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The Application of the Ceric Sulphate-Arsenious Acid Reaction to the Detection of Thyroxine and Related Substances

By C. H. BOWDEN, N. F. MACLAGAN AND J. H. WILKINSON

Department of Chemical Pathology, Westminster Medical School (University of London), London, S.W. 1

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During a study of the urinary excretion products in connexion with the clinical trials of the antithyroxine compound, butyl 4-hydroxy-3:5-diiodobenzoate (Fraser & MacLagan, 1953; MacLagan & Wilkinson, 1951, 1954) it was observed that the urine from patients receiving the drug decolorized the ceric sulphate-arsenious acid reagent of Kolthoff & Sandell (1934). Although a trace of iodide was present, this reagent could not be used for its quantitative estimation because the principal metabolic product, 4-hydroxy-3:5-diiodobenzoic acid, appeared to catalyse the reduction of ceric sulphate by arsenious acid almost as effectively as iodide. Subsequently it was found that many diiodophenols, including thyroxine and diiodotyrosine, gave similar reactions, and it seemed possible that, with the employment of this reagent, a highly sensitive method for the detection of thyroxine and related substances might be developed.

Existing methods for the detection of these compounds, e.g. in paper chromatography, depend either upon the reactions of their amino acid side chains with ninhydrin or diazotized sulphanilic acid, or on autoradiography for ¹³¹I-labelled materials only. The former procedure is not sufficiently sensitive to make visible spots containing less than 10 µg. of thyroxine or other iodine-containing amino acids. Moreover, the presence of metabolites such as iodide, which are not amino acids, cannot be demonstrated by this method. The use of the ceric sulphate-arsenious acid reagent would overcome these disadvantages, and the present paper describes the development of such a method, a brief account of which has already appeared (Bowden & MacLagan, 1954).

EXPERIMENTAL

Preparation of reagents. Unless otherwise specified, reagents of the 'AnalaR' grade were used, and all solutions were prepared with 'iodine-free' water (Riggs & Man, 1940). In our experience, water which had been distilled from glass apparatus proved satisfactory without further treatment.

Three primary solutions have been used, the final reagents being made by diluting or mixing these as described below:

(1) Ceric sulphate (10%, w/v) was prepared by adding Ce(SO₄)₂ · 4H₂O (B.D.H. 'low in other rare earths'; 10 g.) to N-H₂SO₄ (100 ml.), previously cooled to 0–5°. The cloudy solution was refrigerated for 1 hr., then clarified by centrifuging or filtration and stored in a refrigerator.

(2) The arsenious acid solution (5%, w/v) was prepared by adding sodium arsenite (Hopkin & Williams's laboratory reagent grade; 5 g.) in small portions to vigorously stirred N-H₂SO₄ (100 ml.) at 0°. If the temperature was allowed to rise or if stirring was omitted, dehydration occurred leading to the precipitation of arsenious oxide. Alternatively, the sodium arsenite (5 g.) was dissolved in N-NaOH (30 ml.) and the solution run dropwise into mechanically stirred 2N-H₂SO₄ (65 ml.) at 0–5°. The volume was then adjusted to 100 ml. with water.

(3) Ceric sulphate (2%, w/v) was prepared by adding Ce(SO₄)₂ · 4H₂O (2 g.) to 4N-H₂SO₄ (100 ml.).

For ordinary paper chromatograms the reagent was prepared by mixing equal volumes of solutions 1 and 2 immediately before use (reagent A).

If the paper chromatograms are required for photoelectric scanning, then solutions 1 and 2 were diluted to one-fifth the strength with N-H₂SO₄ before mixing (reagent B).

For detection of iodine-containing compounds in aqueous solutions, the same one-fifth strength reagents are used as described. For the detection of iodide in urine, however, solution 3 is used in conjunction with the 1% (w/v) sodium arsenite produced by dilution of solution 2 with N-H₂SO₄.

Aqueous solutions

The method used is a modification of one previously described (Foote, Mackenzie & MacLagan, 1952) for the detection of iodide in urine. Sodium arsenite reagent (1 ml. of 1%) and $\text{Ce}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$ reagent (1 ml. of 2%) were added to 1 ml. of the aqueous iodine-containing solution. The time taken to give distinct fading as compared with a blank using 1 ml. of iodine-free distilled water instead of the solution was noted. A standard time of 5 min. was taken as giving minimum detectable quantities at room temperature.

Alternatively, by placing the tubes in a boiling-water bath immediately after mixing, total decolorization in tubes after 4 min. was taken as giving minimum detectable quantities.

It was essential to clean the glass apparatus thoroughly by washing with chromic acid and rinsing with iodine-free distilled water.

Paper chromatograms

The application of the method to paper chromatograms was also studied. One-dimensional chromatograms on Whatman no. 1 filter paper (40×10 cm.) were run by vertical ascent for 16 hr. at $20 \pm 4^\circ$ using the butanol-dioxan-ammonia mixture of Gross, Leblond, Franklin & Quastel (1950). The chromatograms were dried in a cabinet fitted with an extraction fan and soda lime traps to remove atmospheric iodine.

Preliminary attempts at spraying the mixed reagent *A* on to the paper were hazardous for the operator and gave very poor results. It appeared that the limiting factor was the time allowed for the reagents to react in the wet state, and after numerous trials the following technique was adopted.

The mixed ceric sulphate-arsenious acid reagent *A* (ca. 10 ml.) was applied evenly to a sheet of chromatographic paper of the same size as the original chromatogram by laying it flat on a sloping, clean glass plate and allowing the reagent to flow down the paper so that it was evenly wetted and free from wrinkles and air bubbles. The glass plate was then placed on a flat surface and the wetted paper covered by the dry chromatogram. A second glass sheet was placed on top and firmly pressed down, so that the chromatogram was also evenly wetted. The papers were allowed to remain in this position for varying periods (usually 30 min.) according to the sensitivity required. The plates were then separated and the papers dried in an iodine-free atmosphere. The positions of iodine-containing compounds were indicated by white spots on a yellow background. Each chromatogram thus produced two similar records.

Permanent records. The dry chromatograms faded after a few days as the result of action of atmospheric iodine, and the strongly acid nature of the reagent caused the paper to disintegrate within a few weeks, hence it was necessary to devise some means of preparing more permanent records. For many purposes, simply tracing the spots on a second sheet of paper was adequate, but photographic methods were much to be preferred, as they provide a permanent record of the relative intensity of the spots. Direct photography using an Ilford tricolour blue filter no. 304 and paper gave a record of white spots on a black background. It was simpler and quicker to back the chromatogram with Ilford reflex document paper no. 50 and expose for a few seconds to ultraviolet light from an Osram black glass MBW/V lamp. The paper stained by ceric sulphate blocked the

transmission of ultraviolet light, whilst the white spots did not. Thus the record consisted of black spots on a white background.

Semi-quantitative measurements. A scanning device based upon that of Nanninger & Bink (1951) was employed so that rough quantitative measurements of the amounts of iodine compounds present could be determined. The dry chromatogram was interposed between a source of ultraviolet light (Osram MBW/V lamp) and a Spekker fluorimeter photoelectric cell connected to a Cambridge reflecting galvanometer (sensitivity $2200 \text{ mm.}/\mu\text{A}$ at 2 m.). The photoelectric cell was mounted in a block of wood on which was placed a series of wooden sheets each bored with a hole, the diameter of which was equal to that of the cell. These wooden sheets enabled the length of the light path to be altered so that convenient deflexions of the galvanometer could be obtained. The spots on the chromatograms were placed in position on the top of these blocks and covered with a thin Perspex sheet bearing an outline of the cell to facilitate alinement of the spots being measured, the light source and the cell. The apparatus is illustrated in Fig. 1.

Chromatograms for use in this apparatus were treated with the one-fifth strength ceric sulphate-arsenious acid reagent (reagent *B*). This increases the sensitivity of the estimation at low concentrations without seriously altering the blank reading. It was important to standardize rigidly the amount of reagent used and also the development and running times for this method. The chromatograms had to be completely dry—this was achieved in 30 min. at 20° /1 mm. in a vacuum oven.

Determination of iodine liberated by treatment with ceric sulphate from iodine-containing compounds

The sensitivity of the reaction for a number of organic iodine compounds was studied, and an attempt was made to correlate the results with the rate of liberation of iodine from the compounds when treated with ceric sulphate under standard conditions as follows.

The iodine-containing compound (100 mg.) was dissolved or finely suspended in *tert.*-butanol (2 ml.) and vigorously shaken mechanically at $22 \pm 2^\circ$ for 40 min. with 10%, w/v, ceric sulphate solution in $\text{N-H}_2\text{SO}_4$ (10 ml.). The liberated

Fig. 1. Apparatus for scanning paper chromatograms (half-section).

iodine was then extracted into benzene (20 ml.) by shaking for a further 5 min. The benzene layer was washed 3 times with water (10 ml., 5 ml., 5 ml.) to remove H_2SO_4 and extracted with 0.1N- $\text{Na}_2\text{S}_2\text{O}_3$ (10 ml.). The benzene residue was washed twice with water (5 ml.) and the washings were added to the $\text{Na}_2\text{S}_2\text{O}_3$ extract, which was then back-titrated with 0.1N- I_2 . The results obtained with a number of substances are discussed below.

The nature of the oxidation products of the iodophenols has been subjected to preliminary investigation, but we are not yet able to define their structure. We are, however, indebted to Dr I. D. P. Wootton of the Postgraduate Medical School of London for examining the infrared spectrum of one of the products. This has shown that aromatic character was lost on oxidation.

RESULTS AND DISCUSSION

Aqueous solutions

Table 1 shows the minimum concentrations of potassium iodide, thyroxine, triiodothyronine and diiodotyrosine in aqueous solution required for fading and decolorization under the conditions described above. The reaction is more sensitive at 100° than at room temperature, and very small amounts can be detected.

However, for detection of iodide in urine the reagents need slight modification. The 2 % $\text{Ce}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$ is made up in 4N- H_2SO_4 instead of N- H_2SO_4 . The increase in acid is necessary to prevent the precipitation of ceric phosphate from some urines. This method is similar to that of Foote *et al.* (1952) but is preferred, as the reagents are more stable and slightly more sensitive, giving a minimum detectable concentration of 40–60 $\mu\text{g. I}^-/100 \text{ ml.}$ at room temperature.

Paper chromatograms

Still smaller amounts of iodine-containing compounds can be detected on paper chromatograms. The minimum concentration detectable by spraying was 3.0 $\mu\text{g.}$ potassium iodide, whereas the plate technique was about 300 times more sensitive (limit, 0.01 $\mu\text{g.}$).

Kolthoff & Sandell (1934) have pointed out that the effect of iodides on the reduction of the ceric ion by arsenite is catalytic. It would appear that when the reagent is sprayed on the chromatogram it dries too rapidly for the effect of small traces of iodide to become visible, whilst in the alternative procedure sufficient time is allowed for the catalytic effect to be obvious.

Typical results are shown in Table 2. Fig. 2 shows the range within which the semi-quantitative method may be used. For thyroxine, in the range 0.1–0.4 $\mu\text{g.}$ the correlation between concentration and galvanometer reading is linear with some scatter. The upper limit is fixed by the diameter of the photoelectric cell. Since the amount of light

reaching the cell depends on the area of the spot, spots must not be overloaded with material. Thus it was found necessary to dilute stronger solutions so that the 10 $\mu\text{l.}$ applied to the paper contained about 0.3 $\mu\text{g.}$ thyroxine or 0.09 $\mu\text{g.}$ potassium iodide.

Mechanism of the reaction

Some information has been obtained concerning the nature of the iodine compounds which respond to the reagent. In preliminary experiments it was found that the presence of a free phenolic hydroxyl or similar group was essential, for no derivatives of iodophenols in which the hydroxyl group was protected by methylation or esterification gave the reaction in solution. Similar results were obtained on paper. When present in stoicheiometric amounts,

Table 1. Detection of iodine compounds in aqueous solution

Compound	Minimum concentration causing fading at room temperature ($\mu\text{g./ml.}$)	Minimum concentration causing decolorization at 100° in 4 min. ($\mu\text{g./ml.}$)
Potassium iodide	0.2	0.02
Diiodotyrosine	0.4	0.08
Thyroxine	2	0.1
Triiodothyronine	2	0.2

Fig. 2. Photoelectric scanning of paper chromatogram by transmitted ultraviolet light.

Table 2. Detection of iodine compounds on paper chromatograms

Compound	Smallest amount detectable by	
	Visual or direct photography. u.v. photoelectric measurement ($\mu\text{g.}$)	u.v. photography or u.v. visual ($\mu\text{g.}$)
Potassium iodide	0.01	0.04
Diiodotyrosine	0.02	0.06
Thyroxine	0.1	0.3
Triiodothyronine	0.1	0.3

several phenols which did not contain iodine also decolorized the reagent, but this effect was not catalytic and was easily distinguished from the behaviour of iodides and iodophenols. The catalytic effect was also shown by 4-amino-3:5-diiodobenzoic acid, so it would appear that the presence of an electron-donating substituent is necessary.

In order to examine the possibility that the effects observed with iodophenols were due to liberation of iodine, various iodophenols or their derivatives were treated with excess ceric sulphate under standard conditions and the liberated iodine was titrated with sodium thiosulphate. The results (Table 3) show that those compounds (nos. 1-15) which liberated any significant quantity of iodine had a free hydroxyl group in a position *ortho* to an iodine atom. Conversely, those substances (nos. 16-24) which did not possess this structural feature released little or no iodine on treatment with ceric

sulphate, with the single exception of 3:5-diiodo-tyrosine (no. 16). Since 3:5-diiodotyrosine (no. 8) gives up its iodine more readily than most diiodophenols, it would seem that the alanine side chain may be partly responsible.

From the figures in the last column of Table 3, it is clear that there is a correlation between the smallest amounts of a number of compounds detectable on paper chromatograms and the percentage of iodine set free by ceric sulphate. Qualitatively, the iodo compounds fall into two groups: those which readily release iodine and give the reaction on paper, and those which are resistant to oxidation to iodine and do not give the reaction on paper. The conclusion may therefore be drawn that the detection of iodophenols by the ceric sulphate-arsenious acid reagent is due to the ease of liberation of iodine and not to any specific property of the molecule as a whole. The only exception to this conclusion which

Table 3. Iodine liberated from derivatives of iodophenols by treatment with excess 10% (w/v) ceric sulphate at 20° for 40 min.

Compound						Iodine liberated		Smallest amount of compound detectable on paper chromatograms (μg.)
No.	R	R'	R''	X	Ref.	mg./100 mg. compound	Percentage of total iodine	
1	HO	H	CH ₃	I	—	29.9	42.4	0.01
2	HO	H	COOH	I	—	7.2	11.0	0.02
3	HO	H	COOCH ₃	I	—	11.4	18.1	0.02
4	HO	H	COOC ₄ H _{9-n}	I	a	6.4	11.2	0.01
5	HO	H	CHO	I	—	8.9	13.1	0.05
6	HO	H	COC ₂ H ₅	I	b	23.5	37.2	0.02
7	HO	H	NO ₂	I	—	13.9	21.7	0.02
8	HO	H	CH ₂ .CH(NH ₂).COOH	I	—	26.6	45.3	0.02
9	HO	H	†	I	c	5.2	7.9	0.1
10	HO	H	†	H	c	6.4	10.9	0.1
11	HO	H	COOH	H	—	6.3	13.1	0.1
12	HO	H	COOC ₄ H _{9-n}	H	d	4.4	11.1	0.2
13	HO	H	CHO	CH ₃ O	—	10.2	22.3	0.02
14	H	HO	COOC ₄ H _{9-n}	I	a	2.5	4.4	0.05
15	H	HO	CHO	I	—	15.2	22.4	0.02
16	*	H	CH ₂ .CH(NH ₂).COOH	I	c	5.7	11.8	0.02
17	CH ₃ O	H	COOH	I	—	0	0	>10.0
18	CH ₃ O	H	COOCH ₃	I	—	0	0	>10.0
19	CH ₃ O	H	CN	I	e	0	0	>10.0
20	CH ₃ .COO	H	COOCH ₃	I	e	0.6	1.1	0.5
21	CH ₃ .COO	H	CHO	I	b	0	0	5.0
22	C ₂ H ₅ O.COO	H	CHO	I	b	0.6	1.1	5.0
23	*	H	CN	I	f	0	0	2.0
24	H	CH ₃ O	CHO	I	b	0	0	>10.0
Potassium iodide						71.2	93.0	0.01
Potassium iodate						0	0	0.03

a Sheahan, Wilkinson & MacLagan (1951).

b Wilkinson, Sheahan & MacLagan (1951a).

c Kindly provided by Glaxo Laboratories Ltd.

d MacLagan, Sprott & Wilkinson (1952).

e Wilkinson, Sheahan & MacLagan (1951b).

f Harington & Barger (1927).

The remaining compounds are described in Beilstein's *Handbuch der Organische Chemie*, 4th ed.

we have investigated is the case of potassium iodate, which reacts with the ceric sulphate-arsenious acid mixture, but does not yield any iodine with ceric sulphate alone (Table 3). It has been shown by adding arsenious acid before ceric sulphate, that this anomaly is due to preliminary reduction of potassium iodate by the arsenious acid.

The method as described is relatively insensitive to aliphatic iodine-containing compounds such as iodoacetic acid, but we are informed by Dr I. R. Bush (St Mary's Hospital, London) that spots of such compounds are detectable if the chromatograms are previously treated with alkali. This causes hydrolysis of the compound with the liberation of iodide. Since alkaline solutions of thyroxine contain iodide after prolonged storage, we tried the effect of alkali on chromatograms bearing spots of this substance, but were unable to demonstrate any increased sensitivity.

SUMMARY

1. Methods are described for the detection of inorganic and organic iodine-containing compounds in solution and on paper chromatograms using ceric sulphate and arsenious acid.

2. In solution 0.2 μ g. potassium iodide and 2.0 μ g. thyroxine are detectable at room temperature. On paper chromatograms the method is sensitive to 0.01 μ g. potassium iodide and 0.1 μ g. thyroxine.

3. Methods for making permanent records of the chromatograms are described. The general procedure can be adapted for the semi-quantitative estimation of thyroxine in the range 0.1–0.4 μ g.

4. The mechanism of the reaction has been studied and the conclusion reached that the method depends upon the liberation of iodine from the compounds by oxidation with ceric sulphate. With

one exception (potassium iodate), only those compounds which readily yield iodine when treated with ceric sulphate can be detected on paper with this reagent.

5. The reaction is given by all the aromatic compounds studied which contain one or more iodine atoms in positions *ortho* to an electron-donating substituent such as the hydroxyl or amino group.

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The Inhibition of Horse-Liver Esterase by Rhodamine B

By JANE BURCH

*Department of Biochemistry, University of Cambridge, and Department of Biochemistry, University of Leeds**

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Horse-liver esterase is inhibited by low concentrations of several fluorescent dyes (Burch, 1954). It was considered that this inhibition might be of special interest for the theory of enzyme action, since the combination of small fluorescent molecules with proteins may be studied by an independent

physical method: that of the polarization of fluorescence, introduced by Weber (1952a, b), and applied by Laurence (1952) to the study of the reversible adsorption of dyes by serum albumin. Combination between enzyme and substrate or inhibitor is fundamental to the accepted mechanisms of enzyme action and its reversible inhibition; but, except in the important case of the haem

* Present address.