- Kiessling, W. (1934). *Biochem. Z.* **273**, 103.
 King, E. J. & Delory, G. E. (1939). *Biochem. J.* **33**, 1185.
 King, H. & Pyman, F. L. (1914). *J. chem. Soc.* **105**, 1238.
 Kumler, W. D. & Eiler, J. J. (1943). *J. Amer. chem. Soc.* **65**, 2355.
 Martin, J. B. & Doty, D. M. (1949). *Analyt. Chem.* **21**, 965.
 Meyerhof, O. & Suranyi, J. (1926). *Biochem. Z.* **178**, 427.
 Morton, C. (1930). *Quart. J. Pharm.* **3**, 438.
 Nimms, L. F. (1933). *J. Amer. chem. Soc.* **55**, 1946.
 Nimms, L. F. (1934). *J. Amer. chem. Soc.* **56**, 1110.
 Oesper, P. (1950). *Arch. Biochem.* **27**, 255.
 Paolini, V. (1911). *R.C. Accad. Lincei*, **20**, i, 807.
 Paolini, V. (1912). *R.C. Accad. Lincei*, **21**, ii, 350.
 Royal Society, Symbols Committee Report (1951).
 Toal, J. S. & Phillips, J. I. (1949). *J. Pharm., Lond.*, **1**, 869.
 Walker, D. G. & Warren, F. L. (1951). *Biochem. J.* **49**, xxi.

Micro-estimation of 'Formaldehydogenic Steroids' by Bismuthate Oxidation

By R. W. H. EDWARDS* AND A. E. KELLIE†

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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Oxidation of the C-17 steroid side chain,

to formaldehyde forms the chemical basis for the quantitative determination of the so-called 'formaldehydogenic steroids'. The method can be considered in three stages—the oxidation, the transfer of formaldehyde, the colorimetric determination of formaldehyde—and the completeness of each stage may be examined independently. In current methods the oxidation is carried out at room temperature by periodic acid, excess of this reagent being removed by stannous chloride (Lowenstein, Corcoran & Page, 1946; Corcoran & Page, 1948). Although in early methods the formaldehyde was estimated in the oxidized solution, it is advantageous to isolate the formaldehyde by distillation before assay (Daughaday, Jaffe & Williams, 1948). This has been carried out conveniently, on a micro scale, by isothermal distillation in Conway units (Bassil & Hain, 1950; Hollander, Di Mauro & Pearson, 1951). The formaldehyde produced is estimated by heating with chromotropic acid reagent (MacFadyen, 1945).

Whilst present methods of assay are sufficiently reliable for most clinical purposes, the published methods, even when applied to pure compounds, lack precision. Stage by stage examination showed that whilst the determination of formaldehyde was quantitative, the recovery of formaldehyde by isothermal distillation in conventional Conway units was neither complete nor reproducible. When periodate was used as the oxidant, the high blanks encountered limited the usefulness of the method at

low concentrations. In the present method, sodium bismuthate (Rigby, 1950) replaces periodic acid as the glycol fission agent and is subsequently removed by centrifuging; the formaldehyde is determined after isothermal distillation in a modified Conway unit. This unit permits the transfer of the formaldehyde directly into the chromotropic acid reagent at 100°, the transfer and simultaneous colour development being complete in half an hour. The procedure is applicable in the range of 1–10 µg. of steroid (accuracy ± 0.05 µg.).

The use of bismuthate for the oxidation of corticosteroids has been suggested independently by Brooks & Norymberski (1952) as a basis for the determination of formaldehydogenic steroids and of 17-ketogenic steroids. By studying the action of this reagent upon compounds representing the different types of C-17 side chain encountered in corticosteroids these workers have demonstrated the value of such methods for the determination of corticosteroid metabolites in urine (Brooks & Norymberski, 1953; Norymberski, Stubbs & West, 1953).

EXPERIMENTAL

Recovery of formaldehyde by isothermal distillation

The formaldehyde solution (100 µl. containing 0.5 µg.), prepared by dilution from distilled formalin, was placed in the outer compartment of a no. 2 Conway unit (Conway, 1947), and isothermally distilled into the chromotropic acid reagent (400 µl.) in the inner compartment. At the end of the distillation the contents of the inner compartment were transferred to a small test tube and heated at 100° for 30 min. The percentage recovery figures (Table 1) were based upon the colour density so obtained, taking as 100% that recovery given by heating the same proportions of chromotropic acid reagent and formaldehyde for the same period of time after direct mixing.

* Research Fellow of the London Fever Hospital Board.

† Research Fellow of the British Empire Cancer Campaign.

Although the recovery of formaldehyde was high and consistent at 90–95°, the no. 2 Conway units were not convenient to use under these conditions, for not only was it difficult to raise the units rapidly to this temperature, but special precautions were necessary to preserve the grease seal and prevent a loss of vapour. To overcome these difficulties a modified Conway unit was designed in which the transfer of formaldehyde was $95 \pm 1.2\%$ complete. As formaldehyde recovery in sealed tubes approached 100%, it is possible that the performance of the modified unit could be further improved by effecting the transfer in an autoclave instead of at atmospheric pressure.

Table 1. Recovery of formaldehyde (0.5 μ g.) by isothermal distillation in no. 2 Conway units at various temperatures

(Number of estimations, 4, except for the experiment marked *.)

Temperature (°)	Duration (hr.)	Recovery (%)	Mean error ($\pm$ %)
Room	17	79	5.5
37	17	89	9.5
37	41	84*	4.0
50	17	83	5.7
Rising to 90–95	2.5	94	1.1

* 3 estimations.

Apparatus

Method

The oxidation of the steroid is carried out in a small glass tube (0.5 ml.) with a shallow conical bottom sufficiently thick to withstand centrifuging (Fig. 1b). This tube contains a stirrer of length about 4 cm. made of glass rod 0.5 mm. in diameter and shaped as shown in Fig. 1c. Stirring is achieved by spinning the shaft between forefinger and thumb.

The modified Conway unit. The original no. 2 Conway unit is somewhat large for the small quantities measured, and the modified unit (Fig. 1a) has a centre well of about 0.25 ml. capacity. The units employed are blown from borosilicate glass and fitted with B10 standard socket and stopper. Lugs on both socket and stopper are fitted with springs to withstand the small internal pressures produced on heating.

Fig. 1. (a) Stoppered Conway unit, (b) oxidation tube, (c) stirrer for oxidation tube. The B10 stopper and springs are omitted. Approximately to scale.

Reagents

Water. All water used in the preparation of reagents and in the determinations is distilled from alkaline KMnO_4 .

Chloroform. CHCl_3 (laboratory reagent grade) is washed with a solution of NH_4OH , HCl (5%, w/v), made alkaline with ammonia, and then washed three times with distilled water. After drying overnight over anhydrous CaCl_2 , the CHCl_3 is distilled, rejecting the first and last 10% of the distillate. Ethanol (2%, v/v) is added and the reagent is stored in amber glass bottles.

Propionic acid. Propionic acid treated with about 1% (w/v) CrO_3 is warmed to complete solution and allowed to stand overnight. It is distilled under reduced pressure below 55° and the distillate is subsequently redistilled twice at atmospheric pressure through an efficient fractionating column. The constant-boiling fraction distilling at about 142° is collected, again treated with CrO_3 , and distilled under reduced pressure. The purified propionic acid is stored in the dark under N_2 at 0°.

Chromotropic acid reagent. If necessary, chromotropic acid is purified by treatment with charcoal and by recrystallization from aqueous acetone. Pure chromotropic acid (20 mg.) is dissolved in water (1.30 ml.) and, whilst cooling, H_2SO_4 (76%, w/w, 8.62 ml.) is added. The volume is made up to 10.0 ml. with water. The required concentration of H_2SO_4 may be prepared by running conc. H_2SO_4 (A.R., 10 ml.) into water (4.8 ml.). This chromotropic acid reagent will absorb one-quarter of its own volume of water to become 9M with respect to H_2SO_4 . The reagent is stored at 0°.

Formaldehyde standard. Formalin solution is redistilled and standardized as detailed in *Analar Standards for Laboratory Chemicals* (1951).

Solvents used in dissolving steroid fractions must be free from interfering chromogenic material. In general, solvents distilled before use are suitable.

Procedure

The steroid to be assayed (5–25 m- μ moles) is transferred in solution to the oxidation tube and dried below 40° in a stream of N_2 . The dry residue is then washed down to the tip of the tube with CHCl_3 and the solvent removed under N_2 . The residue is dissolved in propionic acid (10 μ l.) by rotation of the tube and, after standing 10 min., diluted with water (150 μ l.). Some urine extracts give a cloudy solution at this stage but if the solution is stirred during dilution, duplicate determinations agree closely.

The solution is cooled to 10° by immersion in a chilled-water bath and finely powdered sodium bismuthate (A.R., 0.5 mg. approx.) is added. (A convenient spatula may be made by flattening the end of a no. II hypodermic needle.) The temperature is maintained and the mixture, stirred at intervals, is allowed to react in the dark for 45 min. The stirrer is then withdrawn and the tube is centrifuged at 2000 rev./min. for 5 min. to remove the NaBiO_3 and any cloudiness present. The amounts of reagents and steroid may be increased in proportion if required.

The modified Conway units are prepared by pipetting chromotropic acid reagent (400 μ l.) into the outer compartment, using a 1 ml. graduated pipette, the last centimetre of which is bent 45° off axis. The supernatant liquid (100 μ l.) from the oxidation mixture is pipetted into the centre compartment of the unit, and the stopper, lubricated with a trace of 9M- H_2SO_4 , is fixed in position by the springs.

The units are placed on a platform out of bright light, in a simmering-water bath (95–100°) so that the top of the chamber is just immersed. After 30 min. the units are removed and stood to a similar depth in cold water (~15°) for 5 min. Within 30 min. of cooling, the fluid from the outer compartment is transferred to a micro-colorimeter cell of a Spekker Absorptiometer (0.5 ml., optical depth 1 cm.) and the optical density read against 9M-H₂SO₄ as blank, using a yellow light source (Ilford Spectrum Yellow light filter no. 606; transmission 5600–6000 Å).

When assaying extracts of biological material an ‘unoxidized determination’ is carried out by the above procedure, substituting an equal amount of kieselguhr for NaBiO₃. This inert substance also removes any cloudiness. The difference between the ‘oxidized’ and the ‘unoxidized’ determination is due to the formaldehyde produced by the oxidation of the steroid. A blank determination is also made by following this procedure but omitting the steroid. The optical density of this blank should lie between 0.02 and 0.05 (≡ 0.7–1.7 m-μmoles).

Calibration. A calibration graph may be prepared using formaldehyde or any other compound yielding formaldehyde quantitatively on bismuthate oxidation. Calibration points for formaldehyde and for pregn-4-ene-17α:21-diol-3:11:20-trione (cortisone) are shown in Table 2 to lie sufficiently close to a straight line for a calibration factor to be employed in calculations. This factor, in the case of formaldehyde, has been calculated statistically (Youden, 1951) to be an extinction of 0.0302/m-μmole. When pure cortisone was used, the calibration data were not significantly different (*t* test) from those of formaldehyde and showed a standard deviation of ± 1.5% at the mid-range of the calibration.

Table 2. Statistical data comparing the calibration of cortisone and formaldehyde by the procedure described

	(No. of calibration points, 6 per compound.)	
	Cortisone	Formaldehyde
Standard deviation of points (units of optical density)*	0.0084	0.0064
Slope (optical density/m-μmole)	0.0285	0.0302
Standard deviation of slope (optical density/m-μmole)†	0.00069	0.00052

t calculated, 2.96.
t – 1% level, 8 degrees of freedom, 3.36.
No difference is demonstrated between the slopes.
* The standard deviation of points expresses the deviation of points from the statistically calculated best line.
† The standard deviation of slope expresses the possible deviation of slope from that of the statistically calculated best line.

Other steroids, pregn-4-en-21-ol-3:20-dione (11-deoxycorticosterone), pregn-4-en-11β:21-diol-3:20-dione (Kendall’s compound B), pregn-4-en-11β:17α:21-triol-3:20-dione (Kendall’s compound F), pregn-4-en-17α:21-diol-3:20-dione (Reichstein’s compound S) and pregnane-3α:12α:20ξ:21-tetrol all gave one mole of formaldehyde/mole of steroid on bis-muthate oxidation.

Results have been expressed as m-μmoles rather than μg., as this arrangement makes the calibration independent of the molecular weight of the compound oxidized.

Notes on the method

Sources of error. The quantity of formaldehyde measured is so small that low blanks are essential to the method. High blanks arise from the presence of volatile material, in solvent or sample, which reacts with the chromotropic acid as though it were formaldehyde. The blanks are considerably higher if light is not excluded during the oxidation. The source of interfering material may be investigated by carrying out the determinations shown in Table 3.

The ‘unoxidized’ reagent blank is high with solvents which react with chromotropic acid and which are therefore unsuitable. The difference between the two reagent blanks shows the production of interfering volatile material resulting from the action of bismuthate upon the solvent. It is primarily these factors which dictate the choice of a solvent for the steroid and it is upon this basis that highly purified propionic acid has been selected from a large number of solvents tried. With freshly prepared propionic acid the difference between the ‘oxidized’ and ‘unoxidized’ reagent blanks at 10° is so small that the latter can be dispensed with. The propionic acid keeps well at 0° under N₂ but an ‘oxidized’ reagent blank should be carried out with each batch of determinations. Reducing the amount of propionic acid in the oxidation solution lowers the reagent blank, and the 6% aqueous propionic acid solution used in the method represents a compromise between good dissolution of the steroid and low blank values.

A second source of error may arise from the presence of volatile chromogenic material in the steroid preparation. Such interference is removed or minimized by standing all preparations over conc. H₂SO₄ and P₂O₅ in a vacuum desiccator (<1 mm. Hg); any material not removed by this treatment is reflected in the ‘unoxidized’ determination. The difference between ‘oxidized’ and ‘unoxidized’ determinations, less their respective blanks, represents the true formaldehydogenic steroids. It follows that when the reagent blanks are low and almost identical it is immaterial whether they are subtracted or not. It is, however, necessary to carry out an ‘oxidized’ reagent blank as a check on the quality of the reagents. When no volatile impurity is present, the reading of the ‘unoxidized’ determination becomes identical with that of the reagent blank and only the latter determination is necessary.

Table 3. Combinations of reagents necessary for the investigation of the sources of high blank values in the procedure

Factors in system	Reagent blanks		Determination	
	‘Oxidized’	‘Unoxidized’	‘Oxidized’	‘Unoxidized’
Steroid	.	.	+	+
Solvent	+	+	+	+
Bismuthate	+	.	+	.
Kieselguhr	.	+	.	+

Table 4. *Recovery of cortisone added to portions of urine extract*

(The extract was obtained by CHCl_3 extraction of a urine sample hydrolysed by β -glucuronidase. Cortisone (8 m- μ moles) was added in ethanol solution and the solvents were evaporated. The residue (not desiccated *in vacuo* with H_2SO_4 and P_2O_5) was dissolved in propionic acid (10 μl). The ratio of the amounts of 'formaldehydogenic steroid' found in the two unaugmented extracts is 2.58 as compared with 2.50, the ratio of volumes taken. Ox. and unox. refer to the 'oxidized' and 'unoxidized' procedures detailed in the text.)

		Extinctions			'Formalde- hydrogenic steroid' (m-μmoles)	Cortisone recovered (m-μmoles)
Material	Procedure	Observed	Less			
			Blank	Difference (Ox. - unox.)		
Blank	Ox.	0.041	.	.	.	.
	Unox.	0.043	.	.	.	.
Cortisone solution (0.1 ml.)	Ox.	0.279	0.238	0.238	.	7.94
	Unox.	0.041	0.000			
Extract (0.2 ml.)	Ox.	0.323	0.282	0.257	8.51	8.0
	Unox.	0.068	0.025			
Extract (0.2 ml.) + cortisone solution (0.1 ml.)	Ox.	0.548	0.507	0.497	16.5	7.9
	Unox.	0.053	0.010			
Extract (0.5 ml.)	Ox.	0.733	0.692	0.659	22.0	7.9
	Unox.	0.076	0.033			
Extract (0.5 ml.) + cortisone solution (0.1 ml.)	Ox.	0.971	0.930	0.898	29.9	
	Unox.	0.075	0.032			

Bismuthate oxidation. The quantity of sodium bismuthate used in the oxidation is of the order of a 100-fold excess; large variation in the amount used is without effect upon the result. This reagent is a very insoluble substance and frequent stirring of the oxidation mixture is necessary. Formaldehyde production from the C-17 side chains of three typical formaldehydogenic steroids—

—was substantially complete after 30 min. and there is no reason to suppose that the 'glycerol type' of side chain $\text{>COH.CO.CH}_2\text{OH}$ would require longer.

Purity of standards. Samples of cortisone and 11-deoxycorticosterone from several sources failed to yield formaldehyde quantitatively without preliminary purification. Several batches of cortisone, obtained by hydrolysis of cortisone acetate (Mattox & Kendall, 1951) without further purification, gave 0.7–0.96 mole of formaldehyde/mole. Moreover, when this material was desiccated *in vacuo* at 110°, in an attempt to obtain dry material, a further fall in formaldehydogenic capacity occurred (0.5 mole, based on final weight) and a reduction in weight was observed.

The cortisone used in the calibration procedure was obtained by hydrolysis of the acetate, but was purified by partition chromatography on Celite using 50% methanol as the stationary phase. The column was developed with benzene containing 10% ethyl acetate and the early fractions containing unhydrolysed acetate were rejected. The free cortisone was recrystallized from ether-methanol and dried over conc. H_2SO_4 at room temperature and 1 mm. Hg pressure. Prepared in this way the yield of formaldehyde was quantitative and the cortisone could be heated *in vacuo* at 110° without change.

Application of the method to urine extracts

The method has been applied to CHCl_3 extracts of urine hydrolysed by incubation at pH 5.0 with a preparation of calf-spleen β -glucuronidase. Table 4 shows the results of a typical determination at two levels and the good recovery of cortisone added to the extract. The values given for 'oxidized' and 'unoxidized' blanks show the magnitude of these figures in relation to the optical density due to the formaldehydogenic compounds measured.

DISCUSSION

The chief disadvantage of the use of periodic acid and stannous chloride in the determination of formaldehydogenic steroids lies in the high and variable blank figures obtained. This interference which arises, at least in part, from the end products of the reaction, is avoided by the use of bismuthate. As excess of this oxidant can be removed by centrifuging, it is possible, in the following stage, to heat the Conway units and so accelerate and improve the transfer of formaldehyde into the colour reagent. Inspection of the units during the transfer showed that although 30 min. are necessary for this full development of colour, the inner compartment containing the oxidized steroid is dry within 5–10 min. The increased speed of transfer in the modified Conway units is not accompanied by any loss of accuracy; on the contrary, the recoveries are high and show less variation than in determinations carried out in the no. 2 Conway units at lower temperatures.

As applied to extracts of biological material the value of results obtained by the formaldehydogenic method is, however, greatly reduced by lack of

information about the precursors of the formaldehyde; it is possible that some are non-steroid in nature. With the methods of steroid analysis available at the present time, it is not possible to determine what proportion of the formaldehyde formed during the oxidation is of steroid origin. The method has been of value in following extraction and concentration procedures.

SUMMARY

1. A micro method for the determination of 'formaldehydogenic steroids' using sodium bismuthate as the oxidant has been described.

2. The isothermal distillation and subsequent colorimetric determination of the formaldehyde takes place at 100° in a stoppered Conway unit. The determination is complete within 3 hr. and the method is suitable for batch determinations.

3. The method has an accuracy of $\pm 1.5\%$ in the range 5–25 m- μ moles.

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REFERENCES

- Analar Standards for Laboratory Chemicals* (1951). London: B.D.H. and Hopkin and Williams.
- Bassil, G. T. & Hain, A. M. (1950). *Nature, Lond.*, **165**, 525.
- Brooks, C. J. W. & Norymberski, J. K. (1952). *Chem. & Ind.* p. 804.
- Brooks, C. J. W. & Norymberski, J. K. (1953). *Biochem. J.* **55**, 371.
- Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric error*. 2nd ed. London: Crosby Lockwood and Son Ltd.
- Corcoran, A. C. & Page, L. H. (1948). *J. Lab. clin. Med.* **33**, 1327.
- Daughaday, W. H., Jaffe, H. & Williams, R. H. (1948). *J. clin. Endocrin.* **8**, 166.
- Hollander, V. P., Di Mauro, S. & Pearson, O. H. (1951). *Endocrinology*, **49**, 617.
- Lowenstein, B. E., Corcoran, A. C. & Page, L. H. (1946). *Endocrinology*, **39**, 82.
- MacFadyen, D. A. (1945). *J. biol. Chem.* **158**, 107.
- Mattox, V. R. & Kendall, E. C. (1951). *J. biol. Chem.* **188**, 287.
- Norymberski, J. K., Stubbs, R. D. & West, H. F. (1953). *Lancet*, **264**, 1276.
- Rigby, W. (1950). *J. chem. Soc.* p. 1907.
- Youden, W. J. (1951). *Statistical Methods for Chemists*. New York: Wiley.

The Biological Action of Substances Related to Thyroxine

7. THE METABOLISM OF BUTYL 4-HYDROXY-3:5-DIODOBENZOATE

BY 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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The antithyroxine compound, butyl 4-hydroxy-3:5-diiodobenzoate (BHDB) (Sheahan, Wilkinson & MacLagan, 1951), has been subjected to clinical trial on patients with thyrotoxicosis (Fraser & MacLagan, 1953). Advantage was taken of the opportunity thus presented of studying the nature of the substances excreted in the urine, in the hope of gaining some information concerning the mode of action of this drug.

It has been shown, in a preliminary account of this work (MacLagan & Wilkinson, 1951), that approximately half the urinary iodine appeared somewhat surprisingly as 3:5-diiodo-4-methoxybenzoic acid, most of the remainder being accounted for as 4-hydroxy-3:5-diiodobenzoic acid. The study has now been extended to a series of seven patients with thyrotoxicosis, two with myxoedema under

treatment with thyroxine, and to one normal volunteer. Similar excretion studies were also performed on rabbits and rats, in the hope of finding a suitable animal for further investigation of the unusual methylation process.

EXPERIMENTAL

Micro-analyses by Weiler and Strauss, Oxford. Melting points are uncorrected.

Isolation and identification of iodine-containing organic acids

(a) *From human urine.* The method employed may be illustrated by the following typical extraction procedure:

A 40 ml. sample for total iodine determination was removed from a 24 hr. specimen of urine (1260 ml.). The