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.

We should like to express our thanks to Prof. E. C. Dodds, F.R.S., for his interest in this work and to Dr V. Petrow and colleagues, Research Department, Messrs British Drug Houses, for the gift of pregnane-3 α :12 α :20 β :21-tetrol. We also wish to acknowledge the advice of Messrs Aimer Products, Camden Town, who manufactured the modified Conway units used in this investigation.

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The Biological Action of Substances Related to Thyroxine

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

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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

remainder was made acid to Congo red with HCl (20 ml.) and extracted with ether (800 ml.). The ether layer was separated and shaken with 2N-NaOH (50 ml.), and the ether used again for another seven similar extractions of the urine. The alkaline extracts were combined, boiled to expel ether, and acidified with HCl. The mixture was then set aside for 1 hr. to complete the precipitation of the acids, which were washed with water and dried at 100°. (Yield 780 mg., equiv. to 806 mg./day.) In this instance, further extraction of the urine furnished no additional acids, but in some cases it was necessary to extract as many as twelve times to remove all the excretion products. No allowance was made for the acids and phenols normally present in urine, but most of these are appreciably more soluble in water than are the iodinated benzoic acids, and it is unlikely that the crude acids isolated would contain more than traces of iodine-free materials.

The mixed acids were heated under reflux with anhydrous methanol (10 ml.) and H_2SO_4 (0.5 g.) for 3 hr.; the hot solution was then filtered through charcoal. After concentration to about 4 ml., the filtrate was treated with ice (20 g.) and saturated sodium acetate solution (20 ml.) to precipitate the mixed methyl esters, which were collected in a tared sintered-glass crucible. After washing with water, the well-drained solid was extracted three times with N-Na₂CO₃ (5 ml.) and the residue washed with water until free from alkali. The alkali-insoluble material was then dried at 60° to constant weight (283 mg.). Its m.p. was 82–90°, raised by crystallization from methanol to 95°, when colourless needles of methyl 3:5-diiodo-4-methoxybenzoate were formed. Admixture with an authentic specimen prepared by the method of Wheeler & Liddle (1910) did not depress the m.p. (Found: C, 26.1; H, 1.9; I, 60.6. Calc. for $\text{C}_9\text{H}_8\text{O}_3\text{I}_2$: C, 25.85; H, 1.9; I, 60.8%.)

The combined Na₂CO₃ extracts and washings were acidified without delay with acetic acid to precipitate methyl 4-hydroxy-3:5-diiodobenzoate (387 mg.), which was washed with water and dried at 100°. After crystallization from methanol it formed colourless needles, m.p. 168°. Wheeler & Liddle (1910) report 166–167°. Admixture with an authentic sample caused no depression of m.p. (Found: C, 24.2; H, 1.6; I, 62.7. Calc. for $\text{C}_9\text{H}_6\text{O}_3\text{I}_2$: C, 23.7; H, 1.5; I, 62.8%.)

In a preliminary experiment an attempt was made to separate the crude acids by fractional crystallization from aqueous ethanol. The less soluble product (m.p. 247°, decomp.) was at first thought to consist of the expected 4-hydroxy-3:5-diiodobenzoic acid (Paal & Mohr (1896) report m.p. 237° (decomp.), while Hartman & Rahrs (1943) found m.p. 278–279° (decomp.)), but the analytical results indicated that it was probably slightly impure 3:5-diiodo-4-methoxybenzoic acid. (Found: C, 24.4; H, 1.6; I, 62.2. Calc. for $\text{C}_9\text{H}_6\text{O}_3\text{I}_2$: C, 23.7; H, 1.5; I, 62.8%.) Repeated fractional crystallization finally raised the m.p. to 261° (decomp.), not depressed by admixture with an authentic sample of the methoxy acid (m.p. 261°, decomp.). Wheeler & Liddle (1910) reported m.p. 255–256° (decomp.). Unfortunately the losses at each stage in the crystallization procedure were such that there was insufficient of the pure material for analysis.

Methanol was selected for the esterification of the acids because methyl 4-hydroxy-3:5-diiodobenzoate is a readily crystallizable solid with a relatively high m.p. When it became clear that the metabolic product of BHDB consisted of the two acids, methanol still remained the alcohol

favoured for their separation because of the wide difference between the m.p.'s of the two methyl esters. However, in order to exclude the possibility that some unexpected methylation of the phenolic hydroxyl group occurred during esterification of the mixed acids with methanol, another experiment was performed in which the crude acid (1.69 g.) was treated with anhydrous ethanol (30 ml.) and H_2SO_4 (1 g.) by the procedure used for the methyl esters. The Na₂CO₃-insoluble residue (376 mg.), after crystallization from light petroleum (b.p. 60–80°), had m.p. 95°, not depressed by admixture with authentic ethyl 3:5-diiodo-4-methoxybenzoate (Wilkinson, Sheahan & MacLagan, 1951). Admixture with the corresponding methyl ester (m.p. 95°) produced a depression of 20°, and when mixed with ethyl 4-ethoxy-3:5-diiodobenzoate (m.p. 71°, Wilkinson *et al.* 1951), it melted at 60–65°. Acidification of the Na₂CO₃ extract gave ethyl 4-hydroxy-3:5-diiodobenzoate (1.043 g.), which crystallized from aqueous ethanol in colourless needles, m.p. 123°, not depressed by admixture with an authentic specimen (Sheahan *et al.* 1951).

(b) *From rabbit urine.* A young female rabbit (about 1 kg.) was injected intraperitoneally with 4 ml. BHDB (5% (w/v) in arachis oil) daily for 3 days. The urine (680 ml.) was collected over a period of 1 week from the time of the first injection. A sample removed for determination of total iodine indicated that the total urinary excretion during the period was 202 mg., i.e. 59% of the total dose. The remainder of the urine (620 ml.) was acidified to pH 2 with HCl and evaporated to dryness under reduced pressure. The dry residue was then extracted six times with 60 ml. of hot *n*-butanol. The combined extracts were then shaken five times with 20 ml. of 0.5N-NaHCO₃, and the combined aqueous layers were then boiled to expel butanol. The solution was cooled, acidified with acetic acid, and set aside for 1 hr. The precipitated acids were collected, washed with water and dried at 100° (254 mg.). An appreciable proportion of this solid was iodine-free and insoluble in methanol, but the methanol-soluble fraction was esterified as described for human urine. The alkali-soluble ester obtained (70 mg.) was identified as methyl 4-hydroxy-3:5-diiodobenzoate, but the alkali-insoluble material (40 mg.) was very impure and no crystalline product was isolated from it.

In an attempt to discover the fate of the iodine-containing material not accounted for in the above experiment, a female rabbit of 1400 g. was injected intraperitoneally with 10 ml. arachis oil containing 0.5 g. BHDB labelled with ¹³¹I (Wilkinson, 1953). The urine was collected daily and counted in a Veall liquid counter. The daily rates of excretion for the 4 days following the injection were 64, 8, 2 and 4% of the administered drug. The first day's urine (40 ml.) was investigated according to the following scheme, the values in parentheses being the percentages of the total radioactivity of the urine, and the weights of material isolated.

It can be seen that a sample of acid (255 mg.) containing 90% of the urinary radioactivity was obtained by combining the solid precipitated with HCl with that produced by extraction of the filtrate with butanol and ether. Extraction with butanol was repeated until the radioactivity of the aqueous layer ceased to be reduced (about twelve times). Extraction was then continued with ether, but this produced no significant further yield. Only about 1% of the residual radioactivity remained in the aqueous fraction, which would be expected to contain iodide if present; treatment with AgNO₃ and HNO₃ removed about half of this radioactivity, indicating that about 0.5% of the urinary

iodine consists of iodide. It thus appears that losses in the previous experiment with the 1 kg. rabbit were due to incomplete extraction. When esterified with methanol, as described above, the acids (255 mg.) gave 200 mg. of the hydroxy ester; 20 mg. of unchanged acids were recovered, losses due to solubility accounted for a further 17 mg., whilst the alkali-insoluble product of esterification weighed 12.4 mg. This was gummy, and attempts to purify and identify its components failed.

(c) *From rat urine.* A supply of urine (100 ml.) from rats fed on a diet containing 0.8% BHDB was kindly provided by Mr W. E. Sprott. This was extracted by the method used for the rabbit urine, a total of 219 mg. of acid being obtained. On esterification this gave 193 mg. of the hydroxy ester and 26 mg. of an alkali-insoluble gum from which no positive evidence for the presence of the methoxy ester could be obtained.

RESULTS

Table 1 summarizes the results of the investigations into the nature of the urinary iodine. The acids isolated from the urine were separated by conversion into their methyl esters followed by extraction of the soluble methyl 4-hydroxy-3:5-diiodobenzoate with alkali. The residue consisted of the corresponding 4-methoxy ester.

Practically all the iodine found in the urines can be accounted for by the presence of these iodinated benzoic acids, and consequently the amount of BHDB degraded to iodide must have been relatively small. This observation was confirmed by the failure to detect iodide by any chemical method tried. The limit of sensitivity of the oxidizing agent:starch test in the presence of relatively large quantities of organically combined iodine appears to be about 20 mg./l. of urine. The ceric sulphate:arsenite test (Kolthoff & Sandell, 1934) is not applicable to

urines containing 4-hydroxy-3:5-diiodobenzoic acid, since this, and many other iodophenols such as 3:5-diiodotyrosine, thyroxine and 4-hydroxy-3-iodobenzoic acid are almost as effective as iodide in catalysing the oxidation of arsenite by ceric sulphate. None of the urines gave any colour with ferric chloride under a variety of conditions, thus excluding the presence of any appreciable quantity of gallic acid (3:4:5-trihydroxybenzoic acid), which would presumably have been formed if the organic iodine were converted by hydrolysis into inorganic iodide.

Through the kindness of Dr J. D. Kendall, of Ilford Ltd., one sample was shown by electrometric titration using a silver:silver iodide electrode to contain an amount of iodide corresponding to the excretion of about 19 mg. I/day. This particular specimen had been stored for several days, and it is not known to what extent decomposition of urinary acids had contributed to this figure. Fresh urines tested in our laboratory by the same method gave values in the range 5–10 mg./day, but difficulties were experienced as a result of 'poisoning' of the electrode by an unidentified constituent present in several urines. Since this work was completed, it has been found that much better results are obtained when a silver:silver sulphide electrode is employed (Wilkinson, Sprott, Bowden & MacLagan, 1954).

Most of the patients treated with BHDB were thyrotoxic, but results with some non-hyperthyroid subjects are included for comparison. It will be seen from Table 1 that all the specimens of urine examined contained 3:5-diiodo-4-methoxybenzoic acid and, although the series is too short for

Table 1. *The nature of the urinary iodine after the administration of BHDB (1-3 g./day)*

(The values for acids recovered are based upon the ether-extractable, alkali-soluble solid obtained and those for esters isolated have been calculated from the weights of methyl 4-hydroxy-3:5-diiodobenzoate and methyl 3:5-diiodo-4-methoxybenzoate obtained when samples of the mixed acids were esterified with methanol and the products separated. The iodine content of the mixed acids has been calculated on the assumption that the mixture consists of 4-hydroxy-3:5-diiodobenzoic acid and its methyl ether in the same proportions as the esters isolated from it.)

Patient and sex	Diagnosis	Acids recovered (mg./day)	Esters isolated		Methoxy esters (% of total esters)	Iodine content of acids (calc.) (mg./day)	Total iodine (found) (mg./day)	Organic iodine (% of total I)
			Hydroxy (mg./day)	Methoxy (mg./day)				
1, F.	Thyrotoxicosis	920	414	418	50	589	610	97
2, M.		—	130*	188*	59*	—	—	—
3, F.		806	399	296	42	518	545	95
4, F.		1810	1118	403	27	1160	1230	94
5, F.		855	707	104	13	556	592	94
6, M.		1178	792	246	24	757	713	106
7, M.		456	169	212	56	292	282	103
		1060	—†	—†	30-40†	678	664	102
		570	—†	—†	30-40†	364	362	101
8, M.	Simmond's disease, with secondary myxoedema, under treatment with thyroxine	627	392	137	26	387	446	87
9, F.	Myxoedema, under treatment with thyroxine	454	351	38	10	279	296	94
		222	173	17	9	136	164	83
		626	427	83	16	387	416	93
10, M.	Normal volunteer	—	264	89	25	—	—	—

* Urine not extracted quantitatively.

† Solutions accidentally discarded. Values quoted were estimated from incomplete data.

a definite conclusion to be drawn, there is a suggestion that the degree of methylation is higher in hyperthyroid patients than in the others. Thus the percentage of the total esters methylated varies from 13 to 59% in the thyrotoxic group (average 39%), while the other cases gave values between 9 and 26% (average 21%).

In contrast with the observations on human urine, no methoxy acid was isolated from rabbit or rat urine, though there is a possibility that it may have been present in very small amounts.

DISCUSSION

Since BHDB is readily hydrolysed *in vitro* (Wilkinson, 1953), the presence of 4-hydroxy-3:5-diiodobenzoic acid in the urines of patients treated with the drug was not unexpected. The degree of breakdown to inorganic iodide was also of interest. This was detectable, but must have been very slight in view of the good recovery of total iodine in the forms of 4-hydroxy-3:5-diiodobenzoic acid and its methyl ether.

The presence of 3:5-diiodo-4-methoxybenzoic acid in all the human urines examined was surprising in view of the absence of references in the literature to the methylation of phenolic hydroxyl groups in the animal body. Such processes have long been recognized in plants, for instance during

the formation of lignin, although Lederer (1949) has drawn attention to the absence of similar examples in animals. But the methoxy acid has been shown in the present work to be a true metabolic product and not an artifact.

There is some suggestion that the degree of methylation may be related to the degree of thyroid activity (Table 1). If this is so, it might be correlated with the well-known occurrence of creatinuria in thyrotoxicosis. Although this is usually considered to be due to thyrotoxic myopathy, an alternative explanation may be suggested. Du Vigneaud, Cohn, Chandler, Schenck & Simmonds (1941) have shown that the biosynthesis of creatine involves the methylation of guanidoacetic acid, and it seems possible that in thyrotoxic conditions there may be either a greater availability of methyl donors, e.g. methionine, choline or betaine, or an increase in the reactivity of the enzymes concerned. The observations of Kinsell, Hertz & Reinfenstein (1944), who reported that the degree of creatinuria exhibited by patients with thyrotoxicosis was reduced by the administration of testosterone but increased by methyl testosterone, could be interpreted on similar lines.

Our experiments with rats and rabbits did not demonstrate any significant methylation, so that these animals could not be used to provide further information on this process.

SUMMARY

1. A study has been made of the substances excreted in the urine after the administration of butyl 4-hydroxy-3:5-diiodobenzoate (BHDB) to man, rabbits and rats.

2. The principal excretory products found in the urine of seven patients with thyrotoxicosis, two patients with myxoedema under treatment with thyroxine, and one normal volunteer were 4-hydroxy-3:5-diiodobenzoic acid and its methyl ether. These two acids accounted for 90% or more of the total urinary iodine. Less than 2% of the urinary iodine consisted of iodide.

3. The excretion of the methoxy acid appeared to be greater in patients with thyrotoxicosis than in other subjects studied. The implications of this observation in relation to processes of biological methylation are discussed.

4. In rabbits and rats treated with BHDB, only the hydroxy acid was isolated from the urine.

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8. THE EFFECTS OF BUTYL 4-HYDROXY-3:5-DIODOBENZOATE ON THE DEIODINATION OF DIODOTYROSINE AND THYROXINE IN RATS

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The demonstration of the presence of 3:5:3'-triiodo-L-thyronine in the thyroid gland and in circulating blood by Gross & Pitt-Rivers (1952*a*, 1953*a*) and Roche, Lissitzky & Michel (1952*a*, *b*), along with the parallel observations that it has several times the physiological activity of thyroxine (Gross & Pitt-Rivers, 1952*b*, 1953*b*; Gross, Pitt-Rivers & Trotter, 1952; Tomich & Woollett, 1953), has led to a reassessment of the nature of the thyroid hormone. Gross & Pitt-Rivers (1952*c*, 1953*b*) have suggested that triiodothyronine is the peripheral thyroid hormone. This substance may be produced by the condensation of diiodotyrosine with monoiodotyrosine in the gland, or alternatively, as a result of the deiodination of thyroxine, a process which may take place either in the gland or elsewhere (Gross & Pitt-Rivers, 1952*a*, 1953*b*; Michel, 1952; Roche, Lissitzky & Michel, 1953).

Experiments in which the thyroxine inhibitor, butyl 4-hydroxy-3:5-diiodobenzoate (BHDB), depressed the oxygen consumption of mice treated with thyroxine, but actually enhanced that produced by treatment with triiodothyronine (Sheahan, Wilkinson & MacLagan, 1951; MacLagan, Sprott & Wilkinson, 1952), support the latter hypothesis. It was suggested that by exercising a restraint on deiodination processes, BHDB would reduce the rate of formation of triiodothyronine thereby causing partial inhibition of its effects, whilst at the same time by retarding the breakdown of the triiodo compound it would extend its period of effective biological action. The experiments described in this paper were designed to test the first part of this hypothesis.

We decided first to use 3:5-diiodo-L-tyrosine in model experiments in rats, the results of which have