LXIX. CAPON COMB GROWTH-PROMOTING SUBSTANCES ("MALE HORMONES") IN HUMAN URINE OF MALES AND FEMALES OF VARYING AGES

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(Received 18 January 1937)

SOME years ago we used to test the urine from men for comb growth-promoting substances by extracting it with benzene and by determining the growth of the comb of capons produced by such extracts. The amount found varied from more than 12 to less than 4 units per litre of urine, the average being 4-6 units per l. (The unit is $\frac{2}{8}$ of the quantity which, when divided into 8 portions of which 2 portions are injected daily on 4 consecutive days, causes an increase of the comb surface of more than 15% in the majority of capons used for one experiment; cf. Freud *et al.* [1932] and Gradstein [1935].)

Unpublished experiments carried out in this laboratory by K. David have proved that the response of capons to the comb growth-promoting preparations shows seasonal variations. The sensitivity is low during March, April and May, but remains constant for the rest of the year. The experiments described in this paper, with few exceptions, were carried out during the winter months. During the past 18 months we have always controlled our test by comparing the result with the effects of equivalent quantities of pure androsterone on 6 capons.

Our unit has approximately the same effect as 0.1 mg. of androsterone, which amount is accepted as 1 international unit of male hormone. The comb growth-promoting potency of an extract was the same, whether the urine was faintly acid or faintly alkaline during the extraction, and alkaline extraction which yields a smaller dry residue was adopted by preference.

The urine was sometimes used fresh but was more often kept for some days, during which time it had become more or less alkaline. Funk *et al.* [1929] noted that extraction of highly acidified urine gave a better yield than extraction of neutral or weakly acid urine. According to Gallagher & Koch [1934] shaking with strong acid increases the yield. Adler [1934] obtained 27-40 units of comb growth-promoting potency per l. by benzene extraction of strongly acidified male urine. He found that a physiologically inactive extract could be obtained with butyl alcohol, and that by subsequent heating with strong acid, the inactive extract could be converted into a potent material, which promotes the growth of the comb of capons.

Very recently Callow [1936] stated in a short preliminary note that by extracting strongly acidified urine from normal men and women, 26 and 29 international units respectively per l. of urine were obtained. We found that extraction of acid-hydrolysed human male urine by the method described below usually yielded 30-50 units per l., although as little as 15 and as much as 170 units were occasionally obtained. Human female urine similarly gave an average

of 40 units per l. By treatment of neutral urine with boiling benzene or trichloroethylene not more than 2–12 units could be extracted from a quantity which was known to contain 30–50 units per l.

The following method of extraction was used.

1–2 l. of urine, or more if required, were neutralized with HCl. (When urine of one individual was tested, the total urine passed during 4 days was usually used.) The urine was treated with 150 ml. of 25 % HCl per l., and then heated on the water-bath under reflux for 6 hours with one-third to one-half of its volume of benzene or alternatively trichloroethylene. The extraction was repeated with a similar volume of fresh solvent for the same time. The combined extracts were repeatedly washed with water until the last portion of water was neutral. The solvent was evaporated and the residue taken up in 10 ml. of benzene. The benzene solution was taken up in oil and the benzene removed with a current of air. This solution in oil was injected into capons.

The active form of comb growth-promoting substances of urine may be readily separated from the inactive one by extraction of the neutral urine with benzene. After benzene extraction the urine is reduced in volume, treated with alcohol and filtered. The benzene fraction contains the active, and the alcoholic filtrate, the inactive substances; the latter may be rendered physiologically active by treatment with strong acid.

It is not certain that active substances are excreted at all by the kidneys; perhaps the first slight conversion of the inactive precursors occurs in the bladder. (The comb growth-promoting substances in the testes seem only to occur in the active form, for the same quantity of hormone is extracted whether the extraction is carried out from neutral or acid solution.)

Urines from men and women were treated with varying amounts of HCl, and then extracted by the method as described, in order to find the concentration of acid which gave the best yield. These experiments are shown in Table I.

Table I

Exp.	Sex	Without addition of acid	After addition of 25 % HCl per l. urine			
			20 ml.	50 ml.	150 ml.	300 ml.
1	♂	<10 units	30 units	30 units	40 units	40 units
2	♂	<10 "	20–30 "	40 "	50 "	40 "
3	♀	<10 "	30 "	30 "	40 "	40 "

In Exp. 1 the maximum yield was reached after the addition of 150 ml. of HCl, in Exp. 2 after addition of 50 ml. of acid. In Exp. 1 the urine, which had been treated with 300 ml. of HCl per l. and extracted, was then treated with a further 300 ml. of 25 % HCl and then again extracted twice for 6 hours with boiling trichloroethylene, but no more active material could be recovered. The maximum yield from the female urine was attained by addition of 150 ml. 25 % HCl per l. In other experiments one portion of urine was extracted after treatment with 70 ml. 25 % HCl per l. and another portion of the same urine was extracted after treatment with 300 ml. 25 % HCl per l. In all these experiments no more active material was extracted from the more strongly than from the less strongly acidified urine; within the experimental error the yields were the same.

The following experiment shows that the addition of less than 20 ml. of 25 % HCl per l. of urine distinctly increased the yield compared with that obtained by extraction from neutral solution. Three portions of mixed urine

from men collected during 2 days were extracted with hot benzene, one without the addition of HCl, another after being made acid to Congo red and the third after the addition of 150 ml. of 25 % HCl per l. The results are given in Table II.

Table II

Exp.	Without addition of acid	Acid to Congo red	After addition of 150 ml. of 25% HCl
202	<8 units	30 units	100-80 units
204	<8 „	12½ „	75 „

In the first of these two experiments about 30 % of the inactive substances had been activated in the urine made acid to Congo red ; in the second experiment about 20 % had been activated. Sometimes it may happen that activation by acid does not proceed in urine made acid to Congo red ; in such cases even after prolonged heating no appreciable increase in the yield occurs.

The following experiment shows that the inactive fraction is easily hydrolysed. One portion of a sample of male urine was extracted 3 times under reflux with boiling benzene for 4 hours. The combined benzene extracts contained less than 4 units of comb growth-promoting material per l. of urine extracted. The residue from the benzene extraction was now extracted after being strongly acidified and yielded 35 units per l. Another portion of the same sample of urine was autoclaved for 5 hours at 10 atmospheres and then extracted without acidification 3 times for 4 hours with boiling benzene. The benzene extract contained 25 units per l. of urine. By extracting the residue after strong acidification less than 10 units per l. of urine were obtained. Therefore by heating the neutral urine at 10 atmospheres the benzene-insoluble inactive substances were almost completely converted into the benzene-soluble form with some loss, perhaps due to decomposition.

Other experiments have shown that the comb growth-promoting extracts soluble in fat solvents give no better yield when the urine is kept, as is the case with oestrogenic extracts from urine.

By extraction of fresh male urine without acidification 7 comb growth units per l. were obtained. A portion of the same urine kept for 4 weeks and extracted in the same way gave similarly 7 units per l.

For quantitative estimation of the potency of extracts no material advantage was derived from purification. It may be mentioned however that considerable purification was obtained by shaking the benzene extract with a 10 % solution of KOH, all the active material remaining in the benzene layer.

It may be seen from Table III that comb growth-promoting substances were found in almost every urine examined. Healthy men of 20-34 years of age excreted an average of 40-50 units per l. of urine, the values varying from 15 to 170 units per l. In spite of the smaller number of urine samples tested, it may be concluded, however, that women's urine also during the active sexual period contains a quantity of comb growth-promoting substances similar to that of men's urine. Boys and girls of 5 years of age excrete 5-15 units per l. At puberty the excretion rises to the value found in adults during the active sexual period. Old and senile men also excrete the hormone, but, as is shown in Table III, to a less extent than middle-aged men. Old women excrete 5-10 units per l. of urine. Pregnant women, and women 1 year after ovariectomy excrete detectable quantities of comb growth-promoting material.

The regularity with which comb growth-promoting material is excreted by human beings of both sexes at varying ages suggests its alimentary origin. The

Table III. *The comb growth-promoting and oestrogenic substances of human urine from males and females of varying ages*

U./l. = units per l. of urine. U./T. = units per day

The figures not bracketed are the numbers of comb growth-promoting units per l. and the bracketed figures are the numbers of oestrogenic units found. The oestrogenic potency is expressed in international units, the unit being the quantity giving the oestrogenic effect of 0.1 γ oestrin.

A. Males

Various mixed urines from 10 to 20 men	No.	1	10	12	15	H. 62	17	106	116	120	202
	U./l.	30 or more	20	30	25	45	35	50	40	50	80

From results of N. V. Organon, mixed urine from ± 200 men, in many 1000 l.;average yield U./l. = ± 40

Urines from individual males of 20-34 years of age	No.	Fr.	Fr.	O.	K.	H.	H. I	H. II	de J.	D.	M.
	U./l.	35-40	45	15	20	60	170	130	50	80	70
	U./T.				20	90	225	145	65	80	70

Urines from boys 5-15 years of age	No.	L.	v. d. L.	M.	D.	S.	K.
	U./l.	8	15	30	50	10-14	20
	U./T.	8	9	30	50	± 6	—
	Age	10	15	14	14	14	5

Mixed urine from boys of 5 years of age: 15 units per l.

Urine from very old men (senile)	Pa. 92 years of age:	<15 units per l. (50)					
	No.	Gr. I	Gr. II	v. d. H. I	v. d. H. II	v. d. H. III	
	Age (years)	80	80	79	79	79	
	U./l.	35 (<25)	50 (25)	<20 (250)	5 (100)	<20 (20)	
	U./T.	40	45	—	—	—	
Urine from men 59-67 years of age	No.	We. I	We. II	Di.	Kr. I	Kr. II	Kr. III
	Age (years)	59	59	67	66	66	66
	U./l.	40 (>200)	5 (125)	30 (30)	15 (10)	12	12
	U./T.	40	5	35	15	7	20

B. Females

Mixed urines	No.	11	13	16	18	113	
	U./l.	45	50-60	75	70	40	
Urine from individual females of 20-26 years of age	No.	W. I	W. II	Me. B. I	B. II	W. III	S. v.d.L. K.
	U./l.	15	17	70	60	40-50	16 60 30 60-65
	U./T.	22½	21	—	65	55-70	20 45 20 65-70
Girls	No.	W. 5	L. 5	L. 5			
	Age (years)	12	14	14			
	U./l.	12	10	12			
	U./T.	12	5	8			

Mixed urine from girls of 5 years of age: 5 units per l. (50)

Urine from old women	No.	Ch.	L.	Ma.	Ma. I	Ma. II	Ma. III
	Age (years)	65	67	72	72	72	72
	U./l.	10 (10)	7 (75)	11 (80)	<15	<13	<9
	U./T.	—	—	11	—	—	—
Urine from pregnant women (6-8 months pregnant)	No.	K.	de J.	W.	P.		
	U./l.	8	30	25	10		
	U./T.	8	45	16	—		
Urine from women 1 year after ovariectomy	No.	K. I	Kr.	Mu.			
	U./l.	9 (<10)	12 (<10)	<6 (60)			

results given in Table IV, however, make it unlikely that such is the case. The active material in the urine of 2 old men, fed on exactly the same food, was estimated over a period of 2 months.

Table IV

Period during which urine was collected	Units of male sex hormone in urine of			
	de Kr., 87 years of age		Fr., 79 years of age	
	Per l.	Per day	Per l.	Per day
18-20. ix	30 (75)	22.5	—	—
22-25. ix	25 (135)	19	—	—
26. ix.-3. x	40	—	< 10 (20)	—
4-8. x	—	—	< 8 (40)	< 5
9-12. x	20	10	< 8 (75)	< 5
13-16. x	15	15	—	—
17-20. x	15	10	< 10	—
23-28. x.	15	10	< 5	< 3
29. x.-2. xi	30	25	< 12	< 6
3-6. xi	20	15	< 8	< 5
7-11. xi	40	20	< 8	< 5
12-15. xi	30 (70)	10	< 12 (50)	< 6
16-19. xi	25 (90)	17	< 12 (50)	< 8
20-23. xi	20 (60)	15	< 8 (50)	< 5

The figures in brackets are the oestrogenic activities of the urines in international units.

Table IV shows that the comb growth-promoting material excreted by de Kr., 87 years of age, was 10-25 units daily, the urine being collected as a rule over periods of 3-5 days. The tests in the urine of Fr., 79 years of age, carried out on the expectation of a content of 8-10 units per l., yielded completely negative results, so that probably there was no active material present at all.

A further series of experiments was carried out to determine if the excretion of comb growth-promoting substances varied during the menstrual cycle in women. The urine from a 21-year-old normal woman was collected in 5-8 days' portions over a period of 1½ months. The results are tabulated in Table V.

Table V

No.	Date	Times during which urine was collected	Units of comb growth-promoting substances	
			Per l.	Per day
K 1	12-17. vi	12-7 days before onset of menstruation	35	42
K 2	17-24. vi	7-1 days before onset of menstruation	15	20
K 3	24-29. vi	5 days during menstruation	35	35
K 4	29. vi-7. vii	24-16 days before onset of menstruation	55-60	55-60
K 5	7-15. vii	16-8 days before onset of menstruation	15	15
K 6	15-23. vii	8-1 days before onset of menstruation	30	22½

The highest excretion of comb growth-promoting substances occurred during the post-menstrual period. It would be necessary, however, to carry out further experiments before expressing definite views about the excretion of comb growth-promoting material during the various phases of the genital cycle in healthy women.

A similar experiment was carried out with a healthy man. The excretion of comb growth-promoting substances in the urine was estimated regularly during 1½ months. The results are given in Table VI.

No cyclic variations could be recognized in the comb growth-promoting material excreted by this man.

Table VI

Date ...	3-4. vi	5. vi	7. vi	12-13. vi	14-15. vi	17-18. vi	19. vi
No. of units:							
Per l.	40	35	70	70	70	100	40
Per day	30	35	50	60	65	80	30
Date ...	20. vi	22. vi	24. vi	25-26. vi	28-29. vi	3. vii	4. vii
No. of units:							
Per l.	40	60	±70	30	80-100	>40	±30
Per day	35	55	±55	50	64-80	>40	±35
Date ...	7-8. vii	9. vii	10-11. vii	15-16. vii	17. vii	18. vii	
No. of units :							
Per l.	30	75	50	<50	60	60	
Per day	30	75	45	<45	60	60	

The urines of several animals were tested, but the quantities of active substances found were never of the same order as those present in the urine of human beings. The urines from 5 male dogs, one gelding, one mare and two pregnant mares were examined. Tests which would have shown the presence of 5 units per l. were completely negative.

Recently, Kober, in the research laboratories of Organon, Oss (unpublished experiments) found 1 unit per l. of urine, by working up large quantities of the urine from pregnant mares.

The bloods of both steers and cows always contained less than 1 unit of comb-augmenting material per l. Human blood contained less than 20 units per l.

The behaviour of the lipid-insoluble comb growth-promoting substances towards alkali is very different from that of lipid-insoluble oestrogenic substances. The lipid-insoluble fraction of oestrogenic substances is saponified by alkali to yield the lipid-soluble active extract; the lipid-insoluble comb growth-promoting substances in urine remain lipid-insoluble and inactive after the same treatment. The course of a few experiments with male urine may make this clear.

1. Out of a sample of 10 l. urine 2 l. were heated in the neutral condition with benzene under reflux 3 times for 4 hours. The combined benzene extracts were evaporated, taken up in oil and tested as usual. This fraction contains the lipid-soluble comb growth-promoting material present in the urine. $7\frac{1}{2}$ units per l. were found. The remainder of the urine was acidified as usual to Congo red, then 150 ml. of 25% HCl were added per l. urine and the whole heated under reflux with benzene 3 times for 4 hours. The combined benzene solutions were washed with water until the last portions of water were neutral. The benzene was evaporated and the residue taken up in oil. 50 units per l. were found after the acid extraction, making $57\frac{1}{2}$ units in all.

2. Out of the same sample of urine 2 l. were heated for 4 hours with 200 g. KOH under reflux, then neutralized and heated as usual with benzene under reflux 3 times for 4 hours. $7\frac{1}{2}$ units per l. were found. The remainder of the urine was divided into 2 parts. To 1 part 150 ml. of 25% HCl were added as usual and the whole heated under reflux 3 times for 4 hours. In the benzene extract 50 units per l. were found. The second part (= 1 l.) of the remainder of urine was heated under reflux 10 hours longer after addition of 100 g. KOH. After neutralization the urine was heated again with benzene 3 times for 4 hours. Less than 10 units per l. were found in these combined benzene extracts.

This experiment proves that no increase in yield of the lipid-soluble active fraction results from heating the alkaline urine for 10 hours.

The cause of the low yield under these circumstances cannot be the destruction of the hormone by alkali, because after the alkaline extraction an acid extraction with heating gave a yield of 50 units per l., which is the same as that

obtained by direct extraction from acid solution without previous treatment with alkali.

The possibility remained that alkaline hydrolysis might still be obtained by boiling with alcoholic KOH. The following method was used to investigate this possibility.

4½ l. fresh urine from the same sample were heated with butyl alcohol 3 times for 6 hours in the neutral condition. The combined butyl alcoholic extracts, after evaporation of the solvent, were dissolved in water. The mixture containing the free and lipoid-insoluble active materials was heated 3 times for 4 hours with benzene. In the pooled benzene extracts 7½ units per l. were again found. The watery residue, amounting only to 150 ml., was divided into 2 parts. One part (=50 ml. corresponding with 1½ l. urine) was heated again with strong acid under reflux 3 times for 4 hours. In the combined benzene extracts 50 units were found per l. urine.

To the other part (=100 ml. corresponding with 3 l. urine) 3 vols. alcohol (300 ml.) were added and the mixture was heated after addition of 6 g. KOH for 4 hours under reflux, then neutralized and the alcohol was nearly completely evaporated. This last residue was heated with benzene 3 times for 4 hours under reflux. Less than 7½ units were present in the benzene extracts. However if the watery residue were treated afterwards with 25% of HCl as usual, 50 units were found again per l. urine.

From these results it is apparent that even with alcoholic KOH no increase in yield of comb growth-promoting substances could be obtained. The fact that after extraction of the same residue acidified 50 units per l. were found, again confirms the assumption that no active material has been destroyed by the treatment with alcoholic KOH.

All extractions were made on a boiling water-bath.

SUMMARY

1. Like the oestrogenic substances, the comb growth-promoting substances contained in urine exist in at least two modifications of which the greater part is water-soluble and the lesser part fat-soluble. The water-soluble part is converted into the fat-soluble by treatment with acid, or by heating under pressure but not by treatment with alkali. As far as these experiments go only the fat-soluble form causes growth of the comb of capons.

2. On keeping the urine at room temperature the amount of fat-soluble active fraction does not increase, in contrast with the oestrogenic substances.

3. The urine of men up to 40 years of age usually contained 40–50 units per l., but the values varied from 15 to 170 units per l.

4. The urine of women during the active sexual period contained about the same quantity of comb growth-promoting material as did male urine, usually 30–60 units per l.

5. Even after the disappearance of sexual activity, both in men and in women, the presence of comb growth-promoting substances was demonstrable in the urine.

6. Similarly boys and girls excrete comb growth-promoting substances in their urine before puberty. Boys excrete about 15 units per l., and girls about 5 units per l.

7. The urine of women 6–8 months pregnant contained 8–30 units per l. of comb growth-promoting substances.

8. Women 1 year after ovariectomy excreted as much as 12 units of comb growth-promoting substances per l. of urine.

9. It is unlikely that the comb growth-promoting substances found in the urine arise, in appreciable quantity, from the food.

10. The urine of a healthy woman examined during two succeeding menstrual periods showed an increased comb growth-promoting potency during the post-menstrual period. No cyclic type was apparent in the variations in the urinary excretion of comb growth-promoting substance in a healthy man whose urine was examined regularly during 1.5 months.

REFERENCES

- Adler (1934). *Nature, Lond.*, **133**, 798.
Callow (1936). *Lancet*, ii, 565.
Freud, de Fremery & Laqueur (1932). *Pflüg. Arch. ges. Physiol.* **229**, H. 6.
Funk, Harrow & Lewja (1929). *Proc. Soc. exp. Biol., N.Y.*, **26**, 569.
Gallagher & Koch (1934). *Endocrinology*, **18**, 107.
Gradstein (1935). *Arch. int. Pharmacodyn.* **51**, 1.

Note added 8 March 1937. Quite recently Kochakian [1937, *Endocrinology*, **21**, 60] describes "male hormone" estimation in the urine of males. His results are not comparable with ours on account of his different method of assay without comparison with 0.1 mg. androsterone (the international unit).