On the determination of reducing corticosteroids*

by

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The adrenocortical steroids reducing properties were demonstrated by Reichstein and col. (10) as been due to the presence of an α-ketol group in the side chain.

Talbot and col. (14) used Nelson's alkaline cupric reagent (8) on the determination of reducing corticosteroids, but the slight solubility of these compounds in the reagent gives low results. Nikolaichuk (9) has also used Nelson's reagent in blood and adrenal corticosteroids assay.

The reduction of blue tetrazolium was introduced by Chen and Tewell (1) and it seemed to be specific for α-ketol groups; recently, Sulkowitch and col. (13) have used this method for the estimation of urinary reducing corticosteroids.

Heard and Sobel (4) working with Folin-Wu's phosphomolybdic reagent (3) in acetic acid medium have concluded that reduction to molybdenum blue is given by steroids with a primary or secondary α-ketol function, an α, β-unsaturated 3-ketone group, or both.

Heard, Sobel and Venning (5) have employed this same reagent in the estimation of reducing corticosteroids in urine and adrenal extracts with good results.

We have observed, however, that Heard's method (4) (5) is not very sensible in samples containing less than 50 μg of reducing corticosteroids (expressed as 11-desoxycorticosterone).

On trying several reagents for reducing groups we found that Folin-Ciocalteu's phosphotungsticmolybdic acid (2) is reduced proportionally by amounts of 11-desoxycorticosterone between 0 and 50 μg.

In this paper we report the method used by us, and results obtained in the determination of reducing corticosteroids in human urine and in adrenals of rats and guinea pigs.

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* This work is dedicated to Prof. Henrique B. Aragão.

MATERIAL AND METHOD

Reagents:

12N H_2SO_4.

Ethyl ether, chloroform, and ether-chloroform mixture — all prepared according to Heard, Sobel and Venning (5).

0.1N NaOH.

Na_2SO_4, anhydrous.

Glacial acetic acid — according to Heard and Sobel (4).

Standard solution of 11-desoxycorticosterone (DOC) — 25 mg DOC/25 ml in glacial acetic acid. This solution must be kept in the refrigerator.

Folin-Ciocalteu's reagent (2).

Na_2CO_3 — aqueous solution 20 g 100 ml.

Method:

Urine — 20 ml (10 ml or less when high values are expected) of a 24 hour urine stored in the refrigerator without preservative, are acidified to pH 1.0 with 12N H_2SO_4 and extracted with 10 ml of ether-chloroform and three times with 5 ml portions of the same mixture in a separatory funel (siliconated stop-cock). The solvents combined extracts are washed with 10 ml (5 x 2 ml) of chilled 0.1N NaOH and with 10 ml (5 x 2 ml) of distilled water.

The extract is dried with Na_2SO_4 then tansferred quantitavely to a Kitasato flask and the solvent evaporated in vacuum.

The residue is transferred to a Pyrex test-tube (11 x 101 mm) with 2 ml of ether. The solvent is evaporated to dryness (37°C), and the transfer procedure is repeated twice more (2 x ml).

Adrenal Glands — The adrenals of an adult rat or a single guinea pig gland are weighed, triturated in a mortar, then extracted with 20 ml of ether-chloroform (4 x 5 ml). The extract is washed with 6 ml of chilled 0.1N NaOH (3 x 2 ml) and with 6 ml of distilled water (3 x 2 ml). The next steps are the same as for urine.

Colorimetry — To the residues in the test-tubes are added 0.05 ml of glacial acetic acid, 0.5 ml of undiluted Folin-Ciocalteu's reagent, and 0.5 ml of H_2O. The tubes are heated in a constant volume boiling water bath. It is important to keep the test-tubes at the same depth in all determinations (4).

After 60 minutes the tubes are removed from bath, cooled, and then 2 ml of H_2O and 2 ml of Na_2CO_3 solution are added. The test-tubes are shaken, the volume adjusted to 5 ml with H_2O, and after 15 minutes the mixtures are filtered and the optical densities read at 650 mμ. We have used an Unicam spectrophotometer with 10 mm tubes, adjusting the zero optical density with a blank made of glacial acetic acid instead of the extract.

Standard Curve — In a series of 11 x 101 mm Pyrex test-tubes 0, 10, 20, 30, 40 and 50 μg of DOC are added and volume adjusted to 0.05 ml with glacial acetic acid. Colorimetry is made as previously described.

RESULTS AND DISCUSSION

Fig. 1 shows the curve obtained with the described technique.

The results of determinations made in urines of normal adult women appear in Table I, and they are comparable with those obtained by Heard, Sobel and Venning (5) and Sprechler (11).

Table II shows the results of simultaneous determinations made in two urines by the method of Heard, Sobel and Venning (5) and the present method.

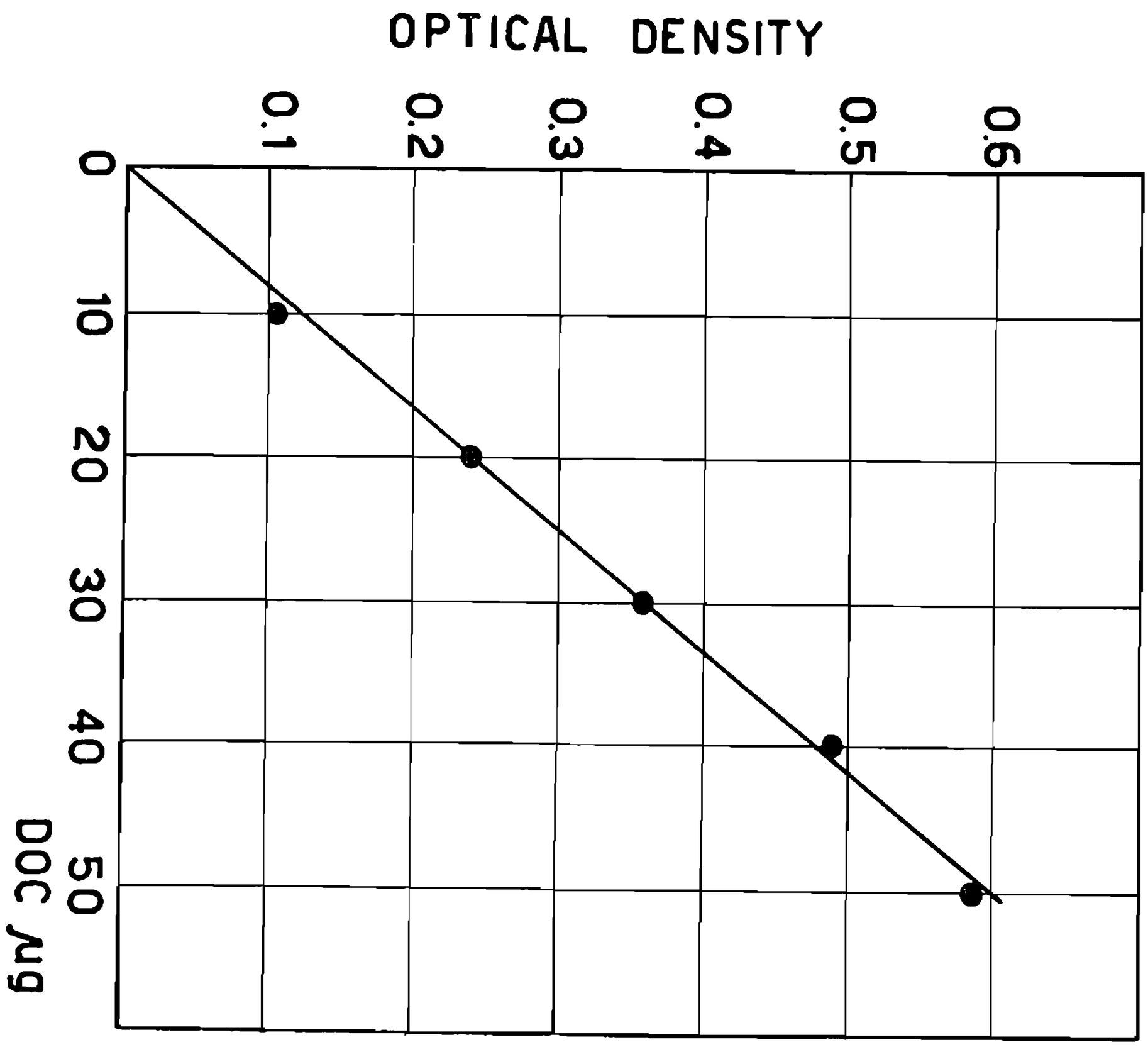

Fig. 1 — Standard curve of 11-desoxycorticosterone with Folin-Ciocalteu's reagent

It must be said that the results obtained by both methods do not express total reducing corticosteroids, which are only determinated after hydrolysis of conjugates with β-*glucuronidase* (6) (7).

The determinations made in adrenals of normal male rats and guinea pigs are presented in Tables III and IV, respectively. These data

are in accordance to those of Staudinger and Schmeisser (12) who have employed phosphomolybdic acid and 500 mg of tissues.

The effect of ACTH on the reducing corticosteroids in adrenal glands of rats and guinea pigs will be reported in a future communication.

TABLE I

Daily urinary reducing corticosteroids excretion.

urine	*mg 24 hours*
A	2.72
B	0.83
C	1.90
D	1.90
E	1.96
F	0.88
Arith. Mean	1.69

TABLE II

Comparison between the method of Heard and col. (5) and the present method.

urine	reducing corticosteroids mg 24 hours	
	Heard and col.	present method
A	1.87	1.86
B	2.99	2.72

TABLE III

Reducing corticosteroids in adrenals of rats.*

rat	weight of 2 glands mg	reducing corticosteroids mg 2 glands	mg 100 g
A	30.5	15.5	50.8
B	39.5	9.2	23.5
C	27.0	6.5	24.1
D	31.2	11.5	36.8
E	32.4	5.3	16.3
F	19.5	9.1	46.6
G	17.7	5.3	29.9
Arith. Mean	28.2	8.9	32.5

* Determinations were made with both adrenals of each animal.

TABLE IV

Reducing corticosteroids in left adrenal of guinea pigs.

guinea pig	adrenal weight mg	reducing corticosteroids µg adrenal	mg/100 g
A	186.0	23.4	12.6
B	169.5	23.4	13.8
C	72.9	24.2	33.2
D	111.0	35.9	32.3
Arith. Mean	134.8	26.7	22.9

SUMMARY

1. The authors preconize the use of Folin-Ciocalteu's reagent in the colorimetric determination of reducing corticosteroids.

2. The reaction follows Beer's law in the range 0-50 µg of 11-desoxycorticosterone.

3. Determinations made in human urine and adrenal glands of rats and guinea pigs are comparable with results obtained by other methods.

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SUMÁRIO

Os autores preconizam o reagente de Folin-Ciocalteu na determinação colorimétrica dos corticosteroides redutores.

A reação segue a lei de Beer na faixa de 0 a 50 µg de 11-desoxycorticosterona.

A excreção urinária de corticosteroides redutores, em 24 horas, de indivíduos normais variou de 0.83 mg a 2.72 mg com a média de 1.69 mg, sem hidrólise prévia pela β-glicuronidase. A intensidade da reação permite efetuar a dosagem em quantidades de urina sensìvelmente menores às usadas por outros métodos baseados nas propriedades redutoras dos corticosteróides.

Os resultados, obtidos com suprarrenais de ratos, variaram de 16.3 mg a 50.8 mg de corticosteróides redutores por 100 g de glândula, com uma média de 32.5 mg, fazendo-se as determinações com as duas glândulas em cada animal.

A taxa média observada nas suprarrenais de cobaias foi 22.9 mg de corticosteróides redutores por 100 g de glândula, com uma variação individual de 12.6 a 33.2 mg, fazendo-se as determinações sòmente com uma glândula.

Os resultados obtidos são comparáveis aos descritos na literatura.

BIBLIOGRAPHY

1. Chen, C., and Tewell J., H. E., 1951, Corticosteroid determination ffiith blue tetrazolium BT, Fed. Proc. *10*, 377-378.
2. Folin, O., and Ciocalteu, V., 1927, On tyrosine and tryptophane determinations in proteins, J. Biol. Chem. *73*, 627-650.
3. Folin, O., and Wu, H., 1920, A simplified and improved method for determination of sugar, J. Biol. Chem. *41*, 367-374.
4. Heard, R. D. H., and Sobel, H., 1946, A colorimetric method for the estimation of reducing steroids, J. Biol. Chem. *165*, 687-698.
5. Heard, R. D. H., Sobel, H., and Venning, E. H., 1946, The neutral lipide-soluble reducing substances of urine as an index of adrenal cortical function, J. Biol. Chem. *165*, 699-710.
6. Kinsella Jr., R. A., and Glick, J. H., 1953, Hydrolysis of conjugates of urinary corticoids with β-glucuronidase, J. Biol. Chem. *203*, 1003-1011.
7. Mason, H. L., 1954, Hydrolysis of conjugates and extraction of urinary corticosteroids, Recent Progress in Hormone Research *9*, 267-278.
8. Nelson, N., 1944, A photometric adaptation of the Somogyi method for the determination of glucose, J. Biol. Chem. *153*, 375-380.
9. Nikolaichuk, S. P., 1954, A method for the determination of corticosteroids in the suprarrenals and in blood, Biokhimiya *19*, 619-625, cited in Chem. Abst. *49*, 1857g.
10. Reichstein, T., and Shoppee, C. W., 1943, The hormones of the adrenal cortex, Vitamins and Hormones *1*, 345-413.
11. Sprechler, M., 1950, Investigations on the chemical determination of corticoids in urine, Acta Endocrinologica *4*, 205-228.
12. Staudinger, H., und Schmeisser, M., 1948, Quantitative chemische Bestimmung der Nebennierenrindehormone, Z. Physiol. Chem. *283*, 54-63.
13. Sulkowitch, H., Rutenburg, A. M., Lesses, M. F., Gargill, S., and Seligmam, A. M., 1955, Estimation of urinary reducing corticosteroids with blue tetrazolium, New England J. Med. *252*, 1070-1075.
14. Talbot, N. B., Saltzman, A. H., Wixom, R. L., and Wolfe, J. K., 1945, The colorimetric assay of urinary corticosteroid-like substances, J. Biol. Chem. *160*, 535-546.