SUMMARY

1. The steam-volatile metabolic products of *Daedalea juniperina* Murr. have been investigated.

2. A new fungal metabolic product 5-(α -propynyl)-2-formylthiophen, m.p. 80°, has been isolated and characterized. It has been given the trivial name of junipal.

3. On oxidation with the Doeuvre reagent junipal affords junipic acid, $C_8H_6O_2S$, m.p. 180°.

4. Oxidation of junipic acid with $KMnO_4$ gives 2:5-thiophendicarboxylic acid, identified as the dimethyl ester, m.p. 145–6°.

5. The ultraviolet and infrared absorption spectra of junipal have been determined and are consistent with the structure advanced.

6. Indications of a second metabolic product closely related to junipal have been obtained.

7. A third metabolic product has been identified as anisaldehyde.

The authors wish to thank Dr W. P. K. Findlay of the Forest Products Research Laboratory, Princes Risborough, for supplying the cultures used, and Dr K. E. Almin of the Swedish Forest Products Research Laboratory, Stockholm, for the determination of the infrared absorption spectrum of junipal. The ultraviolet absorption spectra were determined by means of a Hilger Uvispek spectrophotometer. For the

grant for the purchase of this instrument the authors are indebted to the Central Research Fund Committee of London University.

REFERENCES

- Anchel, M. (1952). *J. Amer. chem. Soc.* **74**, 1588.
 Anchel, M., Polatnick, J. & Kavanagh, F. (1950). *Arch. Biochem.* **25**, 208.
 Birkinshaw, J. H. (1953). *Annu. Rev. Biochem.* **22**, 372.
 Birkinshaw, J. H., Bracken, A. & Findlay, W. P. K. (1944). *Biochem. J.* **38**, 131.
 Birkinshaw, J. H. & Morgan, E. N. (1950). *Biochem. J.* **47**, 55.
 Birkinshaw, J. H., Morgan, E. N. & Findlay, W. P. K. (1952). *Biochem. J.* **50**, 509.
 Celmer, W. D. & Solomons, I. A. (1952). *J. Amer. chem. Soc.* **74**, 1870.
 Challenger, F. & Holmes, J. L. (1953). *J. chem. Soc.* p. 1837.
 Doeuvre, J. (1927). *Bull. Soc. chim. Fr.* (iv), **41**, 1145.
 Griffing, J. M. & Salisbury, L. F. (1948). *J. Amer. chem. Soc.* **70**, 3416.
 Hartough, H. D. (1952). *Thiophene and its Derivatives*. New York: Interscience Publishers.
 Hartough, H. D. & Kosak, A. I. (1947). *J. Amer. chem. Soc.* **69**, 1012.
 Johnson, E. A. & Burdon, K. L. (1947). *J. Bact.* **54**, 281.
 Zechmeister, L. & Sease, J. W. (1947). *J. Amer. chem. Soc.* **69**, 273.

Micro-determination of Bromide in Body Fluids

By G. HUNTER

Stoke Mandeville Hospital, Aylesbury, Bucks

(Received 11 December 1954)

A micro method for the estimation of bromide in presence of chloride and in the absence of organic matter has been described by Hunter & Goldspink (1954). It is based on the quantitative conversion of bromide into bromate by hypochlorite: $Br^- + 3 OCl^- \rightarrow BrO_3^- + 3 Cl^-$, but instead of determining by iodometric titration as already described (Hunter, 1953), bromine is freed under suitable conditions according to: $BrO_3^- + 5 Br^- + 6 H^+ \rightarrow 3 Br_2 + 3 H_2O$ with quantitative conversion into tetrabromorosaniline, which is measured colorimetrically. The method has now been applied to the estimation of bromide in blood and other body fluids and it would appear to be applicable to other tissues. The essential problem here is to destroy the organic matter without loss of bromine. A procedure will be described for the treatment of blood and body fluids which permits precise determination of bromide in many samples in a short time. The material for analysis should not contain more than 100 and preferably less than 25 μg . Br; 1 μg . Br suffices.

EXPERIMENTAL

Reagents. These are described and discussed by Hunter & Goldspink (1954) and are as follows: sodium hypochlorite, N in 0.1N-NaOH, and bromine-free; sodium formate, 50% (w/v); buffer solution (pH 6.35) made by mixing 10 vol. 40% (w/v) NaH_2PO_4 , 2H₂O, 7 vol. 2N-KOH and 5 vol. water; rosaniline solution, 6 mg. in 100 ml. 2N-H₂SO₄; bromide-molybdate mixture, i.e. 0.15 g. KBr and 3 g. ammonium molybdate made to 100 ml. with water; sulphuric acid, 14N; *tert.*-butanol, with 5% (v/v) ethanol; potassium hydroxide, 2N.

Methods

Preparation of the test solution. Place in a nickel crucible (26 mm. diam., 10 ml. cap.) 1 ml. or less of serum or cerebrospinal fluid, add 2 drops 2N-KOH and dry in an oven at 110°. Remove to a muffle furnace at 600° and ignite for 30 min. Remove crucible, cool and add exactly 4 ml. water. Stir thoroughly with a small glass rod and transfer the solution to a test tube (16 $\times$ 125 mm.). Centrifuge. Remove 3.5 ml. of the supernatant fluid with a pro-pipette into another test tube (16 $\times$ 125 mm.) and add 1 ml. buffer, pH 6.35. Add 0.25 ml. sodium hypochlorite solution, mix

Table 1. *Percentage loss of bromide on ignition at different temperatures for varying times*

Into each of twelve crucibles were put 10 μ g. Br, as KBr, with two drops 2N-KOH. All were dried at 110° and then transferred to a muffle furnace at 600°. At 30 min. intervals, up to 2 hr., three crucibles were removed. After cooling, 4 ml. water were added to each and the determination was continued as described in the text. Similar sets were run at 650° and 700°, and with each set three controls, without ignition.

Temp. (°)	Time of ignition (min.)							
	30		60		90		120	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
600	3.6	2.5-5.0	11.1	9.3-12.1	12.8	11.8-14.3	12.7	11.8-13.2
650	9.9	8.9-10.7	14.4	13.6-15.7	19.4	19.3-19.6	26.8	26.4-27.1
700	19.7	16.9-22.8	36.8	35.3-37.9	47.9	46.4-48.6	48.8	47.9-50.0

and heat for 10 min. in boiling water. Then add 0.25 ml. formate solution, mix and heat for further 5 min. in boiling water. Remove, cool and add 0.2 ml. water (the amount lost by evaporation under our conditions). This is the test solution. There is enough of it for several colorimetric observations as described below.

With whole blood and erythrocytes, the above ignition procedure leaves much carbon. This is readily overcome by removing the crucible after it has been 10 min. in the muffle, cooling and breaking down the charred mass with about 1 ml. water, re-drying at 110° and re-igniting for 20 min.

Colour reaction. In a test tube, or colorimeter tube, place 0.1 ml. bromide-molybdate mixture, 0.5 ml. rosaniline solution, 0.4 ml. 14N-H₂SO₄ and 1 ml. of the test solution. Mix and leave this reaction mixture for 3 min. at 20-30°, then add 2 ml. *tert.*-butanol and 1 ml. 14N-H₂SO₄. Mix the solution and measure the optical density in a suitable absorptiometer at about 570 μ .

If there is present in the portion of the test solution used much more than 5 μ g. Br as BrO₃⁻, the colour of the final solution will be brownish owing to destruction of the bromorosaniline formed by excess bromine. In such circumstances the amount of test solution should be reduced but the volume should be kept constant by the addition of a corresponding amount of water before addition of the test solution. If there is less than about 1 μ g. Br present in the test solution, readings rather lower than the correct ones will be obtained. To get a more precise value the test should be repeated using approximately 0.1 ml. rosaniline solution/ μ g. Br estimated by the preliminary observation. The volumes of the bromide-molybdate solution and of 14N-H₂SO₄ are constant in all tests. The volumes of rosaniline and test solutions are variable but the total volume before addition of butanol should be 2.0 ml.

The amount of Br present is readily calculable from the optical density reading, the slope of the linear calibration curve for the absorptiometer used, the amount of material represented by the test portion including the use of 3.5 out of 4.0 parts.

RESULTS

The organic matter in 1 ml. blood is destroyed with a negligible carbon residue if the dried blood is ignited at 600° for 30 min. If the temperature is much below 600°, e.g. 550°, the destruction of carbon is much prolonged. It was thus decided to

determine the loss of bromide by volatilization from 10 μ g., Br as KBr at 600°, 650° and 700° over the time intervals shown in Table 1.

Our main interest in Table 1 is the amount of Br lost in 30 min. at 600°. Other observations have been made with findings between 2 and 5%; indeed the loss is hardly perceptible. Some difference of temperature may be expected at different locations in the furnace.

Table 2. *Recovery of bromide in presence of glucose*

A 10% (w/v) solution of glucose was made with the addition of 1 mg. bromide/100 ml. Portions of 0.25, 0.50 and 1.0 ml. were taken in duplicate and treated as described in the text. Here the crucibles were heated for 10 min. in the muffle, removed, cooled and the charred mass was broken down with a little water and a glass rod. The contents were re-dried at 110°, and re-ignited at 600° for a further 20 min.

Amounts taken for analysis

Glucose (mg.)	Br (μ g.)	Recovery (%)
25	2.5	101
50	5.0	100
100	10.0	99

It was next necessary to test the ignition procedure, i.e. 600° for 30 min., in presence of organic matter. The results of such a test are shown in Table 2. The recovery is indeed rather better than expected, possibly owing to a trace of bromide in the glucose, although no measurable amount was found in controls.

Recovery of bromide added to serum is shown in Table 3. Substantially the same results are obtained with amounts of serum varying from 0.25 to 1.0 ml. It is evident that there is no appreciable loss of bromide associated with the destruction of the organic matter of serum, and the results indicate that, perhaps for most purposes, it is unnecessary to use such prolonged procedures as that described by Francis & Harvey (1933) for ashing the blood.

Table 3. *Recovery of bromide added to sera*

Analysis of the original sera and the same sera to which was added 1 mg. Br/100 ml. was carried out in duplicate with the different amounts shown as taken for analysis.

Serum (no.)	Amt. taken for analysis (ml.)	Br (mg./100 ml.)			Recovery (%)	
		Found	Range	Mean		
1	0.5	1.44	1.44-1.44	1.44		
	1.0	1.44				
	Added 1.0 mg. Br/100 ml.					
	0.25	2.46	2.32-2.46	2.41		97
0.50	2.40					
1.00	2.36					
2	0.5	0.48	0.48-0.50	0.49		
	1.0	0.50				
	Added 1.0 mg. Br/100 ml.					
	0.25	1.52	1.43-1.54	1.47		98
0.50	1.46					
1.00	1.44					
3	0.5	0.99	0.98-0.99	0.98		
	1.0	0.98				
	Added 1.0 mg. Br/100 ml.					
	0.25	1.94	1.86-1.96	1.93		95
0.50	1.95					
1.00	1.91					

Table 4. *Distribution of bromide in a blood*

Duplicate amounts of 1.0 and 0.5 ml. were taken in each case. The calculated value for whole blood is based on a 37% packed-cell volume.

	Br (mg./100 ml.)		
	Found	Range	Calculated
Whole blood	1.26	1.20-1.32	1.26
Corpuscles	0.98	1.40-0.92	
Plasma	1.43	1.43-1.42	

A further test of the method is shown by the analysis of whole blood, plasma and erythrocytes given in Table 4. The amount in whole blood is accurately accounted for by the separate analyses of plasma and of erythrocytes.

The method is equally applicable to the direct determination of bromide in urine as shown from the results in Table 5.

The accuracy of the entire procedure was assessed from twelve determinations on a serum in duplicate or triplicate on 2.0, 1.0, 0.5, 0.25 and 0.1 ml. amounts. The mean was found to be 4.38 mg. Br/100 ml., s.d. 0.15, and extreme range of values 4.52-4.16.

Table 5. *Determination in, and recovery of bromide from untreated urine*

Urine (no.)	Vol. taken for analysis (ml.)	Bromide found (mg./100 ml.)	Mean (mg./100 ml.)	Recovery (%)
1	4	0.19	0.21	
	3	0.21		
	2	0.22		
	1	0.21		
Added 1.0 mg. Br/100 ml.				
	1.0	1.19	1.18	97
	0.5	1.11		
	0.25	1.25		
2	4	0.25	0.26	
	2	0.27		
Added 1.0 mg. Br/100 ml.				
	1.0	1.29	1.22	96
	0.5	1.18		
	0.25	1.18		

DISCUSSION

The use of bromide-free sodium hypochlorite solution is desirable. For example, if the hypochlorite solution contains 0.25 mg. bromine/100 ml. the use of a 1 ml. test portion from the test solution prepared as described above, will give an EEL absorptiometer reading of about 2 divisions (Evans Electroselenium, Harlow, Essex, i.e. approx. 0.02 optical density in 1 cm. path) when the analysis is carried out with the reagents alone and with 1 drop of rosaniline solution present. The blank value of two divisions should be subtracted from the reading obtained in any determination when a 1 ml. test portion is used. If a smaller test portion is used a proportionately smaller blank should be subtracted. But if the hypochlorite is free

from bromine there. no blank value to bother about.

The ashing procedure as described here would obviously not be satisfactory for volatile bromine compounds; nor has it been adequately tested for non-volatile organically bound bromine. In such circumstances it would seem that the bromine might be fixed by heating in sealed tubes with alkali as used by Hardwick (1942).

The method has been found to be useful where the amount of material available for analysis is very limited. It may be regarded as 100 times more sensitive than the titration method described earlier (Hunter, 1953), and it would appear from preliminary observations that it is unnecessary to administer bromide to patients to test the permeability of the barrier between blood and cerebrospinal fluid (see Taylor, Smith & Hunter, 1954), as the method can determine satisfactorily the bromide normally circulating in these fluids. It may further be allowed that the distribution of bromide in plant and animal tissues has not been adequately studied for lack of a precise micro method, and it would appear that the method described here might be adapted to such purposes.

The method has been used to determine bromide in many hundreds of samples of serum and cere-

brospinal fluid and has given rise to no difficulties. We commonly carry out twelve to eighteen determinations in a batch in a few hours.

SUMMARY

1. Details are given for the application of the method of Hunter & Goldspink (1954) to the determination of bromide in body fluids.
2. As little as 1 μ g. Br is enough for analysis, so that less than 1 ml. of serum from people who have received no medicinal bromide, is adequate.
3. The standard deviation of the method is less than 5 %, and the extreme range of variation less than 10 % of mean values.
4. It is suggested that the method might be useful in the determination of bromine in plant and animal tissues.

I am indebted to Mr J. G. Bowser for technical assistance.

REFERENCES

- Francis, A. & Harvey, C. O. (1933). *Biochem. J.* **27**, 1545.
Hardwick, P. J. (1942). *Analyst*, **67**, 223.
Hunter, G. (1953). *Biochem. J.* **54**, 42.
Hunter, G. & Goldspink, A. A. (1954). *Analyst*, **79**, 467.
Taylor, L. M., Smith, H. V. & Hunter, G. (1954). *Lancet*, p. 700.

Fractionation of Phosphates by Paper Ionophoresis and Chromatography

By H. E. WADE AND D. M. MORGAN

Microbiological Research Department (Ministry of Supply) Porton, Wiltshire

(Received 20 December 1954)

Since the importance of phosphorus to cell metabolism was established, several schemes have been described for the fractionation of acid-soluble phosphates. Those most widely used depend upon the different solubilities of the alkaline earth and heavy metal salts in water and ethanol (LePage & Umbreit, 1945; Kaplan & Greenberg, 1944; Sacks, 1949). Even in mixtures of known composition, however, such schemes are liable to give imperfect separations owing to the solubility of some salts being influenced by the presence of others. Another disadvantage of such methods is the relatively large quantity of material required, a disadvantage they share with methods based upon ion exchange (Cohn, 1950; Cohn & Carter, 1950; Volkin, Khym & Cohn, 1951), solvent distribution (Plaut, Kuby & Lardy, 1950) and conventional ionophoresis (Bock & Alberty, 1951).

To overcome these disadvantages the fractionation of phosphates on filter paper is used, but most of the schemes reported are designed for the fractionation and analysis of phosphates of similar constitution, e.g. nucleotides (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950; Davidson & Smellie, 1952; Deutsch & Nilsson, 1953) sugar phosphates (Cohen & McNair Scott, 1950), polyphosphates (Ebel & Volmar, 1951) or for the analysis of adenosine phosphates and phosphates concerned in glycolysis (Hanes & Isherwood, 1949; Bandurski & Axelrod, 1951; Mortimer, 1952). The increasing number of reports of newly discovered phosphates occurring in living material, emphasizes the need for a scheme of analysis which, in addition to giving a good general separation of known phosphates, would facilitate the location, recovery and identification of unknown ones. The following fractiona-