Indirect Analysis of Corticosteroids

4. A NOTE ON THE ROLE OF CHLORIDE AND UREA IN THE BISMUTHATE TREATMENT OF URINES

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Smith & Tompsett (1955) recently described some reactions of sodium bismuthate in an aqueous acetic acid (50 %, v/v) solution of sodium chloride (0.75 %, w/v). They found that the reaction product obtained under these conditions from androstane-diol (presumably 5 α -androstane-3 β :17 β -diol) was strongly chromogenic in the Zimmermann reaction. Under identical conditions *allopregnanediol* (3 β :20 α -diol?) gave a weakly chromogenic product. These results were demonstrated to depend upon the presence of chloride, and were interpreted as arising from the oxidation of chloride to chlorine and the latter agent's ability to oxidize 17-hydroxyandrostanes and 20-hydroxypregnanes to the corresponding ketones. In another group of experiments the authors submitted extracts of urinary steroid conjugates (Edwards, Kellie & Wade, 1953) to treatment with bismuthate followed by hydrolysis with hot hydrochloric acid. They found that the presence of chloride during the oxidative step caused a substantial decrease of the isolated chromogens, and they attributed this finding to an increased destructive effect during the hydrolytic procedure 'due to the formation of unstable chlorine addition or oxidation products'. They concluded that, since urine contains varying and often considerable (up to 1 %) amounts of sodium chloride, treatment of the urine with sodium bismuthate followed by acid hydrolysis will introduce the following two variable factors affecting the measurement (as Zimmermann chromogens) of native 17-oxosteroids and those formed from 17-hydroxycorticosteroids: (i) the formation of additional chromogens by oxidation of native 17-hydroxyandrostanes, and (ii) the destruction of chromogens during the hydrolytic procedure. The purpose of the present communication is to examine Smith & Tompsett's (1955) conclusions in the light of data already published and those now presented.

The reliability of the treatment of urine with sodium bismuthate as a means of converting native 17-hydroxycorticosteroids into 17-oxosteroids or of cleaving glucuronides was tested by criteria designed to reveal any gross interference with the specificity or efficacy of the reagent. The following instances may be cited.

(i) Addition to the urine of a 17-ketone (dehydroepiandrosterone), or of various 17-hydroxycorticosteroids followed by treatment with bismuthate (or by a sequence of reactions terminating with bismuthate treatment) and then by acid hydrolysis, in all cases resulted in the isolation of an amount of Zimmermann chromogens reasonably close to that expected and comparable to that obtained in experiments carried out in aqueous acetic acid (Norymberski, 1952; Norymberski, Stubbs & West, 1953; Norymberski & Stubbs, 1954; Appleby, Gibson, Norymberski & Stubbs, 1955; Norymberski & Stubbs, 1956). Clearly, the destructive effect of urinary chloride was in no case manifested.

(ii) When a pregnancy urine was treated with sodium bismuthate and then extracted, pure pregnane-3 α :20 α -diol was isolated in the same yield as from a sample of the same urine that was hydrolysed with hydrochloric acid (Norymberski & Sermin, 1953). Here then, any release of chlorine from urinary chloride failed to oxidize the 20-hydroxyl group.

(iii) Addition of dehydroepiandrosterone to eleven randomly chosen urines, followed by treatment with borohydride, then with bismuthate, and finally with hot hydrochloric acid, in no case produced a significant increase in the chromogenic values of the extracts (Appleby *et al.* 1955). Oxidation by chlorine of androst-5-ene-3 β :17 β -diol (from reduc-

Table 1. *Determination of 17-oxosteroids in bismuthate-treated urine after addition of androst-5-ene-3 β :17 β -diol and NaCl*

Added to the urine		Chromogens found (μ g./ml.)†
Androst-5-ene-3 β :17 β -diol (μ g./ml.)*	NaCl (%, w/v)*	
0	0	21.8 $\pm$ 2.6
100	0	22.0 $\pm$ 1.0
100	0.5	22.8 $\pm$ 1.0
100	1.0	20.5 $\pm$ 1.3
100	2.5	21.1 $\pm$ 0.8
100	5.0	21.1 $\pm$ 0.8

* Concentration in the urine.

† Mean values of four measurements and standard deviations in terms of dehydroepiandrosterone.

Table 2. *Determinations of 17-oxosteroids in bismuthate-treated urine after addition of dehydroepiandrosterone and NaCl*

Added to the urine		Chromogens found ($\mu\text{g./ml.}$)†	Recovery of dehydroepiandrosterone ($\mu\text{g./ml.}$)
Dehydroepiandrosterone ($\mu\text{g./ml.}$)*	NaCl (%, w/v)*		
0	0	18.4 $\pm$ 1.1	—
0	1.0	19.6 $\pm$ 0.9	—
22.5	0	37.0 $\pm$ 3.3	18.6 $\pm$ 3.5
22.5	1.0	37.5 $\pm$ 2.2	18.1 $\pm$ 2.4

* Concentration in the urine.

† Mean values of six measurements in terms of dehydroepiandrosterone.

Table 3. *Effect of urea on the oxidation of androst-5-ene-3 β :17 β -diol by bismuthate in the presence of chloride*

NaCl* (%, w/v)	Urea* (%, w/v)	Zimmermann chromogens† ($\mu\text{g./sample}$)‡
0	0	2.2 $\pm$ 0.6 (4)§
0.3	0	27.0 $\pm$ 0.5 (4)
0.6	0	36.3 $\pm$ 0.5 (4)
0.9	0	42.5 $\pm$ 1.2 (4)
1.2	0	48.6 $\pm$ 1.9 (4)
1.5	0	47.2 $\pm$ 4.2 (16)
1.5	0.05	45.0 $\pm$ 1.3 (4)
1.5	0.10	29.9 $\pm$ 1.8 (4)
1.5	0.15	7.6 $\pm$ 0.9 (4)
1.5	0.20	3.9 $\pm$ 0.5 (4)§
1.5	0.25	3.7 $\pm$ 1.4 (8)§
1.5	0.50	4.4 $\pm$ 0.4 (4)§
1.5	0.75	4.7 $\pm$ 0.4 (4)§
1.5	1.0	4.0 $\pm$ 0.8 (8)§
1.5	1.5	3.4 $\pm$ 1.7 (8)§
1.5	2.0	3.9 $\pm$ 1.2 (8)§
1.5	2.5	4.0 $\pm$ 1.0 (8)§
1.5	3.0	3.8 $\pm$ 1.6 (8)§

* Concentration in 50% acetic acid.

† Mean values and standard deviations in terms of dehydroepiandrosterone; figures in parentheses denote numbers of replicate measurements.

‡ Corresponding to 80 $\mu\text{g.}$ of treated androst-5-ene-3 β :17 β -diol.

§ Atypical colour.

Table 4. *Bismuthate oxidation of cortisone in the presence of urea or NaCl or both*

NaCl* (%, w/v)	Urea* (%, w/v)	Recovery of cortisone† (%)
0	0	84 $\pm$ 9
0	3	91 $\pm$ 7
1.5	0	95 $\pm$ 6
1.5	3	95 $\pm$ 7

* Concentration in 50% acetic acid.

† Calculated on the basis of the compound's conversion into androst-4-ene-3:11:17-trione; mean values and standard deviations from six measurements of Zimmermann chromogens.

tion of added dehydroepiandrosterone with borohydride) was not apparent.

Unless the unlikely assumption is made that all urines employed in these experiments contained subnormal amounts of chloride, the evidence cited shows that urinary chloride does not exert the types of interference predicted by Smith & Tompsett. More direct and conclusive evidence has now been obtained by adding androst-5-ene-3 β :17 β -diol and sodium chloride to a urine specimen subsequently treated with bismuthate and then with hot hydrochloric acid. The results (see Table 1) give no indication of the oxidation of the diol to a 17-ketone. Further, the recovery of dehydroepiandrosterone from a urine specimen treated as above was unaffected by addition of chloride (see Table 2).

Urea, the major solute of the human urine, is known to undergo the Hofmann amide reaction with hypochlorites (Schestakow, 1905). It seemed to us that, when present in the system $\text{NaBiO}_3\text{--NaCl--CH}_3\text{.CO}_2\text{H--H}_2\text{O}$, urea may well compete for the effective non-specific oxidizing agent (probably hypochlorite) and consequently inhibit its other reactions, without affecting the specific glycol-cleaving action of bismuthate. This hypothesis was tested by treating androst-5-ene-3 β :17 β -diol with the reagent system given above in the presence of urea. The results (see Table 3) clearly demonstrate that relatively small quantities of urea (0.13 mole/mole of NaCl) effectively inhibit the oxidation of the diol to a 17-ketone. It is reasonable to assume that the native urea of the human urine plays the same protective role when the urine is treated with bismuthate. Finally it was shown that the presence of urea or chloride, or both, had no significant effect on the formation of Zimmermann chromogens from cortisone treated with bismuthate (see Table 4).

All experimental procedures were essentially as previously described (see preceding paper and references cited therein). They were performed on samples of urine (2.0 ml.) diluted with an equal volume of acetic acid, or on samples (2.0 ml.) of aqueous acetic acid (50 %, v/v) solutions, whichever were applicable.

SUMMARY

1. The oxidation of 17-hydroxyandrostanes to 17-ketones in the system $\text{NaBiO}_3\text{--NaCl--CH}_3\text{.CO}_2\text{H--H}_2\text{O}$ (Smith & Tompsett, 1955) was confirmed. It was shown that urea effectively inhibits this reaction.

2. Treatment of the human urine with sodium bismuthate in the presence of acetic acid, followed by the reductive removal of surplus reagent and then by treatment with hot hydrochloric acid, in no case resulted in the oxidation of 17-hydroxyandrostanes to 17-ketones or in the destruction of

17-ketones. The failure of urinary chloride to induce these reactions was attributed to the inhibitory effect of native urea.

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Factors which Interfere in the Manometric Assay of Monoamine Oxidase

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The reaction which monoamine oxidase is believed to catalyse may be represented by

in which one molecule of oxygen is absorbed for each molecule of substrate oxidized. When tyramine is used as substrate the theoretical oxygen uptake is apparently obtained, the observed value in the

present experiments being 1.9 atoms/molecule of tyramine. Previous authors (Kohn, 1937; Pugh & Quastel, 1937; Blaschko, Richter & Schlossman, 1937; Luschinsky & Singher, 1945) have pointed out that crude monoamine oxidase preparations also contain catalase, cytochrome *c* and cytochrome oxidase, aldehyde oxidase and peroxidase, enzymes whose combined action (see Fig. 1) would be

Fig. 1. Reactions likely to occur when tyramine is incubated with crude monoamine oxidase.