Urinary Steroids of Cats

2. CORTICOSTEROID-LIKE SUBSTANCES

By SARA BORRELL

Instituto G. Marañón, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain

(Received 12 November 1962)

Adrenal steroid secretion by cats has been studied by Bush (1951, 1953) and reviewed by Dorfman (1959). The cat adrenal secretes both cortisol and corticosterone. Arteta & Borrell (1958) and Borrell (1958) have reported studies of urinary excretion of 17-hydroxy corticosteroids (Porter-Silber chromogens). Cats appear to excrete in urine very low concentrations of 17-hydroxy corticosteroids, all as free steroid. It was possible to demonstrate an increase after the administration of adrenocorticotrophic hormone (ACTH) (Borrell, 1958).

Taylor & Scratcherd (1960, 1962) have obtained evidence that the major pathway for excretion of metabolites of progesterone and corticosterone is via the bile and the faeces. Borrell (1963) has shown that there is essentially no 17-oxo steroid in cat urine. In the present paper, the results of an investigation into the substances reducing blue tetrazolium (blue tetrazolium chromogens) of cat urine are reported. Steroids among these reducing substances would include the 17-hydroxy corticosteroids, the α -ketolic C_{21} steroids and α, β -unsaturated ketones such as Δ^4 -3-oxo steroids.

METHODS

Animals. Adult male and female cats weighing 2.5–3 kg. were used. All animals were prepared by operation according to the method of Arteta & Borrell (1958) to obtain complete collection of urine.

Treatment of urine. Corticoid extraction was always performed on the day of collection except for the pooled urine experiments reported in Table 2, in which the urine was stored at 4° for 3 days while a large enough pool was collected. Urines were then processed by five different methods:

Method A. The urine was extracted according to the methods used by Borrell (1958). Briefly, the pH was adjusted to 6.5–7, the urine extracted once with chloroform and the chloroform washed with 0.1N-sodium hydroxide. This yields free corticosteroids.

Method B. This method is a slight modification of the procedure of DeCourcy, Bush, Gray & Lunn (1953). The pH was adjusted to 7, the urine extracted three times with chloroform (once with 1 vol. and twice with 0.5 vol.). The combined chloroform extracts were washed with 0.1N-sodium carbonate, water and 0.1N-acetic acid, and dried over anhydrous sodium sulphate. The dried residue from the

first chloroform extraction (total extract) was dissolved in 1% (v/v) methanol in benzene and the benzene extracted ten times with water. The combined water extracts were extracted three times with chloroform to obtain the water-soluble steroids in a form that could be evaporated *in vacuo* to a dried residue. Porter-Silber and blue tetrazolium chromogens were determined in the total extract, the benzene-soluble fraction and the water-soluble fraction.

Method C. The urines were hydrolysed by the two-phase solvolysis procedure of Burstein & Lieberman (1958). After solvolysis, both ether and chloroform were used to extract the steroids. The dried residues were partially purified by the same procedure as that described under method B.

Method D. The procedure described by Edwards (1960) for the hydrolysis and extraction of steroid conjugates was followed except for omission of the enzyme hydrolysis. Colour reactions were run on the benzene and ethyl acetate extracts and after solvolysis.

Method E. Urines were refluxed at 100° for 6 hr., at neutral pH, as described by Borrell (1961). Chloroform extracts were made and separated into benzene-soluble and water-soluble fractions by the same procedure as that described in method B.

Hydrolysis with β -glucuronidase. As in Borrell (1958), 100 Sigma units of bacterial β -glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) plus 10 000 units of penicillin (Merck and Co. Ltd.) were added to each 25 ml. of cat urine. Incubation was carried out at 37° for 22 hr.

Colour reactions. The Porter-Silber reaction was performed by the modification described by Borrell (1958). For the blue tetrazolium chromogen, 2 ml. portions were used. To each of these were added 0.1 ml. of tetramethylammonium hydroxide and 0.1 ml. of the blue tetrazolium solution, prepared by the method of Chen, Wheeler & Terwell (1953). Tubes were incubated for 2 hr. in the dark at room temperature. After incubation, 1 ml. of 95% (v/v) ethanol was added and the extinction read at 510 m μ in a Unicam spectrophotometer (SP. 600). Standards of 10 and 20 μ g. of cortisone were run with each set of samples.

RESULTS

Table 1 shows the unconjugated Porter-Silber-chromogen and blue tetrazolium-chromogen values for urines of normal male and female cats, obtained by two different extraction procedures. There was no demonstrable difference between the values of urines from male and female cats, so all the results have been combined. With chloroform extracts made at pH 7, mean values for both the chromogens in the total extracts obtained by method A agreed

with the values in partially purified water-soluble fractions obtained by method B. Porter-Silber and blue tetrazolium chromogens were then studied in the water-soluble fraction after the administration of ACTH (12 i.u., intramuscularly). In the first 24 hr. after the administration of ACTH the Porter-Silber chromogens increased significantly ($P < 0.01$), and they returned to normal in the second 24 hr. The blue tetrazolium chromogens did not follow this pattern. They did not change in the first 24 hr., and fell significantly in the second 24 hr. Since the blue tetrazolium chromogens include the 17-hydroxy corticosteroids, there would appear to have been a decrease in the other materials reducing blue tetrazolium in the first 24 hr. as well as in the second 24 hr.

The direction of the changes seemed to indicate that the blue tetrazolium chromogens might not be the usual steroid metabolites. DeCourcy *et al.* (1953) showed that all the blue tetrazolium-reducing material in chloroform extracts of unhydrolysed human urine (pH 7) went into the water-soluble fraction when partitioned between water and benzene. The chromogenic material in neutral chloroform extracts of cat urine made by method B did not partition completely into the aqueous phase. Although the mean value reported in Table 1 for the water-soluble fraction obtained by method B agreed with the mean value for total extracts obtained by method A, there was additional material in other urine extracts which was

benzene-soluble. In 12 experiments, 28-31% of the blue tetrazolium chromogen measured in the total chloroform extract from method B was benzene-soluble.

Attempts were also made to determine whether or not there are conjugated blue tetrazolium chromogens in cat urine. Samples of pooled urine were subjected to a variety of procedures. The solvolysis procedure of Burstein & Lieberman (1958) for steroid sulphates, and the acid hydrolysis procedure of Edwards (1960) for total conjugates, were not satisfactory for studying cat urines. Very high blanks were obtained in both chromogenic reactions, and large amounts of interfering pigments could not be removed even by partition between water and benzene. Hydrolysis and extraction by method E (Borrell, 1961) gave values for total and water-soluble blue tetrazolium chromogen that were slightly higher than the values obtained in extracts of other samples of the same pooled urines not hydrolysed (Table 2). This indicated the possibility that there were some conjugated blue tetrazolium-reducing compounds. Method E hydrolyses sulphates.

To ascertain whether some conjugated material was present as glucosiduronate, three pooled urines from groups of four cats were separately incubated with β -glucuronidase and then extracted and partially purified by method B. Control samples were extracted without addition of enzyme and without incubation. In two of the three pooled

Table 1. Porter-Silber and blue tetrazolium chromogens in extracts of unhydrolysed cat urines

Experimental details are given in the text.

Status of cat	Extraction method	No. of cats	No. of determinations	Excretion of chromogens ($\mu\text{g. of cortisone equivalent/24 hr.}$)	
				Porter-Silber chromogen	Blue tetrazolium chromogen
Normal	A	16	59	7.9 $\pm$ 0.48*	—
Normal	A	21	10	—	43.1 $\pm$ 6.30
Normal	B, water-soluble fraction	8	16	7.3 $\pm$ 0.66	40.9 $\pm$ 5.25
Given ACTH (12 i.u.): 0-24 hr. urine sample	B, water-soluble fraction	8	8	20.4 $\pm$ 2.30	38.9 $\pm$ 4.18
Given ACTH (12 i.u.): 24-48 hr. urine sample	B, water-soluble fraction	8	8	6.3 $\pm$ 1.30	21.4 $\pm$ 3.95

* Data of Arteta & Borrell (1958).

Table 2. Distribution of total blue tetrazolium chromogen between benzene-soluble and water-soluble fractions of urinary extracts obtained by different procedures

Experimental details are given in the text.

Method	No. of determinations	Concn. of blue tetrazolium chromogen ($\mu\text{g. of cortisone equivalent/100 ml.}$)		
		Total	Benzene-soluble	Water-soluble
B (unhydrolysed)	3	147	39	85
E (hydrolysed)	3	160	44	101

Table 3. *Water-soluble blue tetrazolium chromogens of pooled cat urines before and after hydrolysis with β -glucuronidase*

Experimental details are given in the text.

Pool	Sex	Concn. of blue tetrazolium chromogens (μ g. of cortisone equivalent/100 ml.)	
		Unhydrolysed	Hydrolysed
1	M	42.4	75.2
2	F	46.4	95.6
3	M	65.5	69.2
4	M	29.6	43.6
5	F	20.4	73.1

urines there was an increase in blue tetrazolium chromogen after hydrolysis by glucuronidase. The results are given in the first three lines of Table 3. Two more pooled urines were similarly treated, except that the control samples that were extracted without incubation also contained buffer and β -glucuronidase. The values in the fourth and fifth lines are therefore all corrected for a small chromogen blank obtained in an extract of glucuronidase and buffer alone. Again an increase was seen after hydrolysis with β -glucuronidase. The differences in cortisone equivalent/100 ml. of urine from one pool to another (Tables 2 and 3) reflect differences in diuresis among the cats.

Because of the interfering yellow chromogen present in the cat urine, it was not possible to determine the 17-ketogenic steroids by the method of Appleby, Gibson, Norymberski & Stubbs (1955). The Allen (1950) correction was not applicable, since the absorption spectrum of the yellow chromogen was not linear over the range 440–600 m μ . In several experiments, no significant increase in absorption at 520 m μ could be demonstrated.

DISCUSSION

Since the cat adrenal secretes approximately five times as much cortisol as it does corticosterone (Bush, 1951, 1953; Hechter & Pincus, 1954; Dorfman, 1959), it is likely that the excretion of 17-hydroxy corticosteroid metabolites would be greater than the excretion of metabolites from 17-deoxy steroids. However, instead of being only slightly higher than the concentration of 17-hydroxy corticosteroids (Porter–Silber chromogens) in urine, the concentration of blue tetrazolium-reducing chromogens was six times as high.

Other unexpected findings with regard to the blue tetrazolium-positive material include: (1) the abnormally large percentage that is soluble in benzene, as compared with the material in human urine (DeCourcy *et al.* 1953); (2) the relatively enormous increase after hydrolysis with β -gluc-

uronidase as compared with the small increase after sulphate hydrolysis; and, most important of all, (3) the failure to increase after the administration of ACTH.

For the 17-oxo steroids (Borrell, 1963) and for the 17-hydroxy corticoids (Borrell, 1958; Taylor & Scratcherd, 1962), the evidence seems to indicate clearly that the urine is not a major pathway of excretion. To evaluate the importance of the urinary pathway of excretion for corticoid metabolites without the dihydroxyacetone side chain, it is necessary to characterize further the blue tetrazolium-chromogenic material, to ascertain whether the conjugated blue tetrazolium-positive material, unlike the free, increases after the administration of ACTH, and to see if there is another quantitatively more important pathway than the urinary one for the excretion of steroids by the cat. Even if all the material reducing blue tetrazolium consisted of corticosteroid metabolites, the amounts excreted per day are in microgram quantities. Bush (1953) has estimated the maximum rate of corticosteroid secretion per day per cat as 15 mg. Therefore, it seems unlikely that the urinary pathway is of importance for this class of compounds either.

SUMMARY

1. In a search for the urinary metabolites of adrenal steroids, the materials reducing blue tetrazolium were measured in extracts made by several procedures, before and after hydrolysis.

2. There is about six times as much free blue tetrazolium chromogen as Porter–Silber chromogen in cat urine.

3. After hydrolysis with β -glucuronidase, an increase in blue tetrazolium chromogens was seen in four out of five experiments.

4. There is some doubt as to whether these blue tetrazolium chromogens are corticosteroid metabolites because about one-third is benzene-soluble when partitioned between benzene and water and because there is no increase after the administration of adrenocorticotrophic hormone.

The author thanks Dr Betty L. Rubin for her advice and assistance in preparing this manuscript.

REFERENCES

- Allen, W. M. (1950). *J. clin. Endocrin.* **10**, 71.
- Appleby, J. I., Gibson, G., Norymberski, J. K. & Stubbs, R. D. (1955). *Biochem. J.* **60**, 453.
- Arteta, J. L. & Borrell, S. (1958). *Rev. Iber. Endocrin.* **5**, 511.
- Borrell, S. (1958). *Biochem. J.* **70**, 727.
- Borrell, S. (1961). *J. clin. Endocrin. Metab.* **21**, 1321.
- Borrell, S. (1963). *Biochem. J.* **89**, 51.
- Burstein, S. & Lieberman, S. (1958). *J. biol. Chem.* **233**, 331.
- Bush, I. E. (1951). *J. Physiol.* **115**, 12p.

- Bush, I. E. (1953). *J. Endocrin.* **9**, 95.
- Chen, C., Wheeler, J. & Terwell, H. E. (1953). *J. Lab. clin. Med.* **42**, 749.
- DeCoursey, C., Bush, I. E., Gray, C. H. & Lunnon, B. (1953). *J. Endocrin.* **9**, 401.
- Dorfman, R. I. (1959). In *Comparative Endocrinology*, p. 613. Ed. by Gorbman, A. New York: John Wiley and Sons, Inc.
- Edwards, R. W. H. (1960). In *Steroids: Chromatographic and Electrophoretic Techniques*, chapter 23, p. 409. Ed. by Smith, I. London: William Heinemann Ltd.
- Hechter, O. & Pincus, G. (1954). *Physiol. Rev.* **34**, 459.
- Taylor, W. & Scratcherd, T. (1960). *Biochem. J.* **77**, 22 p.
- Taylor, W. & Scratcherd, T. (1962). *Proc. int. Congr. Hormonal Steroids, Milano*, p. 142.

Biochem. J. (1963) **89**, 57

The Function of Bile Salts in Fat Absorption

THE SOLVENT PROPERTIES OF DILUTE MICELLAR SOLUTIONS OF CONJUGATED BILE SALTS

By A. F. HOFMANN*

Department of Physiological Chemistry, University of Lund, Lund, Sweden

(Received 10 December 1962)

That bile salts influence the absorption of dietary fat has been known for many years, but the mechanism of their action has remained unclear. Hartley (1936) proposed the term 'amphipathic' to refer to compounds possessing dissymmetric polar and non-polar regions, which, because of their unusual molecular structure, exhibit a certain characteristic behaviour in water under appropriate conditions. Such substances behave as detergents when micellar solutions are formed above their respective Krafft points. Hartley (1936) pointed out that bile salts should be classified as amphipathic compounds. Subsequently, dye-solubility studies by McBain, Merrill & Vinograd (1941) as well as osmotic-pressure measurements (Roepke & Mason, 1940) confirmed Hartley's prediction that micellar aggregation should take place in aqueous bile salt solution above the critical micellar concentration. For reviews on micelle formation, see Hartley (1936, 1955), McBain & Hutchinson (1955), Pankhurst (1953) and Garrett (1961).

Ekwall, Sten & Norman (1956) and Ekwall, Fontell & Sten (1957) have studied the detergent properties of certain of the bile salts, and have compared them with paraffin-chain soaps. The development of greatly improved synthetic methods (Norman, 1955) and chromatographic methods (Norman, 1953; Sjövall, 1959*a*) enabled Norman (1960) to compare the critical micellar concentrations of the glycine- and taurine-conjugated bile acids, which are predominant in human bile.

Micellar solubilization of the products of pancreatic lipolysis has been proposed as an im-

portant step in fat absorption (Bergström & Borgström, 1955), and recent work has demonstrated a micellar phase of bile salt, fatty acid, monoglyceride and cholesterol in human-intestinal content during fat absorption (Hofmann & Borgström, 1962).

The present paper gives observations on the solvent properties of dilute micellar bile salt solutions *in vitro* under conditions similar to those present in the human small-intestinal lumen during fat absorption, with respect to Na⁺ ions (0.15 M), pH (6.3) and temperature (37°) (Borgström, Dahlqvist, Lundh & Sjövall, 1957). With these experimental conditions, micellar solutions are not formed by the salts of the unconjugated bile acids (Hofmann, 1961*a, b*) and the experiments described below deal with only the glycine and taurine conjugates, the forms occurring in man (Sjövall, 1959*b*).

The predominant products of pancreatic lipolysis are amphiphiles, that is, compounds also having dissymmetric polar and non-polar regions, but which are poorly soluble in water (Lawrence, 1959, 1961). They are soluble in certain detergent solutions forming a liquid-crystal phase or co-acervate which contains the compound, detergent and water when the amphiphile is present in excess. Because of the complexity of amphiphile-detergent interactions, it seemed important to characterize the solvent properties of micellar bile salt solutions with respect to the simplest solutes possible. A representative non-polar solute, *trans*-azobenzene (Hartley, 1938*b*), and a polar or amphiphilic compound, glycerol 1-mono-oleate (Hofmann, 1961*a*), were chosen as prototypes. Alkyl alcohols, the amphiphilic substances most commonly employed

* Present address: The Rockefeller Institute, New York 21, N.Y., U.S.A.