0.5–1.0 ml. is taken. With bromine values from 3 to 10 mg./100 ml. the error is within $\pm 5\%$. A minimum of about 50 μg . bromine is desirable with the method, although with special care, fair estimates may be made with considerably less. Many hundreds of determinations have been performed with this method and it has been found very reliable and trouble-free. It is well adapted to the determination of bromine in many samples in a short time.

The method has also been used with whole blood and erythrocytes. In the latter case, haemolysis with an equal volume or 2 vol. of water has preceded precipitation. In the presence of large amounts of protein, methanol yields a more discrete precipitate and may be substituted for ethanol. If whole erythrocytes or haemoglobin solutions are ashed, the potential interference of ferric ions in freeing iodine from iodide should be borne in mind.

However, the concentration of phosphate present under the conditions described adequately suppresses this action.

SUMMARY

1. Details are given for the application of the Van der Meulen reaction—the oxidation of bromide to bromate by hypochlorite—to the determination of bromide in blood, cerebrospinal fluid and urine.

2. The method is not affected by the presence of chloride in moderate concentration, but iodide reacts quantitatively like bromide.

3. This semi-micro method is well adapted to the determination of bromide in body fluids following bromide administration.

4. The accuracy is about $\pm 1\%$ for samples containing 50 μg . bromide in 0.5 ml. fluid.

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A Note on the Hydrolysis of the Urinary Conjugated Adrenocortical Steroids by β -Glucuronidase Preparations

BY R. I. COX AND G. F. MARRIAN

Department of Biochemistry, University of Edinburgh

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It was reported by Kinsella, Doisy & Glick (1950) that the yields of 'reducing lipids' extractable from the urine of human subjects by a chloroform-ether mixture could be increased 3- to 5-fold by a preliminary incubation of the urine with an enzyme preparation obtained from *Escherichia coli* which was rich in β -glucuronidase activity. At about the same time Corcoran, Page & Dustan (1950) reported 10- to 20-fold increases in extractable formaldehydrogenic and glycogenic material after incubation of urine with a commercial preparation of β -glucuronidase. Shortly afterwards it was independently observed by Cox & Marrian (1951) that the yield of chloroform-extractable 'formaldehydegenic steroid' obtainable from urine could be increased about tenfold, compared with that obtained after hydrolysis at pH 1.0 for 24 hr., by

incubating the urine, or butanol-extractable material from urine, with ox-spleen β -glucuronidase preparations. Following these pioneering studies, preliminary treatment of urine with bacterial or animal-tissue β -glucuronidase preparations has been widely used in quantitative studies on the urinary glycogenic, reducing, and formaldehydegenic steroids, as well as in work directed towards the isolation and characterization of such steroids (cf. Venning, 1951, 1952; Kinsella, Baggett & Glick, 1951; Daughaday, Farr & Houghton, 1951; Corcoran, Dustan & Page, 1951; Cohen, 1951; Baggett, Glick & Kinsella, 1952; Schneider, 1952; Romanoff, Wolf & Pincus, 1952; Wilson, 1952; Bayliss, 1952). Cox (1952) has also recently shown that the yield of acetaldehydegenic material, determined by periodate oxidation, which can be

extracted from urine with chloroform can be significantly increased by a preliminary treatment of the urine with β -glucuronidase preparations.

Although it seemed probable that the increased yields reported in the literature of extractable 'adrenocortical steroids' following incubation with β -glucuronidase preparations were largely or wholly due to hydrolysis of urinary steroid glucuronides by β -glucuronidase, it was considered by the present authors that it would be unwise to assume that this was necessarily the case in view of the relative crudeness of the enzyme preparations used and the conspicuous absence of adequate controls in most of the published experimental work. Thus the possibility could not be ignored that the liberation of free steroids might have been due to the hydrolysis of steroid conjugates other than glucuronides by an enzyme in the bacterial or tissue preparation other than β -glucuronidase. Furthermore, the possibility also had to be considered that the observed increased yields of reducing, formaldehydogenic or acetaldehydogenic material might have been the result of hydrolysis by enzymic action of precursors in the enzyme preparation itself: e.g. the liberation of glycerol (formaldehydogenic) from incompletely defatted enzymic preparations by lipase action.

These possibilities have now been investigated in a series of experiments in which the chloroform-extractable formaldehydogenic and acetaldehydogenic substances were determined in the butanol-extractable material from the urine of normal men after incubation with ox-spleen β -glucuronidase (A), and the values compared with those obtained in control experiments in which the urine extracts were incubated with boiled enzyme (B), in which the enzyme was incubated alone (C), and in which the extracts were incubated with the enzyme in the presence of saccharate (D).

EXPERIMENTAL

β -Glucuronidase preparations. Ox-spleen extracts were prepared and standardized for β -glucuronidase activity with phenylglucuronide as described by Cox (1952).

Collection and extraction of urine. Four 24 hr. urine specimens were collected from normal men using 100 ml. butanol as a preservative for each. The pH of each specimen was roughly adjusted to 7 by stirring in 50 g. NaHCO_3 , and the urine was then extracted three times with equal volumes of butanol. The extract was evaporated under reduced pressure in the water bath, the last traces of butanol being removed by azeotropic distillation under reduced pressure after the addition of ethanol. In order to remove the greater part of the free unconjugated steroids from these residues they were washed three times with 400 ml. portions of dry boiling CHCl_3 .

Incubations. Each residue, prepared as described above, was dissolved in 300 ml. water and 30 ml. M-acetate buffer (pH 4.5) added to the solution. After adjustment to pH 4.5 with acetic acid the solution was made up to 400 ml.

with water and divided into three equal portions. The under-mentioned mixtures were then made up and incubated at 37° for 48 hr. after the addition of 15 ml. CHCl_3 .

A, 150 ml. urine extract; 15 ml. water; 5000 glucuronidase units.

B, 150 ml. urine extract; 15 ml. water; 5000 glucuronidase units (boiled).

C, 155 ml. water; 10 ml. M-acetate buffer (pH 4.5); 5000 glucuronidase units.

D, 150 ml. urine extract; 15 ml. 0.1 M-saccharate (adjusted to pH 4.5); 5000 glucuronidase units.

After the completion of the incubation, each mixture was extracted three times with 200 ml. portions of CHCl_3 . The CHCl_3 extract was washed once with 200 ml. 0.1 N-NaOH and three times with 100 ml. volumes of water. The alkali washing and the first two water washings were back-extracted with 100, 50 and 50 ml. respectively of CHCl_3 , and each back-extract added to the main bulk of CHCl_3 before proceeding with the next washing. The washed CHCl_3 extract was dried by standing over Na_2SO_4 (20 g.) for 20 min., filtered, and evaporated to dryness under reduced pressure.

Table 1. *Liberation of formaldehydogenic substances (FS) from urine extracts by incubation with ox-spleen β -glucuronidase preparations*

(A, extract + enzyme; B, extract + boiled enzyme; C, enzyme blank; D, extract + enzyme + saccharate.)

Urine batch no.	FS (as mg. HCHO/24 hr. urine specimen)			
	A	B	C	D
3	0.690	0.093	0.048	0.101
	0.672	0.104	0.032	0.109
4	0.846	0.099	0.060	0.139
	0.786	0.116	0.053	0.149
6	0.401	0.020	0.023	0.066
	0.451	0.035	0.007	0.060
8	0.922	0.129	0.083	0.192
	0.942	0.123	0.070	0.202

Table 2. *Liberation of acetaldehydogenic substances (AS) from urine extracts by incubation with ox-spleen β -glucuronidase preparations*

(A, extract + enzyme; B, extract + boiled enzyme; C, enzyme blank; D, extract + enzyme + saccharate.)

Urine batch no.	AS (as mg. CH_3CHO /24 hr. urine specimen)			
	A	B	C	D
3	0.482	0.092	0.013	0.077
	0.485	0.089	0.010	0.089
4	0.422	0.069	0.013	0.076
	0.443	0.069	0.025	0.063
6	0.253	0.021	0.000	0.025
	0.245	0.023	0.008	0.032
8	0.319	0.044	0.034	0.061
	0.335	0.042	0.027	0.057

Determinations in duplicate of formaldehydogenic and acetaldehydogenic substances were carried out on the residue by the method of Cox (1952). The results of experiments on four separate 24 hr. urine specimens are shown in Tables 1 and 2.

DISCUSSION

On comparing the values obtained from the series *A* incubations with those obtained from the boiled enzyme controls (*B*) it will be seen that the enzymic hydrolysis increased the yields of extractable formaldehydogenic and acetaldehydogenic substances about 7- to 15-fold and 5- to 11-fold respectively. The amounts of formaldehydogenic and of acetaldehydogenic substances determined in the enzyme blank series (*C*) were about half or less than half of those determined in the boiled enzyme controls (*B*), showing that the amounts of extractable formaldehydogenic and acetaldehydogenic substances liberated during the incubations from precursors in the enzyme preparations were of no quantitative significance. By comparing the values obtained in series *D* with those in series *A* and *B* it can be seen that saccharate largely, but not completely, inhibited the liberation of extractable formaldehydogenic and acetaldehydogenic substances by the enzyme preparation. Since Karunairatnam & Levvy (1949) showed that saccharate is a powerful inhibitor of β -glucuronidase, this constitutes strong evidence that the observed liberation of formaldehydogenic and acetaldehydogenic substances involves the hydrolysis of glucuronides by β -glucuronidase. (Levy (1952) has shown that the inhibiting action of saccharate solutions upon β -glucuronidase activity is due to the presence of saccharo-1:4-lactone.) Since the values obtained in series *D* are slightly higher than those in the boiled enzyme controls (*B*) it is possible that enzymic hydrolysis of some other type of conjugate may also be involved to a small extent in the liberation of formaldehydogenic and acetaldehydogenic substances. It seems more probable, however, that the concentration of saccharate employed was not

sufficiently high to inhibit β -glucuronidase activity entirely.

It should be pointed out that the evidence presented here does not exclude the possibility that the observed increases in extractable formaldehydogenic and acetaldehydogenic substances resulting from incubation of urine extracts with β -glucuronidase preparations may be partly due to the hydrolysis of glucuronides of chloroform-extractable formaldehydogenic and acetaldehydogenic substances other than steroids.

SUMMARY

1. The liberation of chloroform-extractable formaldehydogenic and acetaldehydogenic substances on incubation of urine extracts with ox-spleen preparations rich in β -glucuronidase activity has been studied.

2. Control incubations with enzyme preparations but no urine extract have shown that substantially all of the formaldehydogenic and acetaldehydogenic substances liberated arise from precursors in the urine and not by the enzymic decomposition of precursors in the spleen preparation itself.

3. The liberation of formaldehydogenic and acetaldehydogenic substances from urine extracts by incubation with ox-spleen preparations is largely inhibited in the presence of 8×10^{-3} M-saccharate. This provides strong support for the view that this liberation is due to the hydrolysis of glucuronic acid conjugates by β -glucuronidase.

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