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A Method for the Dermination of Aldosterone, Cortisol and Corticosterone in Biological Extracts, Particularly Applied to Human Urine

By P. J. AYRES, O. GARROD, SYLVIA A. SIMPSON* and J. F. TAIT*
Department of Physics applied to Medicine and the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, and the Institute for Clinical Research, Middlesex Hospital, London, W. 1

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Increased amounts of aldosterone have been found in the urine of patients with certain pathological conditions (Gaunt, Renzi & Chart, 1955), and its analysis in human urine and body fluids is therefore of some interest. Methods previously used for the estimation of this compound in biological extracts have been based on these procedures: (*a*) bioassay of crude (Liddle, Cornfield, Casper & Bartter, 1955; Liddle, Bartter, Duncan, Barber & Delea, 1955; Mader & Iseri, 1955; Gordon, 1955) or purified (Luetscher & Axelrad, 1954; Venning *et al.* 1955; Cope & Llaurado, 1954; Genest, 1955; Simpson & Tait, 1955*a*) extracts, partition-column chromatography being used for the separation of the steroids by Simpson & Tait and paper chromatography by the other workers; (*b*) separation in the propylene glycol–toluene system (Zaffaroni & Burton, 1951), followed by chromatography in the Bush C system (Bush, 1952), the resulting purified aldosterone being estimated by visual examination of the soda-fluorescent spot (Neher & Wettstein, 1955).

Both methods have serious disadvantages. The bioassay results can be influenced by the effects of administered impurities or, in some cases, by other corticoids (Luetscher & Curtis, 1955). The double-chromatography method, first used for the analysis of adrenal gland extracts (Simpson & Tait, 1953), has not yet been shown to be either specific or quantitative when relatively large amounts of other corticoids are present. Moreover, the procedure described by Neher & Wettstein (1955) gives low values for aldosterone in normal urine (see below).

The method described here depends on the extensive purification of aldosterone diacetate followed finally by an objective measurement of the fluorescence on a paper chromatogram. Specific measurements of cortisol and, if necessary, of corticosterone, can be made at the same time. It seems possible, by this method, to measure aldosterone in one-third of a 24 hr. specimen of normal urine with a precision and accuracy of 10–15%. It has been applied to the analysis of adrenal homogenate, human urine and peripheral blood. From the stage immediately preceding the silica-gel column (Fig. 1), the method is basically the same for the analysis of urinary, blood or adrenal gland extracts. This paper is concerned only with its use with human urine. A preliminary account of some of this work has already been published (Ayres, Simpson & Tait, 1956).

* External scientific staff, Medical Research Council.

EXPERIMENTAL

Nomenclature

Aldosterone, cortisol, tetrahydrocortisone, tetrahydrocortisol and corticosterone are used as trivial names for 11β:21-dihydroxy-3:20-dioxo-pregn-4-en-18-al, 11β:17α:21-trihydroxypregn-4-ene-3:20-dione, 3α:17α:21-trihydroxypregnane-11:20-dione, 3α:11β:17α:21-tetrahydroxypregnan-20-one and 11β:21-dihydroxypregn-4-ene-3:20-dione respectively throughout this paper.

Materials

Radioactive steroids. [4-^{14}C]Cortisol and corticosterone (specific activity about 1·5 mc/m-mole), supplied by the U.S. Public Health Department, were purified by partition-column chromatography with the solvent systems described later in this paper. About 5% of the radioactivity was found in effluent fractions less polar than would be expected for the pure steroids; these fractions were discarded.

Aldosterone [*carboxy*-^{14}C]diacetate (specific activity about 1·4 mc/m-mole) was prepared as previously described (Avivi, Simpson, Tait & Whitehead, 1954).

Reagents and solvents

Kieselguhr. Celite 545 was used as the supporting medium in the partition columns. This was washed once with conc. HCl, then to neutrality with water and finally dried at 120° for 15 hr.

Silica gel. Purified silica gel (Davison Chemical Co., grade 950, 60–200 mesh) was used untreated. Although it has been reported that this gel can degrade steroids under certain conditions (A. S. Meyer, personal communication), various amounts (0·1–5 μg.) of aldosterone, cortisol and corticosterone have been chromatographed in the Bush & Sandberg (1953) system with over 95% recovery of unchanged steroid (Simpson & Tait, unpublished observations) with one untreated batch of silica gel.

Benzene (May and Baker Ltd.) was redistilled. Chloroform (May and Baker Ltd.) was redistilled over potassium carbonate after addition of ethanol (2% v/v). Methanol (James Burroughs) was redistilled over calcium. Ethyl acetate (B.D.H. Ltd.) was washed with 5% aqueous solution of sodium carbonate, dried, and freshly redistilled over potassium carbonate. Acetic anhydride (AnalaR) was used untreated. Pyridine (B.D.H. Ltd.) was redistilled weekly. Blue tetrazolium, 3:3′-dianisolebis-4:4′-(3:5-diphenyl)tetrazolium chloride (G. T. Gurr Ltd.). Light petroleum, boiling points 80–100° (May and Baker Ltd.).

Methods

The method employed is shown in Fig. 1.

Extraction. Unpreserved urine (1 l.) from a 24 hr. collection is acidified to pH 1 which, as first shown by Luetscher & Axelrad (1954), increases the yield of free aldosterone. An amount (2 μg.) of [4-^{14}C]cortisol and, if required, 1 μg. of [4-^{14}C]corticosterone, is added to the urine to enable the isotope-dilution method to be used for analysing these steroids. The urine is passed continuously through 500 ml. of chloroform for 24 hr. at room temperature, the Cohen-type extractor (Cohen, 1950) being used, and is then further extracted by shaking with 250 ml. of fresh chloroform. The chloroform phases are washed with 150 ml. of 0·05 N-Na_2CO_3 and 100 ml. of water, which are both back-extracted with 100 ml. of chloroform. The combined chloroform extracts are taken to dryness *in vacuo* at 40° and further purified by passing through a 3 g. silica-gel column (Bush & Sandberg, 1953). The dried residue from the ethyl acetate–methanol eluate is chromatographed on a kieselguhr-partition column (Morris & Williams, 1953; Simpson & Tait, 1953).

Partition-column chromatography. A column 60 cm. long, of 1 cm. internal diameter, is packed with 26 g. of Celite 545, with water–methanol (1:1, v/v) as the stationary and benzene–ethyl acetate (5·2:1, v/v) as the mobile phase. The purified extract is added to the column in a total volume of 2 ml. of mobile phase containing about 50 μg. of Sudan

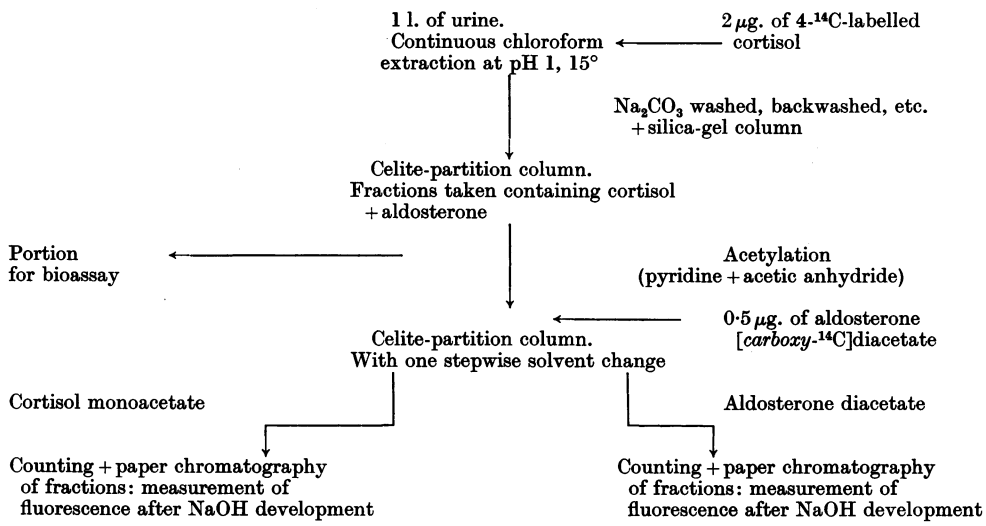

Fig. 1. Summary of the method for the analysis of aldosterone and cortisol in human urine.

red dye. Fractions (3 ml.) are collected. Aldosterone and cortisol have identical partition coefficients in this system and appear together consistently in the same fractions, i.e. fractions 6–12 inclusive from the appearance of red dye. These fractions are pooled, taken to dryness *in vacuo* and acetylated overnight with 0·15 ml. of acetic anhydride in 0·3 ml. of pyridine at room temperature. A portion for bioassay may be taken before acetylation. Aldosterone [*carboxy*-^{14}C]diacetate (0·5 μg.) is then added and the acetylated mixture dissolved in 2 ml. of mobile phase and run on a partition column 30 cm. long, of 1 cm. internal diameter, 13 g. of Celite 545 with methanol–water (4:1, v/v) being used as the stationary and light petroleum–benzene (3:2; v/v) as the mobile phase. When 12 × 5 ml. fractions have been collected after adding the steroids to the column, the mobile phase is changed to light petroleum–benzene (2:11; v/v), to hasten the appearance of the cortisol monoacetate. Portions of the fractions (20% for aldosterone diacetate, 10% for cortisol monoacetate) are evaporated to dryness and analysed for radioactivity. The dried residues are transferred with chloroform to the wells of cavity microscope slides, which are then placed as close as possible to a mica-window counter (G.E.C. type E.H.M. 2). This arrangement has an overall efficiency of about 10% and, hence, 1 μg. of [4-^{14}C]cortisol or 1 μg. of aldosterone [*carboxy*-^{14}C]-diacetate give approximately 850 counts/min., with a background of 10 counts/min. Correction for self-absorption is unnecessary for the counts obtained from the samples containing aldosterone diacetate. The presence, however, of solid material of unknown identity in some of the cortisol monoacetate fractions usually necessitates a self-absorption correction of about 10% of the initial count. This is carried out by eluting the sample from the slide after obtaining the initial count, adding 1 μg. of [4-^{14}C]cortisol and recounting. Fig. 2 shows the results for a typical analysis. Control experiments have shown that eluting and replating the sample reduces the count by less than 2%. All results in this paper have been obtained in this manner. Recently it has been found that, with a Tracerlab flow-counter type SC/16, samples can be counted after transfer with chloroform to a 2 cm. diameter circle of lens tissue mounted on a copper planchet. The count is then increased by a factor of 4·5 with a background of 23 counts/min., and a correction for self-absorption is unnecessary.

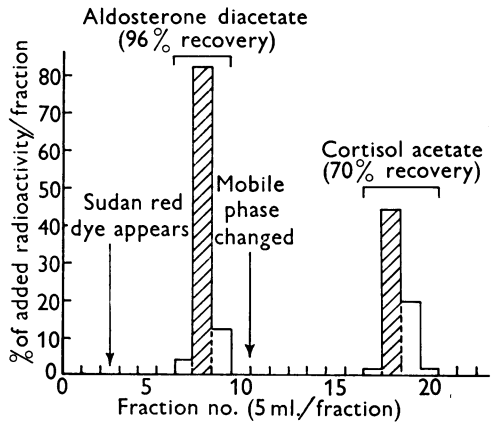

Fig. 2. Analysis of radioactive aldosterone diacetate and cortisol monoacetate in the eluates of a partition column. The radioactivity in the eluates, as measured by a mica-window Geiger counter, is expressed as a percentage of that of the cortisol added initially to the urine or of the aldosterone diacetate added to this column. Portions from the fractions (cross-hatched rectangles) containing peak amounts of each steroid are taken for paper chromatography.

Estimation of aldosterone diacetate and cortisol monoacetate. Portions of the peak radioactive fractions are chromatographed in paper against standards (0·5 and 1 μg. of aldosterone diacetate; 0·75 and 1·5 μg. of cortisol monoacetate) with systems (Bush, 1952) in which the derivatives run at an R_F value of about 0·5. The B 3 system is used for aldosterone diacetate and a modified B 2 system [methanol–water (4:1, v/v) stationary phase] for cortisol monoacetate. The paper is sprayed with a solution of 0·004% blue tetrazolium in 10% (w/v) aqueous NaOH (Bush, 1952), which allows a preliminary quantitative examination to be made of the resulting formazan. The soda fluorescence is developed by heating and the orange-yellow light emitted is measured quantitatively by a fluorimeter described in the Addendum. The specific activity of the steroids is obtained, from which the amounts initially present in the urine can be calculated. The values for cortisol are thus corrected for losses that have occurred throughout the entire procedure, those for aldosterone for losses after addition of the radioactive diacetate.

The fractions less polar than cortisol in the first Celite column are used for the estimation of corticosterone after running on a partition column, 60 cm. long, of 1 cm. internal diameter, methanol–water (4:1, v/v) being used as the stationary and benzene–light petroleum (10:1, v/v) as the mobile phase. The column is analysed for radioactivity as previously described. The fraction containing most radioactivity is divided into two portions which are run against standards of corticosterone in the same paper-chromatographic system as is used for cortisol monoacetate. The corticosterone initially present in the urine is now estimated in the same manner as described for cortisol.

Other compounds of biological interest, such as 11β- and 17α-hydroxyprogesterone and deoxycorticosterone, appear in the fractions less polar than corticosterone in this column. If such fractions are pooled, these three compounds can be analysed in the same column system as is used for the purification of aldosterone diacetate without changing the mobile phase.

These procedures for the analysis of steroids can also be applied without further modification to small-scale preparative work (less than 10 mg.).

RESULTS

In the course of analysing about thirty urine specimens by this method, the only soda-fluorescent spots which have been seen in the final paper chromatogram of the aldosterone diacetate were those running at the same speed as the standards. Occasionally, spots giving blue fluorescence or reacting with the tetrazolium salt have appeared, but not close enough to the aldosterone diacetate to interfere with the development or measurement of the yellow soda fluorescence. The final paper

chromatogram of the purified cortisol monoacetate has shown two positive soda-fluorescent spots in about one-fifth of the analyses. One which appeared in all chromatograms runs at the same speed as the standards of cortisol monoacetate, and gives an equivalent reaction with the tetrazolium salt. The other is more polar than cortisol monoacetate and gives a positive soda-fluorescent reaction only. It is, however, sufficiently separated from the cortisol monoacetate not to interfere when the soda fluorescence of this last compound is being measured. The tetrazolium and soda-fluorescence reactions also give equivalent results in the estimation of aldosterone diacetate and the reacting areas are superimposed symmetrically. For both aldosterone diacetate and cortisol monoacetate the amounts as measured by fluorescence or by the tetrazolium reaction are correlated with corresponding radioactive counts in successive fractions of the final column.

The final paper chromatogram of the purified corticosterone usually shows several spots in addition to that giving both positive tetrazolium and soda-fluorescence reactions and running at the same speed as the standards of corticosterone. One of these extra spots, usually present after fractionating normal urine, gives blue fluorescence on development of the chromatogram. As this compound runs close to corticosterone, it may add to the fluorescence measured from this last compound. Owing to the narrow transmission band of the secondary filters, this blue fluorescence is unlikely to contribute appreciably to the fluorimeter readings, as is indicated by very low values found for corticosterone in the patient with bilateral adrenalectomy (Table 3), when the interfering compound was still present in normal amounts. Nevertheless, because of this potential interference the corticosterone values quoted in this paper should be regarded as maximum estimates only.

Comparison of bioassay and physicochemical methods

As previously described, a portion of the pooled fractions containing aldosterone and cortisol from the first partition column may be taken for bioassay in the sodium/potassium test (Simpson & Tait, 1952). The bioassays were carried out as described previously except that radioactive sodium and potassium were not injected and the urinary sodium/potassium ratio was measured with a flame photometer (Warren, 1952). Log sodium/potassium was used as the response metameter and log (amount of injected material) as the dose metameter. Recent studies have shown this procedure to be preferable to that used in the original method (Jones, Simpson & Tait, 1956). Four-point assays were employed, sixteen rats being injected with the urinary extract and an equal number with pure aldosterone. In the amounts present, cortisol would have no effect on the urinary sodium/potassium excretion. Hence concomitant measurements for aldosterone may be made on the same urine specimen with the physicochemical and bioassay procedures. Fig. 3 shows five such pairs of values for specimens of five normal urines. Statistical analysis gives

$$y = 1{\cdot}07 - 0{\cdot}52x,$$

where $y \equiv$ aldosterone in μg. (bioassay) and $x \equiv$ aldosterone in μg. (physicochemical), and

$$\delta y = 1{\cdot}66, \quad \delta x = 1{\cdot}52,$$

where δy and δx are the standard errors of the estimate. Thus

$$\delta y = \Delta y \sqrt{(1 - r^2)}, \quad \delta x = \Delta x \sqrt{(1 - r^2)},$$

where $\Delta y \equiv$ variance of y, $\Delta x \equiv$ variance of x, the correlation coefficient (r) being $+0{\cdot}986$. There is thus no indication from these data that the values given by the two methods are not equivalent.

Recoveries

Specimens (24 hr.) of urine from male patients with no obvious endocrine dysfunction were used to investigate the recovery of non-radioactive cortisol and aldosterone and [4-^{14}C]corticosterone and cortisol. When necessary, the specimen was diluted to 2 l. with water, two portions of 1 l. being taken, each representing one-half or less of a 24 hr. urinary output. [4-^{14}C]Cortisol (2 μg.) was added to all the portions as for the routine procedure. The initial aldosterone and cortisol were estimated for one of

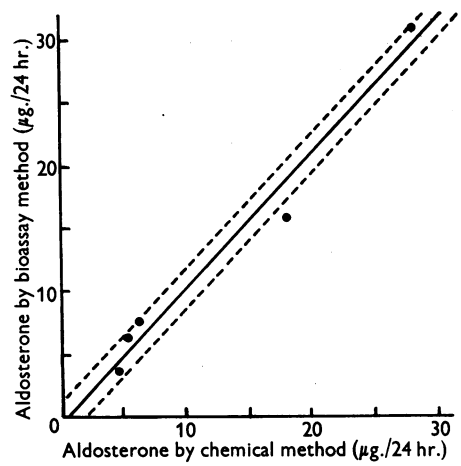

Fig. 3. Comparison of biological and physicochemical estimation of aldosterone on the same specimens of urine (5 male patients). The full line is the best-fitting line as calculated by the method of least squares. The vertical distance from the full line to the dotted line is equal to δy (standard error of the estimate), where $\delta y = \Delta y \sqrt{(1 - r^2)}$, Δy is the variance of the bioassay results and r ($= +0{\cdot}98$ for the five points) is the correlation coefficient.

Table 1. *Recovery of non-radioactive aldosterone and cortisol, and [4-^{14}C]cortisol and [4-^{14}C]corticosterone, after addition of them to the urine of male subjects*

Steroid added	No. of determinations	Mean recovery (%)	Standard error	Coefficient of variation (%) S.D. × 100 / Mean
Non-radioactive aldosterone (15 μg.)	10	77	±6	23
Non-radioactive cortisol (33 μg.)	7	97*	±5	12
[4-^{14}C]Cortisol (2 μg.)	25	70	±1·5	10
[4-^{14}C]Corticosterone (1 μg.)	7	73	±1	2·5

* Measured by isotope-dilution method.

the portions and 15 μg. of aldosterone or 33 μg. of non-radioactive cortisol or both were added to the other sample and the analysis was repeated. After correcting for the initial values, the recovery of the non-radioactive steroids could be calculated.

To each of seven specimens 1 μg. of [4-^{14}C]corticosterone was added as in the routine procedure. The recoveries of the two radioactive steroids, corticosterone and cortisol, were calculated from the radioactivity added to the urine compared with that present in the form of corticosterone or cortisol monoacetate in the fractions of the final partition column.

Table 1 shows the experimental results for all the recovery experiments. Any loss of non-radioactive aldosterone on the final partition column was corrected by applying the isotope-dilution method after adding radioactive aldosterone diacetate. In practice this loss was generally less than 5%. The value of 97 ± 5 (S.E.) % for the estimation of added non-radioactive cortisol obtained by the isotope-dilution method was already corrected for losses throughout the procedure. The actual recoveries of non-radioactive and radioactive cortisol were therefore not significantly different. The recovery values for radioactive cortisol and corticosterone and non-radioactive aldosterone did not differ significantly from one another. They were of the order of 70% from beginning to end of the procedure.

The mean initial values in the portions analysed were 4·0 ± 0·4 (S.E.) μg. of aldosterone and 13·1 ± 2·5 (S.E.) μg. of cortisol. Hence the amounts of non-radioactive steroid added were about three times those initially present. There was no significant difference in the recovery of 2 μg. of [4-^{14}C]cortisol whether the 33 μg. of non-radioactive cortisol was added or not. The procedure described should therefore give a reasonable estimate of recoveries of aldosterone released by acid treatment, and of free cortisol, when applied to normal urine.

Reproducibility

Two equal portions from the same specimen of urine, obtained as described for the recovery experiments, were analysed for cortisol and aldosterone to provide duplicate data. The results for the urine from four normal subjects are shown in Table 2.

Table 2. *Duplicate estimations of cortisol and aldosterone in urine samples from control male subjects*

Cortisol (μg./24 hr.) Values	Cortisol (μg./24 hr.) Mean	Aldosterone (μg./24 hr.) Values	Aldosterone (μg./24 hr.) Mean
86·2, 76·6	81·4	14·6, 13·2	13·9
31·9, 27·8	29·8	18·6, 14·1	16·4
39·4, 40·0	39·7	22·2, 24·8	23·5
28·9, 33·6	31·2	4·3, 6·7	5·5

Values for normal and pathological subjects

Table 3 shows the values for the 24 hr. urinary excretion of aldosterone, cortisol and corticosterone, as measured by this method in a series of male subjects with no obvious endocrine dysfunction. It also shows the value for a single patient after bilateral adrenalectomy who was maintained on a daily dose of 50 mg. of cortisone plus 0·25 mg. of fluorohydrocortisone, cortisone alone having failed to provide adequate electrolyte balance. Data are also included from two patients with primary aldosteronism or Conn's syndrome (the patient reported by Chalmers, FitzGerald, James & Scarborough, 1956, and another patient kindly provided by Dr J. R. Nassim of Stanmore Orthopaedic Hospital) and three patients with other pathological conditions.

Corticosterone and cortisol were measured concomitantly in urine specimens from seven normal male subjects. The values are included in Table 3. The mean ratio of cortisol to corticosterone was 7·4 with a range of 2·7–13·7; these should be considered as minimum values only, because of the possible lack of specificity of the method for the estimation of corticosterone.

Table 3. *Daily excretion of aldosterone, cortisol and corticosterone in a control series of male subjects and some patients with pathological conditions*

Subject	Aldosterone (μg./24 hr.)	Cortisol (μg./24 hr.)	Corticosterone (μg./24 hr.)
Bilateral adrenalectomy maintained on 50 mg. of cortisone + 0·25 mg. of fluorohydrocortisone	0·5	65	0·4
Primary aldosteronism (Chalmers *et al.* 1956):			
(*a*) Immediately before operation	28	21	—
(*b*) After removal of tumour	1	11	—
Primary aldosteronism (Dr J. R. Nassim, unpublished observations):			
(*a*) Immediately before operation	33·3	20·8	0·8
(*b*) After removal of tumour	0·8	11	—
Adrenal carcinoma causing virilization and severe hypertension:			
(*a*) Before operation	5·8	370	34
(*b*) After removal of tumour	15	5·3	4
Cushing's syndrome due to metastasizing adrenal carcinoma (post-adrenalectomy with recurrence of symptoms)	4·0	1200	—
Salt-losing nephritis (maintained in normal electrolyte balance)	127	42·8	3
Mean and range of control male subjects	11 (4·6–23·5) (31 subjects)	35 (13–86) (24 subjects)	5·8 (2·2–9·0) (7 subjects)

DISCUSSION

Of those hormones potent in prolonging the life of adrenalectomized animals, cortisol, on a weight basis, is probably the major one secreted by the normal human adrenal cortex. The main metabolites of this compound in human urine are tetrahydrocortisone and tetrahydrocortisol (Schneider, 1952; Burstein, Savard & Dorfman, 1953). The 20β-hydroxy and 17-oxo products of these two compounds are also present, and to a lesser extent, dihydrocortisone, 17α:21-dihydroxypregnane-3:11:20-trione (Fukushima *et al.* 1955; Dorfman, 1954; Schneider, 1952). Smaller amounts of metabolites retaining the original A-ring structure have been found, including 11β:17α:20β:21-tetrahydroxypregn-4-en-3-one (Reichstein's compound E), 17α:20:21-trihydroxypregn-4-ene-3:11-dione (Reichstein's compound U), cortisone and also unchanged cortisol (Holness, 1956; Burton, Zaffaroni & Keutmann, 1951). Aldosterone is secreted in much smaller quantities than cortisol by the mammalian adrenal cortex under normal physiological conditions. Indirect evidence suggests that the ratio of cortisol to aldosterone in normal human adrenal venous blood is of the order of 100:1 (Simpson & Tait, 1955*b*). This accounts for the present lack of information on metabolites of aldosterone. Tetrahydrocortisone and tetrahydrocortisol in human urine are conjugated with glucuronic acid, but the amounts of cortisone and cortisol extracted by chloroform are not increased by incubation of the urine with β-glucuronidase according to published reports (Gray & Lunnon, 1953), nor by mild acid hydrolysis, i.e. standing at pH 1 for 24 hr. at room temperature (Simpson & Tait, unpublished observations). The extractable free aldosterone, however, is increased by both these procedures, acid hydrolysis being the more productive according to most workers (Axelrad, Cates, Johnson & Luetscher, 1955; Venning *et al.* 1955; Mills, 1954; Neher & Wettstein, 1955). As measured by the method described in this paper, the yield of aldosterone is increased 20–30-fold when this is continuously extracted from urine at pH 1, compared with identical treatment at pH 6 (Simpson & Tait, unpublished observations). Presumably this treatment hydrolyses a conjugate, the nature of which has not yet been established. The urinary excretion of tetrahydrocortisone plus tetrahydrocortisol in normal subjects is of the order of 6 mg./day, the secretion of cortisol by the adrenal cortex being about 20 mg./day; that of aldosterone is probably of the order of 250 μg./day, this being the amount necessary to maintain patients with Addison's disease in adequate electrolyte balance (Mach, Fabre, Duckert, Borth & Ducommun, 1954). The maximal amount of metabolites of aldosterone excreted in the urine would therefore be expected to be 250 μg./day, and that of any tetrahydro products not more than 60 μg./day. The only known measurable metabolite of aldosterone would appear to be the presumed conjugate which, on acid hydrolysis, yields 10 μg. of free aldosterone/day. This should represent an appreciable fraction of the excretion products of this hormone, and it is doubtful whether the analytical procedure could be greatly simplified as a result of the characterization

of any tetrahydro derivatives of aldosterone. The measurement in peripheral blood presents an even greater problem since the concentration of the free compound appears to be 0·08 μg./100 ml. of whole blood (Simpson & Tait, 1955*b*), and this is not increased by acid hydrolysis at pH 1 (Simpson & Tait, unpublished observations). Since only small amounts of free cortisol are excreted in the urine compared with tetrahydrocortisone and tetrahydrocortisol, it is not yet known whether any useful purpose is served by the estimation of this compound when fairly simple methods are available for the specific measurement of the tetrahydro metabolites. However, until this aspect of the problem has been thoroughly investigated, it was thought worth while to incorporate a determination of free cortisol into the method, little extra labour being involved.

The achievement of complete specificity is of paramount importance in measuring quantities of steroid of the order of 10 μg./24 hr. The claim for specificity in determining aldosterone by the method just described is based on the following considerations:

(*a*) The separative methods used purify a compound which behaves like authentic aldosterone in a partition column having resolving power of approximately 400 theoretical plates. After acetylation, the amount of this material in the fractions of the final partition column, as measured by soda fluorescence after paper chromatography, can be correlated with the radioactivity of the same fractions. A small amount of authentic aldosterone [*carboxy*-^{14}C]diacetate is added to the sample before column chromatography. Paper chromatography of a portion of the fraction containing most radioactivity shows a single soda-fluorescent spot running at the same speed as authentic aldosterone diacetate.

(*b*) This spot gives symmetrical reducing and fluorescence reactions when developed with a solution of tetrazolium salt in sodium hydroxide. The two reactions are equivalent in terms of the standards.

(*c*) The amount of aldosterone as measured by the physicochemical technique agrees with the values obtained by concomitant bioassay of the purified free aldosterone before formation of the diacetate.

(*d*) The amount of aldosterone that could be detected in the urine of a bilaterally adrenalectomized patient who was fully maintained on cortisone was very low. The values could also be correlated with other clinical conditions characterized by disturbances in electrolyte metabolism but with normal excretion of cortisol or its metabolites.

(*e*) As was also found by other workers, with different methods, the yield of free aldosterone is greatly increased by acid hydrolysis at pH 1, whereas that of free cortisol is not.

The precision of the measurement of aldosterone and free cortisol can be estimated from the data on the recovery and duplicate experiments shown in Tables 1 and 2. The coefficients of variation based on the recovery of added inert aldosterone and cortisol were 23 and 12 % respectively (Table 1). The recovery values were obtained by subtracting the levels obtained before from those found after the addition of inert steroids to the urine, thus including the errors of both estimations. The coefficients of variation are therefore greater than would be given by repeated measurements on the same sample. Thus the estimated coefficients of variation of the individual aldosterone and cortisol values obtained in the recovery experiments are 15 and 8 % respectively, which are in good agreement with the mean coefficients of variation, 16 and 7 %, given directly by the duplicate data presented in Table 2.

The total variation which these coefficients represent consists of a combination of errors of radioactive counting, errors of the fluorescence measurements on the final paper chromatogram and, with aldosterone, differences in the recovery of this steroid from the stage of urine extraction up to that of adding the radioactive aldosterone diacetate. With cortisol, this last variation is eliminated by applying the isotope-dilution method throughout the entire procedure. As estimated from the variation in the recovery of radioactive cortisol (Table 1; i.e. 10 %, including radioactive counting errors) the error due to differences in recovery would be about 7 %. This could also be eliminated in the measurement of aldosterone if the radioactive free steroid were available for addition to the urine. In the present method, by adding radioactive aldosterone diacetate, it is only necessary to analyse the effluent fraction containing the peak concentration of this steroid in the second column.

The values for the 24 hr. excretion of aldosterone in hospital patients without endocrine disturbances, and in normal students, are higher by this method than those obtained by other workers employing different procedures. Since the lowest individual value in nineteen cases investigated by this method is higher than the mean values given by other workers, the difference is probably statistically significant. This could be the result of one or more of the following factors:

(*a*) The accidental selection of subjects in our series with abnormally high aldosterone values arising from dietary conditions or concealed endocrine disease. However, there is no evidence for this and the electrolyte balance of the subjects appeared to be normal.

(*b*) Decreased destruction of aldosterone in the urine at pH 1 owing to the use of the continuous extraction procedure.

(*c*) Increased recovery of aldosterone in the present method, possibly as a result of more efficient extraction or the use of partition-column chromatography as a separative method, rather than paper chromatography as employed by most other workers.

(*d*) The possible inhibition of the activity of aldosterone by interfering substances in those methods depending on bioassay.

At the present time there is not enough evidence to allow any firm conclusions to be drawn as to the relative importance of the above factors. However, Dr J. J. Walraven of Organon, Oss, Holland (private communication), has recently found that when continuous extraction is used as the first stage of the Neher–Wettstein method (Neher & Wettstein, 1955), the amounts of aldosterone obtained are 2–3 times those with their normal procedure on the same urine.

SUMMARY

1. A method is described for the analysis of aldosterone, cortisol and corticosterone after continuous extraction of human urine at pH 1 by chloroform for 24 hr. at room temperature.

2. An isotope-dilution method is used. Paper chromatography of the steroids or their acetyl derivatives follows their extensive purification by column chromatography. The final spot on the paper chromatogram is measured quantitatively by a fluorimeter after development with a solution of sodium hydroxide.

3. Evidence is presented on the specificity of the method.

4. Recovery of steroids throughout the procedure is of the order of 70 %.

5. The recovery and duplicate data suggest that the coefficient of variation for the determination of aldosterone and cortisol is 15 and 7 % respectively.

6. The mean values for the 24 hr. urinary excretion of aldosterone and cortisol have been found to be 11 μg. (31 cases) and 35 μg. (24 cases) respectively.

7. Some values found in pathological conditions are given.

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ADDENDUM

A Fluorescence Method for the Microanalysis of Δ^4-3-Oxo Steroids on Paper Chromatograms

By P. J. AYRES, SYLVIA A. SIMPSON and J. F. TAIT
Department of Physics Applied to Medicine and the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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The method for the analysis of aldosterone and cortisol, described in the preceding paper, depends finally on the assay of their acetyl derivatives after paper chromatography. The fluorescence of the areas containing steroid is measured without elution, after developing the chromatograms by the method of Bush (1952). The method is applicable to the micro-analysis of other Δ^4-3-oxo steroids, although the intensity of their fluorescence may be weaker, particularly for the more non-polar free compounds such as 11β-hydroxypregn-4-ene-3:20-dione and 17:-hydroxypregn-4-ene-3:20-dione. The apparatus and method have been demonstrated (Ayres, Simpson & Tait, 1956).

EXPERIMENTAL

Preparation of paper specimens

The chromatograms are sprayed on both sides of the paper with a 0·004% (w/v) solution of 3:3′-dianisolebis-4:4′-(3:5-diphenyl)tetrazolium chloride in 10% (w/v) NaOH. The chromatograms are then heated for about 15 min. at a distance of 50 cm. from a 2 kw electric fire with elements distributed evenly over an area 25 cm. square. When the chromatogram is still slightly damp, it is finally dried with hot air from a hair drier. It is essential that the preliminary heating be carried out in still air.

By viewing the chromatogram under ultraviolet light, the head of the template positioner (*g*, Fig. 1) is placed on either side of the paper with the crosswires arranged centrally over

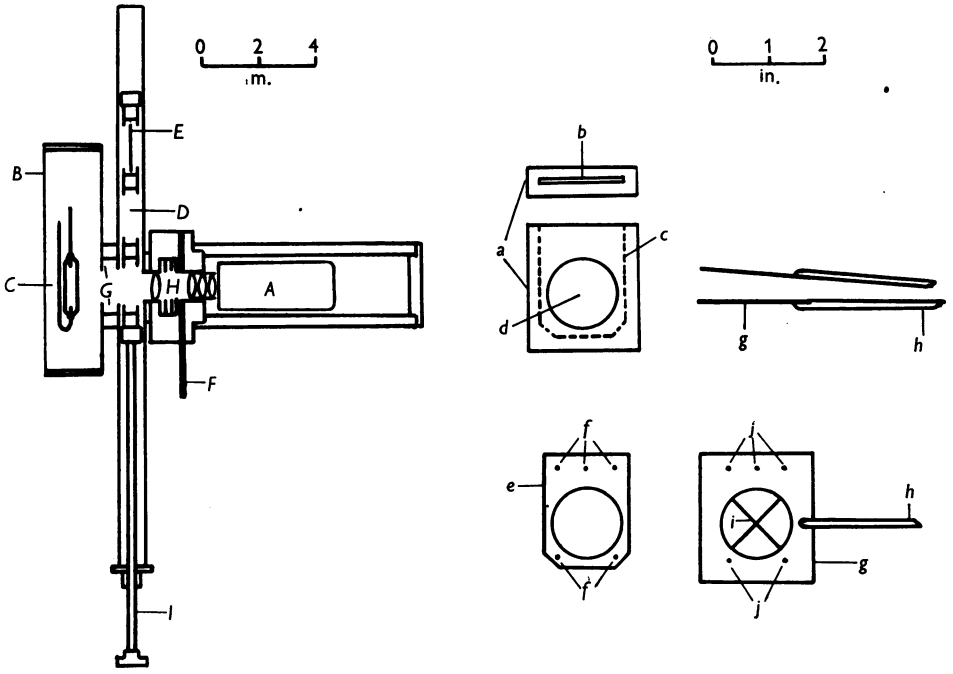

Fig. 1. Fluorimeter and accessories for measuring fluorescence of compounds on paper chromatograms. The diagram on the left shows the fluorimeter schematically: *A*, photomultiplier; *B*, lamp-housing; *C*, mercury-vapour lamp; *D*, compartment for paper holder (*a*); *E*, solid fluorescent standard; *F*, shutter; *G*, primary filter compartment; *H*, secondary filter compartment; *I*, sliding control for paper holders. The diagrams on the right show the accessories for positioning the fluorescent compound centrally: *a*, paper holder; *b*, slot for paper; *c*, outline of paper; *d*, aperture with central fluorescent spot; *e*, cutting template for paper chromatogram; *f*, positioning pins; *g*, template positioner; *h*, tongs; *i*, crosswire; *j*, positioning holes.

a fluorescent spot containing steroid. The handles of the positioner are then locked and a pin is pushed through the positioning holes to puncture the paper. A cutting template with a set of positioning pins is placed over the fluorescent spot with the pins fitting into the holes in the paper. The paper is now cut round the template to the shape shown at *e*, Fig. 1. The other spots are dealt with in the same way and in addition, for routine analysis, a suitable area not containing steroid is taken to serve as a blank measurement. The pieces of paper are then placed in paper holders (*a*, Fig. 1), each of which consists of a slotted square of brass with a circular aperture, 3·2 cm. in diameter. The fluorescent spots should be in the centre of this aperture. The paper holders can now be placed in the compartments (*D*, Fig. 1) of the fluorimeter.

Description of fluorimeter

The fluorimeter has a mercury-vapour lamp, an 80 w Mazda type MB/V, with glass envelope removed, as a light source. The light from this source (after passing through four Ilford 828 filters, total thickness 1·2 cm.) irradiates the paper or an Ilford 805 filter which serves as a solid fluorescent standard (*E*, Fig. 1). A quartz lens of focal length 2·5 cm. is placed 2·5 cm. from the fluorescent spot on the paper, so that the emitted light is made nearly parallel before entering the filter compartment. One Hilger and Watts interference filter (maximum transmission 5975Å), together with Ilford filters 401 and 607, is used as secondary filter. The light is then focused on the cathode (0·95 cm. in diameter) of an E.M.I. photomultiplier Type 6094 by three lenses of focal length 2·5, 3·2 and 3·2 cm. The optical arrangement ensures that fluorescent light emitted from any point in the circular aperture of the paper holder is focused on the photomultiplier cathode. The high-tension supply for the cathode and dynodes of the photomultiplier (usually 1700 v, negative with respect to earth) is supplied by an Atkins Robertson and Whiteford Type 1082A power pack. A Pye Scalamp 7904/S galvanometer measures the current passing between the collector plate and ground potential. With this electrical arrangement it was found essential, if instability were to be avoided, to have a sheath maintained at cathode potential surrounding the glass body of the photomultiplier. The a.c. mains supply was stabilized by an Advance 250 w constant-voltage transformer.

Method for routine fluorimeter measurements

For routine analyses, four fluorescent spots are usually measured. These consist of two standards (0·5 and 1 μg. of aldosterone diacetate or 0·75 and 1·5 μg. of cortisol monoacetate) and two portions, in the ratio 2:1, of the steroid to be analysed. The high-tension supply to the photomultiplier is adjusted so that with the solid fluorescent source in position, the galvanometer gives a certain fixed reading (usually 60 mm. on the most sensitive range). This is repeated after every measurement of the fluorescent spots, to eliminate an error of about 2% due to variation of light-source intensity and photomultiplier sensitivity. The pieces of paper are now inserted in turn and the corresponding galvanometer readings noted. The sliding control (*I*, Fig. 1) moves the three paper holders laterally into position for this purpose, and three positioning notches ensure that the circular apertures are concentric with the photomultiplier cathode. If necessary, the paper holders can be moved laterally until a maximum reading of the galvanometer is observed. However, by using the method described for cutting the paper, this reading is invariably as defined by the positioning notches. The reading for the blank is now subtracted from those of the fluorescent spots. This results in a higher coefficient of variation for the measurement of the smaller amounts of steroids; the readings, corrected for blank, are therefore weighted in the final calculation by taking: [unknown amount of steroid (larger portion)]/[known amount of standard steroid (larger standard)] as (sum of two galvanometer readings of unknown amount of steroid)/(sum of two galvanometer readings for two standard spots).

Fluorescence spectra

Before the secondary filters for the fluorimeter were chosen, the spectral emissions of the soda-fluorescent spots of aldosterone diacetate and cortisol monoacetate, together with that of a blank piece of paper developed in the same way, were obtained. The pieces of paper were placed in front of the entrance slit of a Hilger Uvispek apparatus, which served as a monochromator, and irradiated with ultraviolet light of wavelength 365 mμ. obtained from the source and primary filters of the fluorimeter. The E.M.I. 6094 photomultiplier tube, employed in the fluorimeter, was used as the detector instead of the photocell of the Uvispek apparatus. Galvanometer readings were taken for emergent light of different wavelengths between 400 and 600 mμ. Both slit widths were maintained at 0·06 cm.

Fig. 2 shows the spectral emission of the soda fluorescence of a piece of blank paper and also that of 5 μg. of aldosterone diacetate corrected for this blank fluorescence. The filled-in points show the latter spectrum also corrected for the spectral sensitivity of the photomultiplier cathode by employing the data provided by the manufacturers. These are the major corrections necessary to obtain the true fluorescence spectrum as the dispersion is nearly constant throughout the wavelength range investigated.

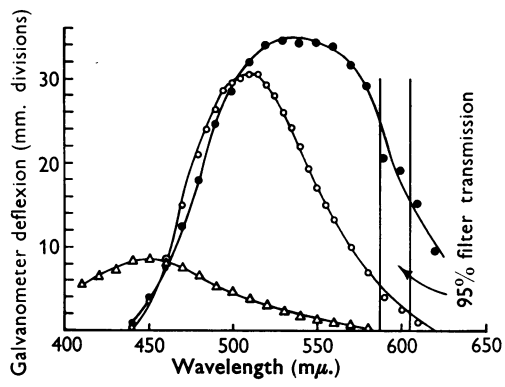

Fig. 2. Emission spectrum of the soda fluorescence of 5 μg. of aldosterone diacetate on a paper chromatogram. ○, The spectrum as measured by the photomultiplier used in the fluorimeter (cf. Fig. 1), corrected by subtracting the fluorescence of a corresponding area of paper not containing steroid; ●, the same spectrum corrected for both paper blank and spectral sensitivity of the photomultiplier cathode; △, the corresponding spectrum from paper not containing steroid as measured by the photomultiplier.

It can be seen that, as measured directly by the photomultiplier, the maximum fluorescence of the steroid occurs at 515 mμ., the corrected maximum being at 540 mμ. Both the corrected and measured maxima for the blank paper were at 450 mμ. Similar curves were obtained for cortisol monacetate. The longer the wavelength, the greater is the ratio of the intensity of fluorescence of steroid compared with that of the blank paper. However, because the sensitivity of the photomultiplier falls off rapidly above 590 mμ., filters were chosen which had 95% of their transmitted light between 585 and 605 mμ., as shown in Fig. 2.

RESULTS

Proportionality of galvanometer reading to quantity of steroid

Five chromatograms, each with four spots of 1, 2, 4 and 8 μg. of aldosterone diacetate, were developed and measured as previously described. In addition, the readings from twenty chromatograms with 0·5 and 1 μg. of aldosterone diacetate were available for analysis. The results are shown in Fig. 3. The values on the ordinate represent the mean values of several galvanometer readings corresponding to a particular quantity of steroid expressed as the ratio of that given by 1 μg. of steroid on the same chromatogram. This procedure was adopted because of the large variation of the galvanometer readings given by different chromatograms with a constant quantity of steroid. This variation is probably due to differences in the conditions of spraying and heating of different chromatograms, although the treatment of the total area of a single chromatogram may be uniform. Examination of the means and standard errors of the experimental points shown in Fig. 3 indicates that the galvanometer readings are proportional to quantity of aldosterone diacetate up to at least 4 μg., but that this may not be so for the readings corresponding to 8 μg. of steroid or more. More rigorous statistical treatment of the data confirms this conclusion.

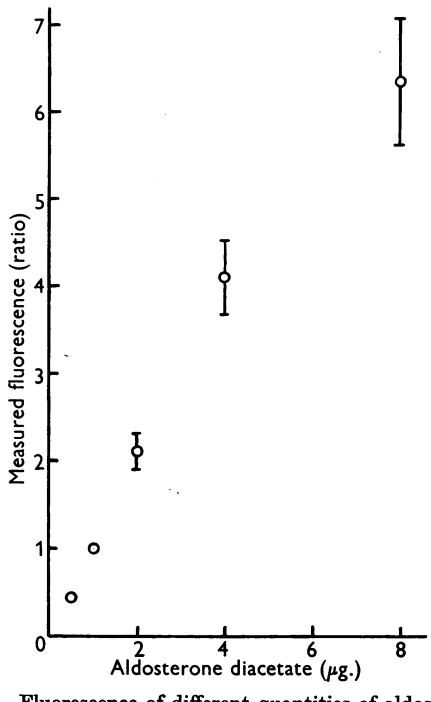

Fig. 3. Fluorescence of different quantities of aldosterone diacetate. Values on the ordinate represent the mean values of several measurements of fluorescence expressed as the ratio of that given by 1 μg. of steroid on the same chromatogram. The length of the vertical lines attached to the points represents twice the standard error of the mean. The point corresponding to 0·5 μg., i.e. $0{\cdot}478 \pm 0{\cdot}01_3$ (S.E.), represents the mean of the results from twenty chromatograms; other points (2, 4 and 8 μg.) are the mean results from five chromatograms.

Effect of impurities

Cortisol was extracted and measured from 1 l. of urine exactly as described in the preceding paper, except that the urine was first adjusted to pH 6. The same urine was then further continuously extracted at pH 1. About 10% of the 2 μg. of [4-^{14}C]cortisol added initially to the urine was extracted in the second process, presumably owing to incomplete preliminary recovery. This enabled a second measurement of the cortisol to be made. However, whereas the first paper chromatogram from the extraction at pH 6 showed no fluorescent- and very few ultraviolet-absorbing impurities, the second paper chromatogram from the extraction at pH 1 showed some running even at the same speed as the cortisol monoacetate spot, although these did not give yellow fluorescence. In the analysis of six urines carried out in this manner, the mean ratio of the values given by extraction at pH 1 and 6 was $1{\cdot}03_5 \pm 0{\cdot}05$ (S.E.).

Sensitivity

Twenty chromatograms, with 0·5 μg. spots of aldosterone diacetate, gave galvanometer readings of $10{\cdot}1 \pm 1{\cdot}1$ (S.D.) after correction for blank fluorescence. Two chromatograms, with four spots of 0·5 μg. on one and four of 0·25 μg. of aldosterone diacetate on the other, were measured. Four blank areas were also measured for each chromatogram. The mean and standard deviations for the readings were 0·5 μg. of steroid, $14{\cdot}1 \pm 1{\cdot}0$, corresponding paper blanks, $7{\cdot}4 \pm 1{\cdot}0$; and 0·25 μg. of steroid, $10{\cdot}1 \pm 1{\cdot}2$, corresponding paper blanks, $6{\cdot}8 \pm 1{\cdot}1$.

Precision

Two batches of aldosterone diacetate were compared in a typical series of determinations. One batch was prepared from a specimen of aldosterone,

kindly supplied by Professor Reichstein, Basle. The other was prepared by the authors from Allen and Hanbury's adrenal extract. Both preparations of aldosterone diacetate were purified by partition-column chromatography and standardized by ultraviolet absorption. Nine chromatograms were used for the comparison with 0·5 and 1 μg. of both specimens on every sheet. They were developed, measured and the results calculated as previously described for routine analyses. The mean ratio of London/Basle aldosterone, as found by this method, was $0{\cdot}98_9$ with a standard deviation of 0·062 for the nine determinations. The mean coefficient of variation for determinations carried out in this manner with pure steroids was therefore about 6 %.

Effect of spot size

Two chromatograms, with four spots of 2 μg. of aldosterone diacetate on both sheets, were run, developed and measured. The final diameter of the spot was varied by altering the area of application of the steroid solution at the starting line of the chromatogram. The final spot diameter and corresponding galvanometer readings (in parentheses), corrected for blank fluorescence, were: 0·6 cm. (20 mm.); 1·0 cm. (21 mm.); 1·4 cm. (20 mm.) and 1·8 cm. (19 mm.) for one chromatogram, which included the range of spot sizes normally encountered in analysis, and 1·0 cm. (14 mm.); 2·0 cm. (12 mm.); 3·0 cm. (8 mm.) and 4·0 cm. (6 mm.) for the other chromatogram.

These results again clearly show the variation of galvanometer reading between chromatograms for the same quantity of steroid.

DISCUSSION

The comparison of the two different specimens of pure aldosterone diacetate gives a coefficient of variation of 6 % for the fluorimetric method. The coefficients of variation in the determination of urinary cortisol and aldosterone by the complete method, described in the preceding paper, are 7 and 15 % respectively. The random errors involved in the analysis of cortisol in urine are probably a combination of those involved in radioactive and fluorescence measurements. As the radioactive counting errors are about 4 % (total counts of approximately 600), the coefficient of variation for the fluorimetric measurement of cortisol monoacetate from urine can be estimated as 6 %. The random errors in the analysis of aldosterone are probably a combination of these plus the variation in recovery, which is not fully corrected for by the isotope-dilution method in the determination of this steroid. An indication of the variation in recovery of aldosterone is given by the coefficient of variation in the recovery of radioactive cortisol, i.e. 10 %. If this figure is taken for the recovery and 4 % for the radioactive errors, the coefficient of variation of the fluorimetric method can be estimated as about 10 %. Thus the coefficient of variation of the method, as applied to the determination of pure aldosterone diacetate or crude cortisol monoacetate and aldosterone diacetate prepared from urine by column chromatography, would appear to be between 6 and 10 %.

The major reasons for likely systematic errors in the method are the possible quenching action of impurities, the effect of spot size and other effects leading to lack of proportionality of galvanometer reading to quantity of steroid.

For the measurement of pure steroids, the results previously described make it likely that these errors will not occur if the spot obtained after developing the chromatogram contains less than 4 μg. of steroid and is less than 2 cm. in diameter. For the measurement of impure fractions, not only must these conditions be satisfied but, in addition, impurities must not enhance or quench the fluorescence. The correlation of the concomitant bioassays and physicochemical results suggests that these criteria are being followed in the application of the method to the analysis of aldosterone in human urine. The agreement between results for the recoveries of inert and radioactive cortisol added initially to the urine indicates that this is also true for measurements of this steroid. However, in both these cases the fluorimetric method has been applied only after extensive purification, and it may be dangerous to assume that systematic errors do not occur for other applications of the method. However, even when many impurities are present in the final chromatogram such as after extraction of free cortisol from urine at pH 1 following extraction at pH 6 as described in the Results section, no interference can be detected. This conclusion is based on the agreement between the measured specific activity of the cortisol after extraction at either pH 1 or 6 and assumes that cortisol is not significantly increased by acid hydrolysis, an assumption which accords with the results of other workers using different methods.

The measurement of Δ^4-3-oxo steroids on paper chromatograms by the soda-fluorescence method has previously been carried out by visual examination of the resultant spots. The present method has the advantage that an objective measurement is made. However, the specificity of the reaction as discussed elsewhere (Bush, 1954) is not affected by this modification.

A reaction which appears to be similar in principle and specificity and which can be carried out in solution has recently been described (Abelson & Bondy, 1955). This involves the development of yellow fluorescence by the action of a solution of

potassium *tert.*-butoxide in *tert.*-butanol on a Δ^4-3-oxo steroid. This solution method may be more convenient for measuring relatively pure steroids. The paper method, however, has the advantage that additional separation of steroids is obtained during the analytical procedure and the presence of compounds, other than the one to be measured, is revealed; this method may therefore be preferable for the analysis of impure fractions.

It seems likely that the method for measuring fluorescent compounds on paper can be applied to substances other than Δ^4-3-oxo steroids. During the preparation of this paper our attention was drawn to the description of such an application by other workers (Mourodineanu, Sanford & Hitchcock, 1955).

SUMMARY

1. A method has been described in which amounts of Δ^4-3-oxo steroids greater than 0·2 μg. can be estimated by measuring their fluorescence after chromatography on paper and development by a solution of sodium hydroxide.

2. The intensity of fluorescence is proportional to the quantity of steroid if the final spot contains less than 4 μg. of steroid and is less than 2 cm. in diameter.

3. If four spots (two of them standards) are used for an analysis, the coefficient of variation of the estimate is between 6 and 10 %.

Financial assistance from the Medical Research Council and the Nuffield Foundation has made this work possible. Dr P. Armitage gave us valuable advice on statistical matters. We have had invaluable technical assistance from Messrs J. Tierney and D. Staples. We wish to acknowledge the encouragement and interest of Professor Sir Charles Dodds, F.R.S., and Professor J. E. Roberts.

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The Reactions of Diazonium Compounds with Amino Acids and Proteins

By A. N. HOWARD and F. WILD
Department of Medicine, University of Cambridge

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The reaction between diazonium compounds and proteins in alkaline solution was first studied in 1904, with sulphanilic acid as the amino compound (Pauly, 1904). Coloured compounds were formed, and the reaction was investigated by allowing the diazonium compound to react separately with all the amino acids then known to be present in proteins. It was found that only tyrosine and histidine gave coloured solutions. Later, Pauly (1915) isolated derivatives from these two amino acids with diazotized *p*-arsanilic acid.

In all azoproteins prepared by treating proteins with an excess of diazotized *p*-arsanilic acid, more arsenic was found on analysis than is to be expected from the combined tyrosine and histidine content (Hooker & Boyd, 1934; Boyd & Mover, 1935; Kapeller-Adler & Boxer, 1936). In an attempt to explain the results it was suggested that diazonium compounds could also react with phenylalanine, tryptophan, proline and hydroxyproline. Kapeller-Adler & Boxer (1936) reported that they had prepared and isolated derivatives from these amino acids with diazotized *p*-arsanilic acid.

The reaction of diazotized *p*-sulphanilic acid with horse serum and compounds with groupings found in proteins has been investigated (Eagle & Vickers, 1936). Indole, the aliphatic amino group of glycine, α-alanine, phenylalanine, ornithine and lysine and the imino group of proline and hydroxyproline were found to deactivate the diazonium compound, from which it was concluded that reaction occurs with aliphatic amino and imino groups in proteins but not with peptide linkages, guanidino or amide groupings. No attempt was made to prepare derivatives of compounds containing the groupings mentioned above: the destruction of the diazonium compound was followed by the addition of alkaline β-naphthol.

Derivatives of glycine have been isolated by treating it with diazotized aniline and nitroaniline (Busch, Patrascanu & Weber, 1934). Subsequently derivatives have been isolated from the products of