

1. A modification of the colorimetric estimation of citric acid is described in which permanganate is replaced by vanadic acid. This gives increased simplicity and speed of operation, and greater freedom from interference by other oxidizable substrates. The yield of pentabromoacetone is almost quantitative.

2. Some recent modifications of the colorimetric estimation of pentabromoacetone are critically examined.

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Studies in Detoxication

27. THE ORIENTATION OF CONJUGATION IN THE METABOLITES OF 4-CHLOROCATECHOL AND 4-CHLORO-RESORCINOL, WITH SOME OBSERVATIONS ON THE FATE OF (+)-ADRENALINE, PROTOCATECHUIC ACID AND PROTOCATECHUIC ALDEHYDE IN THE RABBIT

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Garton & Williams (1948) have shown that catechol gives rise to monoconjugates, *o*-hydroxyphenyl-glucuronide and *o*-hydroxyphenylsulphuric acid, in the rabbit. Quinol and resorcinol also form monoconjugates in the rabbit (Garton & Williams, 1949). The object of the present work was to find out which of the two hydroxyls becomes conjugated if a third group *X* is present in a position *para* to one of the

hydroxyls in catechol compounds. We first studied protocatechuic aldehyde ($X = \text{CHO}$), protocatechuic acid ($X = \text{COOH}$) and (+)-adrenaline

These three compounds formed glucuronides in the rabbit, but we did not succeed in isolating them in a crystalline state. The structure of these glucuronides could not therefore be rigidly proved. With 4-chlorocatechol ($X = \text{Cl}$), however, we succeeded in isolating in high yield a crystalline monoglucuronide whose structure could be determined. The structural

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