A PAPER-CHROMATOGRAPHIC TECHNIQUE FOR THE DETERMINATION OF PLASMA CORTICOSTEROIDS*

BY

BARRY LEWIS

From the Department of Physiology, University of Capetown

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Several methods are now available for the estimation of corticosteroids in plasma. As no reaction specific for corticosteroids is known, these techniques involve complicated procedures for isolating the hormones; it is not surprising that specificity and simplicity have borne an inverse relationship. Bongiovanni and Eberlein (1955) and Mason (1955) have reviewed some aspects of the subject.

The majority of techniques depend on a method, introduced by Porter and Silber (1950), using phenyl-hydrazine. The reaction is not, of course, specific for the dihydroxyacetone configuration of cortisol and of related substances; many carboxyl compounds yield a colour, including numerous drugs (Marks and Leftin, 1954; Silber and Busch, 1955). Inactive breakdown products of corticosteroid, notably tetrahydrocortisone, may also react. In 1952 Nelson and Samuels published the first technique based on the Porter-Silber reaction, whereby an ether-chloroform extract of plasma is finally purified by chromatography on florosil. Cortisol is the main substance determined, and recoveries vary from 55 to 132%. Bayliss and Steinbeck (1953a, 1953b), who modified the method to the extent of running a more reliable blank, consider that only one-third to one-half of the colour is due to cortisol, part of the remainder being due to tetrahydrocortisone.

Further methods have since appeared (Bondy and Altrock, 1953; Kassenaar, Molenaar, Nijland, and Querido, 1954; Silber and Porter, 1954; Wallace, Christy, and Jailer, 1955; Reddy, Haydar, Laidlaw, Renold, and Thorn, 1956) which dispense with chromatography; most methods depend on partitioning between an aqueous alcohol and petroleum or toluene to remove less polar lipids, or merely rely on the selectivity of the extracting solvent. In our experience a single distribution does not adequately separate the corticosteroids from contaminants; Bongiovanni and Eberlein (1955) have had “erratic” results. None of the methods mentioned attempts to estimate individual steroids.

Weichselbaum and Margraf (1955) have introduced a more reliable technique. A somewhat lengthy isolation procedure is followed by partition chromatography on silica.

Polarographic estimation was used by Morris and Williams (1953), who employed reversed phase column chromatography to isolate corticosteroids; steroids were then resolved on two partition columns. For this technique 50 ml. of blood was required. In 1955 they published a modified procedure in which corticosteroids were resolved on a single column using the principle of gradient elution; the mobile phase was changed automatically. The characteristic $\alpha$-ketol side-chain of corticosteroids was determined by reduction of blue tetrazolium, a method of relatively high sensitivity described by Chen and Tewell (1951), Mader and Buck (1952) and Nowaczynski, Goldner, and Genest (1955).

Another grouping present in all corticosteroids, the $\alpha$-$\beta$ unsaturated ketone configuration, may be estimated by a promising fluorimetric procedure (Abelson and Bondy, 1955). The usual range is 0.1–10 $\mu$g. Fluorescence is induced by potassium tert-butoxide, a reagent not easily prepared in sufficiently pure form.

Sweat (1954a, 1954b, 1955) has evolved a fluorescence reaction for cortisol and corticosterone which is highly sensitive and relatively selective. A suitable range is 0.05–5 $\mu$g. cortisol. Cortisol and corticosterone are estimated separately after chromatography of the extract on silicic acid. Sweat’s elution diagram (1955) shows tailing of both steroids; Weichselbaum and Margraf could resolve them only twice in 20 attempts with this technique. The fluorimetric determination has much to recommend it, including great simplicity, and is employed routinely in the method to be described. However, the blue tetrazolium and

*The work reported has been taken in part from a thesis accepted for the degree of Ph.D. by the University of Capetown.
potassium tert-butoxide procedures have also been used successfully.

Dialysis in a three-phase system has been used by Axelrod and Zaffaroni (1954) to isolate plasma corticosteroids in a reasonable state of purity, but it is time-consuming and requires special apparatus.

In the separation of corticosteroid mixtures paper-chromatographic systems possess high resolving power (Bush, 1952; Zaffaroni, Burton, and Keutman, 1950; Pechet, 1953, 1955; Kritchevsky and Tiselius, 1951). Quantitative applications were limited by high blank values, but this drawback has been overcome by washing papers with ethanolic alkali (Lewis, 1956a). Paper strip chromatography has some considerable advantages over column methods; several samples may be analysed with ease at the same time, and the running time with volatile solvents is less than two hours. Paper strips prepared as described constitute micro-columns on which $R_f$ values seldom vary by more than 2%, at least with the solvent system recommended.

In the present study a reliable method of plasma corticosteroid determination was sought which would be simple enough to permit routine use on a large scale. In planning the isolation procedure several solvents were compared with regard to selectivity, corticosteroid recovery, and tendency to emulsify. A reversed-phase paper-chromatographic procedure was developed which efficiently and rapidly separated corticosteroids from less polar contaminants. Qualitative studies on circulating corticosteroids were performed. Physiological variations have been analysed, and some results in a year's experience with the method are presented, enlarging on an earlier communication (Lewis, 1955).

**Experimental**

**Materials and Apparatus.**—Organic solvents are distilled monthly and the middle 80% fraction collected.

A Beckman DU spectrophotometer with fluorimetry attachment and photomultiplier, and a primary filter (Corning 5113) and secondary filter (Wratten gelatine 61) are required. Distilling flasks should be of 100 ml. capacity, with a well to hold 1-2 ml. and a glass still-head bearing a fine air-bleed. Also needed are a mechanical shaker and apparatus for eluting paper strips (Lewis, 1956b).

**Preparation of Paper for Chromatography.**—Strips of Whatman No. 4 paper for chromatography are cut, 50 by 1.5 cm., the greater dimension being in the machine direction of the sheets; they are washed in large chromatography tanks by downward syphoning of the following:

(i) 2N sodium hydroxide in 95% ethanol for 24 hours
(ii) Distilled water, until eluate is neutral
(iii) 95% ethanol for three hours

The method has been discussed in detail elsewhere (Lewis, 1956a). After drying the strips, their lower 5 cm. is exposed for 30 minutes to the vapour of dichlorodimethylsilane at atmospheric pressure in the apparatus illustrated in Fig. 1.

**Method.**—Heparinized blood is centrifuged within 30 minutes of being drawn; 5 ml. plasma is required. It is convenient to work up six samples at a time. Plasma has been stored at \(-15^\circ\) C. for a month without detectable loss of corticosteroids.

The plasma is extracted with 4 volumes of ethyl acetate for 10 minutes. The large excess of solvent is necessary to avoid emulsification. Two extractions are performed.

The combined extract is washed with 1-2 ml. normal aqueous sodium hydroxide, then with the same

![Fig. 1.—Apparatus for exposing ends of paper strips to dichlorodimethylsilane vapour. The halves of the glass lid, with the papers between them, are clamped together by metal bands, each with an adjusting screw.](http://jcp.bmj.com/.../group.bmj.com)
Volume of 2% acetic acid; the aqueous layer is carefully removed. The extract is taken to dryness under reduced pressure at 35 to 40°C. The residue is meticulously washed into the well of the distilling flask with ethyl acetate, which is then evaporated. The residue is taken up in two 0.1 ml volumes of acetone and is applied quantitatively to a 1 cm band in the centre of the silicone-treated part of the paper strip (Fig. 2). A stream of nitrogen ensures compact spotting.

A short ascending chromatogram (R1) is now run, using 85% methanol; the technique differs from that of Bush (1955a) in that the paper is first rendered non-wettable. A blank strip is included at this stage. The front is allowed to reach a point 2 cm. beyond the siliconed region (line X in Fig. 2). In evaluating this reversed phase “defatting” chromatogram, corticosteroids were located by sodium hydroxide fluorescence, cholesterol and fat by brief immersion in a saturated solution of Sudan black in 70% methanol. Corticosteroids migrate at the solvent front, tailing slightly on the unsiliconed region. Cholesterol, neutral fat, and almost all pigment remain at the starting point. The mechanism of this procedure may involve adsorption chromatography, less polar lipids being adsorbed on to the methylated surface of the paper, or elution from the solid phase, corticosteroids being extracted by the advancing solvent. The fact that corticosteroids move at the solvent front suggests that a single distribution operation is taking place, favouring the latter mechanism. After drying, the non-wettable part of the strip is cut away, and the corticosteroids concentrated on line X by pipetting on to the lower edge of the paper a solvent consisting of ethylene dichloride 45, ethyl acetate 45, and methanol 10 parts, v/v, which carries the steroids at the solvent front. This concentrating technique is modified from that of Bush (1952) only in that ethylene dichloride replaces chloroform. The latter may create oxidation products which destroy corticosteroids.

The corticosteroids are now resolved by chromatography in benzene/50% methanol (R2), as described by Bush (1952) except that the ascending method is used. A 36 cm. run takes 90–100 minutes. The tank is kept in a 30° ± 1° incubator during equilibration and running, a fan circulating the air in the incubator. A reference strip is usually included.

The strips are cut as in Fig. 3; 7 cm. lengths are eluted into capillary pipettes by downward syphoning of 0.2 ml absolute ethanol.

Fluorimetry is performed by a slight modification of Sweat’s method, using the Beckman instrument in place of the Farrand, and a tungsten lamp in place of a mercury one. Three millilitres of concentrated sulphuric acid is added rapidly to the eluate, and mixed with a glass rod. The solution is placed in a calibrated 4 by 1 by 1 cm. fluorimetry cell and read 30 minutes after mixing against a solvent blank and cortisol and corticosterone standards of 0.5 and 2 μg. With this technique the calibration curve is linear between 0.1 and 10 μg.; the larger cell size in comparison with the original method does not materially affect the sensitivity. The paper blank is similarly estimated.

**Qualitative Studies on Human Peripheral Blood.**—Blood was drawn from four normal males; A.C.T.H. was not administered. Pooled plasma, 1,200 ml., was immediately worked up as described in the quantitative procedure. A 10 cm. “defatting” chromatogram was run, with 85% methanol, on a 6 cm. wide silicone-treated paper strip. The leading 4 cm. was eluted with methanol, and the solution obtained was divided into four parts.

One fraction was chromatographed in benzene/50% methanol, and another in toluene/propanol. The leading 4 cm. was eluted with methanol, and the solution obtained was divided into four parts.

A third was acetylated and run in toluene-light petroleun/70% methanol (B1 system of Bush, 1952). To the last were added 10 μg each of authentic cortisol,

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**Fig.** 2.—Reversed-phase ‘defatting’ chromatogram (R2), using 85% methanol. The non-wettable part of the paper, below Y, is subsequently cut away; corticosteroids are concentrated on line X and then resolved by chromatography in benzene/50% methanol (R1).

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**BARRY LEWIS**
DETERMINATION OF PLASMA CORTICOSTEROIDS

![Diagram of cortisol and corticosterone bands](image)

(cortisone, and corticosterone; a mixed chromatograph was performed, two-dimensionally, in benzene/50% methanol followed by Bush's C system (toluene-ethyl acetate/50% methanol). The papers were treated by the combined blue tetrazolium and sodium hydroxide fluorescence methods.

In a second experiment, a pooled sample of plasma was worked up similarly and chromatographed in benzene/50% methanol. The eluate from the cortisol region was examined by spectrophotometry in sulphuric acid (Zaffaroni, 1953) and determined by fluorimetry in sulphuric acid, fluorimetry in potassium tert-butoxide (Abelson and Bondy, 1955), and absorptiometry of the diformazan formed with blue tetrazolium (Nowaczynski et al., 1955). Of the eluate from the aldosterone region, a sample was run in Bush's C system, aldosterone being determined by fluorimetry in potassium tert-butoxide. The remainder of the eluate was acetylated and chromatographed in benzene/formamide, spots being located by spraying the paper with the blue tetrazolium—20% sodium hydroxide reagent of Nowaczynski et al.

**Results**

**Qualitative Analysis.**—In experiment 1, the first three strips showed yellow-fluorescent, blue tetrazolium-reducing spots with $R_F$ or $R$ values in

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Fig. 3.—(a) The cortisol (F) and corticosterone (B) bands eluted after chromatography in benzene/50% methanol. (b) Elution diagram obtained after chromatography of 2 μg. cortisol and 2 μg. corticosterone in the same system; 2 cm. sections were eluted and determined fluorimetrically. (c) Elution diagram after chromatography of extract of 10 ml. plasma.
agreement (to within 2–4%) with cortisol, cortisone, and corticosterone or their acetates. The former reaction is specific for α-β unsaturated ketones, while the latter is given by α-ketols. The intensity and area of the spots suggested plasma concentrations of about 7, 2, and 1 µg. per 100 ml. respectively. The two-dimensional chromatogram showed homogenous spots corresponding to these corticosteroids, Rₚ or R values being within 2% of the authentic reference steroids. Of interest was a small spot giving both reactions, containing 0.5–1 µg., which moved slightly more slowly than cortisone in the first system and slightly faster than cortisol in the second, as would be expected of aldosterone.

The second sample contained a substance which on acetylation had an Rₚ value in benzene/formamide in agreement to within 3% with the authentic aldosterone diacetate and which reduced blue tetrazolium. The aldosterone region of the toluene/proplylene glycol chromatogram of this sample contained the equivalent of 0.16 µg./100 ml. of plasma of an α-β unsaturated ketone. This figure is comparable to the semi-quantitative assessment of the aldosterone spot in the first sample, 0.15–0.3 µg./100 ml., and to the bio-assay figures of Simpson and Tait (1953), 0.05–0.1 µg. aldosterone/100 ml. whole blood.

The eluate from the cortisol region of the benzene/50% methanol chromatogram was determined by three procedures possessing different specificities. Sulphuric acid fluorimetry indicated a plasma level of 10.9 µg./100 ml., potassium tert-butoxide fluorescence 10.1 µg./100 ml., and blue tetrazolium reduction 11.1 µg./100 ml. The ultra-violet absorption peaks of the sulphuric acid chromogens corresponded closely to those of authentic cortisol.

In the paper chromatograms a small, highly polar blue tetrazolium-reducing spot, probably tetrahydrocortisone, was constantly present; a white fluorescence at the solvent front and a faint blue-fluorescent spot between cortisol and cortisone were observed. Corticosteroids in amounts below 0.15 µg./100 ml. plasma would not have been detected.

No corticosteroids other than cortisol and corticosterone would therefore be expected in the regions eluted in the quantitative procedure in amounts which would interfere materially with results. Although cortisol and possibly aldosterone and tetrahydrocortisone were present in the pooled sample studied, the first two do not give a strong fluorescence with sulphuric acid, while cortisone and tetrahydrocortisone lie outside the eluted areas. These conclusions are supported by the elution diagram, Fig. 3c; Fig. 3 also shows the absence of tailing of cortisol and corticosterone. This contrasts with Kofrányi’s finding (1955) that quantitative paper chromatography of amino-acids is seriously hampered by occult tailing.

Recoveries.—Cortisol recovery was determined on 20 samples by measurements on duplicate samples of plasma; to one of each pair was added 0.5 or 2 µg. of the steroid. The range was 86–96%, mean 92%. Corticosterone recovery, estimated on 10 occasions, varied from 82 to 96% (mean 87%).

Duplicate cortisol determinations were made on 20 random samples of plasma, both normal and abnormal, the differences varying from 0.0 to 2.2 µg./100 ml. At the probability level, P=0.05; a difference between readings of 2.7 µg./100 ml. is significant.

Normals.—Thirty samples of venous blood were obtained from apparently normal subjects between 15 and 42 years of age. Twenty were males. The effect of diurnal variation was minimized by drawing blood at 8–9 a.m.

The range for cortisol was 6–12 µg. per 100 ml. plasma (mean 9.2, S.D. 1.5). Corticosterone was present only in nine samples, varying from 0 to 6 µg./100 ml. The mean was 1.2 µg.

In the male subjects the mean cortisol level was 8.9 µg./100 ml., while the mean for 10 females was 9.9 µg./100 ml.

Five subjects between 60 and 79 years of age had cortisol levels in the range stated.

Physiological and Pathological Variations.—The response to a single oral dose of cortisol is shown in Fig. 4.

The effect of intravenous infusion of 40 units of A.C.T.H. (Organon) over six hours was studied.

![Fig. 4. Plasma cortisol after oral administration of 120 mg. of the free alcohol of this steroid.](http://jcp.bmj.com/ on September 8, 2017 - Published by group.bmj.com)
Determination of Plasma Corticosteroids

in six subjects 13–63 years of age, plasma cortisol and urinary ketogenic steroids (Norrymberski, Stubbs, and West, 1953) being determined. The plasma cortisol rose significantly within one hour, and exceeded 30 μg./100 ml. at four to six hours in every case. The mean plasma cortisol rose from a basal level of 9 μg./100 ml. to 36 μg./100 ml. at six hours, while urinary ketogenic steroid excretion showed a less pronounced increase, from 1.0 to 2.6 mg./hour. Using a method which determines the plasma level not only of cortisol but also of a major degradation product, tetrahydrocortisone, Bayliss and Steinbeck (1954) found that maximal adrenocortical stimulation led to a threefold rise of plasma 17-hydroxycorticosteroids after six hours, with a further rise after more prolonged infusions. The plasma cortisol therefore appears to provide a slightly more sensitive index of adrenal function than the other two measurements.

Exercise did not affect plasma levels (Table I); this is in agreement with excretion studies by

TABLE I
EFFECT OF EXERCISE ON PLASMA CORTISOL AND CORTICOSTERONE

<table>
<thead>
<tr>
<th>Exercise</th>
<th>Plasma (μg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Two-hour walk</td>
<td>11 (0)*</td>
</tr>
<tr>
<td>Competitive squash</td>
<td>7 (0)</td>
</tr>
<tr>
<td>Six-hour route march</td>
<td>8-9</td>
</tr>
</tbody>
</table>

* Effect on corticosterone in brackets.

Thorn, Jenkins, and Laidlaw (1953), and suggests that if any adrenal hyperfunction takes place during exercise it is accompanied by accelerated clearance of cortisol from the blood stream. The well-known hypercortico-adrenalism during pregnancy is illustrated in Fig. 5; it is pronounced only in the last trimester and wanes rapidly during the puerperium. A marked rise occurs during labour, from 22 to 29 and from 23 to 35 μg. cortisol/100 ml., in two subjects studied. During two water-loading tests on normal subjects (20 ml./kg.) diuresis was unaccompanied by any significant change in plasma cortisol despite a fall in plasma osmolarity in one subject from 413 to 312 m. osmols/litre; a permissive action of 17-hydroxycorticosteroids is indicated.

A significant but minor elevation of plasma cortisol was noted in one of two students immediately before an oral examination; the basal level was 7.7 μg./100 ml.; the figure of 10.9 μg./100 ml. during a state of anxiety was still within the normal range. Hetzel, Schottstaedt, Grace, and Wolff (1955) have reported transitory elevation of the urinary 17-hydrocorticosteroids during "stressful life experiences" in some subjects.

Two out of three subjects studied showed a rise in plasma cortisol after fasting for 24 hours, but the level did not significantly exceed the normal range.

A diurnal variation in adrenal activity has been observed (Tyler, Migeon, Florentin, and Samuels, 1954; Doe, Flink, and Goodsell, 1956). Porter-Silber chromogens in blood and urine were highest at 6 a.m. and lowest at 10 p.m. In the present study the plasma cortisol was found to show a similar fluctuation, 8 a.m. levels being almost double those at midnight, with a steady decline during the day. This is a potential source

TABLE II
EXAMPLES OF PLASMA CORTISOL IN DISEASE

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Plasma Cortisol Level (μg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addisonian crisis</td>
<td>0-5</td>
</tr>
<tr>
<td>Addison's disease, on deoxycorticosterone</td>
<td>0-0</td>
</tr>
<tr>
<td>Total adrenalectomy</td>
<td>0-2</td>
</tr>
<tr>
<td>Subtotal adrenalectomy for phaeochromocytoma; maintained on sodium chloride only</td>
<td>2-0</td>
</tr>
<tr>
<td>Cushing's syndrome (adrenal hyperplasia)</td>
<td>39</td>
</tr>
<tr>
<td>Phaeochromocytoma</td>
<td>8-0</td>
</tr>
<tr>
<td>Gonadal agenesis (age 13)</td>
<td>4-5</td>
</tr>
<tr>
<td>&quot; (,, 11)</td>
<td>10-8</td>
</tr>
<tr>
<td>Ovarian hyperthecosis (Stein-Leventhal syndrome)</td>
<td>4-4</td>
</tr>
<tr>
<td>Ovarian hyperthecosis</td>
<td>6-0</td>
</tr>
<tr>
<td>Diabetic coma</td>
<td>26</td>
</tr>
<tr>
<td>Essential hypertension</td>
<td>7-0</td>
</tr>
<tr>
<td>Surgical shock (compound fracture of femur)</td>
<td>18</td>
</tr>
<tr>
<td>Laparotomy, three hours post-operatively</td>
<td>15</td>
</tr>
<tr>
<td>Partial gastrectomy for carcinoma, one hour post-operatively</td>
<td>27</td>
</tr>
</tbody>
</table>
of error in interpreting clinical data, but as the pattern of variation is reasonably consistent from subject to subject, it would appear that values obtained at the same time of the day are sufficiently comparable.

Levels in some pathological states are shown in Table II.

Discussion

In a year's experience with this method, no serious difficulties have been encountered; it is rapidly performed and is sufficiently reproducible. Zero values in three cases of Addison's disease and high plasma cortisol levels soon after administering this steroid or A.C.T.H. suggest that the technique is of adequate specificity, and qualitative studies on pooled normal plasma support this contention. Despite the large number of substances detected in adrenal vein blood by Hudson and Lombardo (1955), among which cortisone was conspicuously absent, peripheral blood contained detectable amounts of only five steroids, in decreasing order of polarity, tetrahydrocortisone (probably), cortisol, aldosterone, cortisone, and corticosterone.

The presence of cortisone in peripheral blood is reported also by Morris and Williams (1953) and by Bush and Sandberg (1953). Its apparent absence from adrenal vein blood suggests a peripheral origin, possibly from cortisol. The cortisol/corticosterone ratio of 8:1 is consistent with most published data (Bush and Sandberg, 1953; Simpson and Tait, 1955; Mills, 1954). Bush (1955b) has exhaustively reviewed this topic. Morris and Williams (1953, 1955) have consistently found higher corticosterone levels; the mean F:B ratio in the earlier paper was 1.35, and more recently 0.9. Sweat (1955) has obtained a ratio of 2.25. In view of the relatively feeble effect of corticosterone on intermediate metabolism, and the doubt attending methods of determining this steroid, it is suggested that in the routine measurement of adrenal "glucocorticoid" function it is sufficient to determine plasma cortisol alone.

The physiological variations in adrenal function must be borne in mind when interpreting clinical data, but it appears that the plasma cortisol level is less labile than might have been expected.

It is facile to assume, as is so often done, that the plasma corticosteroid level is a precise measure of adrenal function. Based as it is upon a single observation in time, and representing a balance between release of corticosteroids and their removal from the circulation, data may not easily be interpreted; it is possible that excretion studies will retain a place in assessing adrenal activity. An analogy may be drawn with some decalcifying diseases, in which plasma calcium may be normal, with an excessive urinary excretion.

Although experience to date is limited, no plasma cortisol level has yet been inconsistent with clinical and other biochemical data. This claim cannot be made for urinary formaldehydogenic steroid estimations nor for urinary butanol-soluble Porter-Silber chromogens. Plasma 17-hydroxycorticosteroid determinations may give normal results in Cushing's syndrome (Mason, 1955) and Addison's disease (Eik-Nes, Sandberg, Nelson, Tyler, and Samuel, 1954).

Summary

A technique is presented for the determination of plasma cortisol and corticosterone; it is based on conventional solvent extraction followed by paper chromatography and fluorimetry. Recovery data and norms are reported. The method is sensitive, reproducible, and simple, avoiding as it does the technical problems of column chromatography. Qualitative studies of circulating steroids indicate the presence of cortisol, aldosterone, cortisone, tetrahydrocortisone, and traces of corticosterone. Some physiological and pathological variations are described.

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