A Microdialysis Procedure for Extraction and Isolation of Corticosteroids from Peripheral Blood Plasma

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The steroids which have so far been recognized as physiologically active constituents of adrenocortical secretion are present in extremely low concentration in peripheral blood. Since the physical and chemical characteristics by which they are measured are not specific, it is necessary to separate the steroids from the great bulk of other materials present in the blood before attempting to measure them. The difficulties and objectives of such separation have been clearly stated by Levy & Kushinsky (1954). Some simplification of the problem has resulted from the finding that practically all the corticosteroids in freshly drawn blood are in the plasma (Morris & Williams, 1953; Bush, 1954). The difficulties, however, remain considerable.

For large-scale operations, differential solvent-extraction procedures, such as the classical method of Cartland & Kuizenga (1936), are still probably the most satisfactory. A number of methods have been devised for use with smaller amounts of plasma such as can reasonably be drawn from human subjects or large experimental animals. Most of these methods are based on a crude solvent extraction, followed by isolation and purification by means of chromatography on activated fluorosilicate (Nelson & Samuels, 1952), silica gel (Richards & Sweat, 1953) or charcoal (Levy & Kushinsky, 1954). Such methods, as some of the authors have mentioned, should not be used with amounts of plasma smaller than 10 ml., because of the risk of serious proportional errors. Moreover, there is considerable difficulty in obtaining chromatographic materials of uniform reliability (Eik-Nes, Nelson & Samuels, 1953; Weichselbaum & Margraf, 1955).

An important advance in procedure came from the discovery by Szego & Roberts (1946) that oestrogenic steroids could be separated from both proteins and lipids in one step by dialysis. These authors obtained their best results with very small quantities of steroid, such as might be encountered in small samples of peripheral blood. A later modification of this procedure (Zaffaroni, 1953; Axelrod & Zaffaroni, 1954) improved greatly the recoveries of more polar steroids such as the 11-oxygenated corticosteroids. This improvement consisted in diluting the blood or plasma with methanol and dialysing against a two-phase outer layer of aqueous methanol and chloroform. The proportions of methanol and water in the inner and outer layers were found to be of critical importance to the speed and completeness of dialysis. The chloroform, by continuous extraction of the steroids from the outer methanol layer, maintained a steep concentration gradient between the inner and outer methanol layers. Lombardo, Hudson, Mann & Mittelman (1954) substituted dichloromethane for the chloroform, because the former is a more specific solvent for the corticosteroids and has the advantage of a lower boiling point. Extracts obtained by this method are sufficiently clean for resolution by paper chromatography without further preliminary purification.

Axelrod & Zaffaroni (1954) and Lombardo *et al.* (1954) obtained practically complete recoveries of the more polar corticosteroids by these methods from blood samples of 50–100 ml. volume. Because of the finding of Szego & Roberts (1946) that best results in their work with oestrogens were obtained with much smaller samples, this approach seemed to offer distinct possibilities for the measurement of corticosteroids in the peripheral blood of individual small experimental animals, whose total blood volume would not be sufficient for the size of sample required by other methods. This paper describes a microadaptation of the procedure of Axelrod & Zaffaroni (1954), with which it has proved possible to measure the concentration of the larger corticosteroid fractions in as little as 1 ml. of peripheral blood plasma.

EXPERIMENTAL

Dialysis

The method of Axelrod & Zaffaroni (1954) could not be used directly with very small samples of plasma, since quantities of less than 5 ml. of diluted plasma could not be used in a conventional dialysis casing without either a serious proportional loss in handling or poor mixing inside the dialysis sac. Therefore a special cell was devised, as illustrated in Fig. 1. The cells used in the present work were made to special order by the Emerald Glass Co., 544 Rogers Road, Toronto 9, Canada.

Each cell consists of three pieces: an outer chamber, an inner chamber and a stopper for the inner chamber. They can be separated easily for cleaning. All are made of Pyrex glass, and interchangeable ground-glass joints of standard taper are employed at the points of contact. In use, the bottom of the inner chamber is covered with a sheet of cellulose dialysis membrane (Visking Corp.), held tightly in place by loops of white cotton thread, and sealed to the glass surface by a thin layer of collodion. (Dr A. G. Gornall has employed these cells recently, and advises that some brands of collodion cause serious difficulty because of solubility in dichloromethane. It is probably advisable to test the collodion for this before employing it in the dialysis cells.) Several stainless-steel or Monel-metal ball bearings, $\frac{3}{16}$ in. in diameter, roll freely on the upper surface of the membrane to ensure constant mixing of the contents of the inner chamber.

The cellulose membrane and the cotton thread are washed thoroughly in dichloromethane, 50% (v/v) aqueous methanol and water, before they are used in the dialysis cells. Before the assembled cells are employed they are again rinsed thoroughly with water and detergent, followed by water and finally by 50% (v/v) aqueous methanol. They are drained completely, the last drops of liquid being removed with a clean fine-tipped Pasteur pipette connected to a suction pump. The membrane must not be allowed to dry out completely, however, because of the risk of splitting.

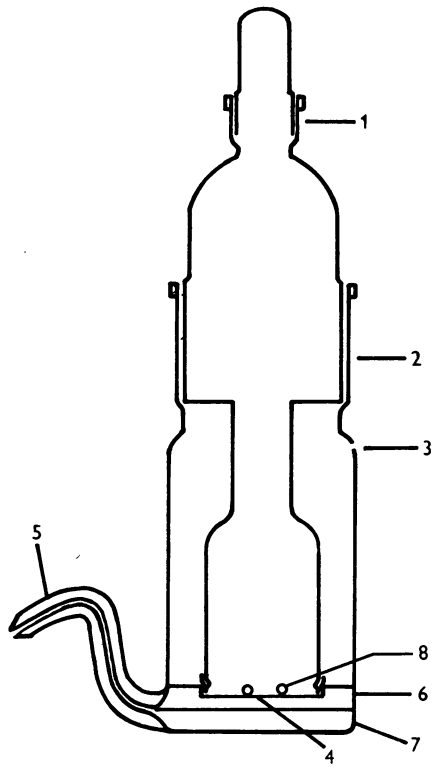

Fig. 1. Microdialysis extraction cell. 1, 2, Standard ground-glass joints; 3, pressure equilization vent; 4, cellulose membrane; 5, capillary spout; 6, 60% methanol layer; 7, dichloromethane layer; 8, stainless-steel or Monel-metal ball bearings, $\frac{3}{16}$ in.

In use, 1 ml. of plasma is pipetted into the inner chamber, directly on to the surface of the cellulose membrane. This is followed at once by addition of 2 ml. of 50% (v/v) aqueous methanol, after which the stopper is inserted. Gentle rocking of the cell causes the ball bearings to mix the plasma and methanol, a very fine suspension of denatured protein, which forms coarser precipitates only after many hours, being produced. In the outer chamber are placed 10 ml. of 60% (v/v) methanol and 8 ml. of dichloromethane. The dimensions of the cell, designed for these volumes, are such that the plasma–methanol layer in the inner chamber is approximately 1 mm. thick and the surface of the two-phase system in the outer chamber is about 2 mm. above the level of the membrane. The tip of the drainage spout is approximately 1 cm. above this level, so that no siphonage occurs through the spout unless the cell is deliberately tipped downwards.

A rack bearing a number of dialysis cells is placed on a hammock-like rocking device, which tilts the cells gently and slowly from side to side. This movement causes the metal ball bearings to stir the plasma mixture constantly, and simultaneously mixes the two liquid phases in the outer chamber very gently so that no emulsification occurs. The rack and cells should be in a cool sheltered spot, shielded from direct sunlight or wide variations of temperature, in order to minimize the tendency to distillation of methanol from the inner chamber to the upper portion of the cell. After an appropriate period of dialysis (discussed below), the dichloromethane layer can be decanted through the capillary spout, which serves as a micro-separating funnel, and fresh dichloromethane can be added through the pressure-equalization vent by means of a syringe and a fine needle. Successive extracts of each sample are decanted into the same conical centrifuge tube, dried under a stream of N_2 in a warm-water bath (35°) and then redissolved in the minimum amount of absolute methanol for quantitative transfer to a chromatography paper.

If the cell is cleaned immediately after use, no protein remains adherent to the dialysis membrane and the cell can be used a number of times with the same membrane in place. When not in use, the cells are stored with water in them to prevent drying and splitting of the membranes.

Paper chromatography

Several different paper-chromatographic systems are available for use with adrenocortical steroids (e.g. Burton, Zaffaroni & Keutmann, 1951; Bush, 1952; Pechet, 1953; Sakal & Merrill, 1953; Schmidt & Staudinger, 1953), and the choice must depend upon the specific requirements of the problem under study. In the problem for which the present micro-extraction procedure was devised, Bush's (1952) benzene–methanol–water system was the most convenient. The steroids to be measured were conveniently separated in a short run, so that ascending chromatography could be used. This was found to offer the advantages of more compact zones and more uniform movement of solvent and steroids on the parallel limbs of each chromatogram.

The solvent mixture, benzene–methanol–water (2:1:1, by vol.), is placed directly in the bottom of the chromatography jar. It separates into an upper benzene-rich phase

and a lower aqueous-methanol phase. The paper lining the tank extends to the bottom and becomes preferentially wetted by the aqueous-methanol phase, thus keeping the atmosphere in the jar saturated with the vapours of these solvents. Equilibration of the vapour phase with benzene can occur directly from the surface of the benzene layer. A small window cut in the paper lining the jar permits direct observation of the lower part of the jar and its contents.

Whatman no. 1 filter paper is used, after being cleaned by exhaustive capillary washing with benzene for 24 hr., followed by 50% (v/v) methanol for 3–4 days. The washing procedure is most important for successful quantitative work and should be done with small batches of paper so that washed papers are not stored for more than a week or two before use. Lewis (1956) has described a capillary-washing procedure employing ethanolic NaOH. He reports that papers cleaned by his method can be stored indefinitely before use in chromatography. For chromatography, the paper is cut as shown in Fig. 2. The limbs are 1 cm. wide and 36 cm. long, and are left joined at top and bottom to prevent tangling and cross-contamination during handling. Each dialysis extract or reference compound is applied to the lower 6 cm. of a separate limb, below the pencilled starting line. The various samples are then concentrated at the starting lines of their respective strips by means of an adaptation of Bush's 'wick method' (Bush, 1952). The lower edge of the paper is immersed in a mixture of equal parts of $CHCl_3$, methanol and ethyl acetate placed in the bottom of a chromatography tank used exclusively for this concentration procedure. This mixture is allowed to advance by capillary ascent just to the starting line, taking about 5 min. to do so. The paper is air-dried for a minute, then the preliminary run is repeated in the same manner. After three or four such runs, the whole of each sample is effectively concentrated in a very narrow band at the starting line. The amount of non-steroid lipid material in the dialysis extract is usually too small to interfere with this preliminary concentration procedure, or with the subsequent chromatography. With some post-prandial blood samples, however, some interference was encountered. This was readily overcome by partitioning the dialysis extract between aqueous methanol and light petroleum. The aqueous-methanol layer was then evaporated, and the residue redissolved in methanol for purposes of chromatography, as described above.

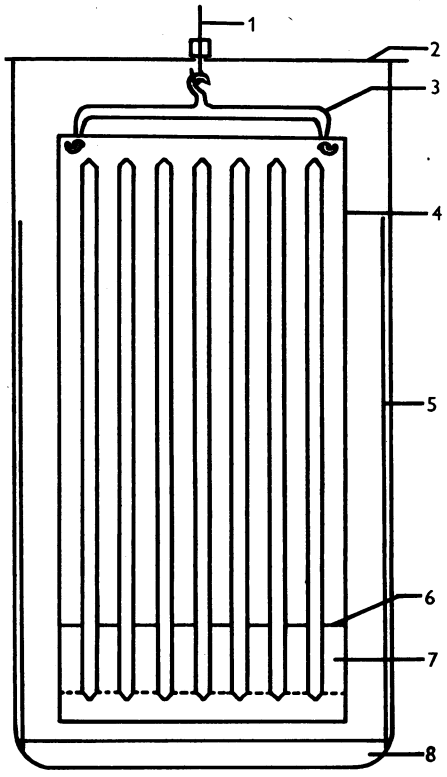

Fig. 2. Apparatus for ascending chromatography of plasma extracts. 1, Adjustable suspension wire passing through rubber stopper; 2, ground-glass lid with $\frac{1}{8}$ in. drilled hole for suspension wire; 3, glass suspension hook for paper; 4, multiple-limbed chromatography paper; 5, paper lining wall of tank; 6, pencilled starting line; 7, area of application of sample; 8, solvent mixture.

Pure samples of the steroids under study are run on each chromatogram. At least two strips on each chromatogram are used for these reference compounds, usually the third and sixth limbs of each eight-limbed paper. One or two strips are left blank. The paper is suspended in the chromatography tank above the surface of the benzene, and left overnight to equilibrate with the vapour phase. In the morning, the suspension hook is lowered until the lower edge of the paper is immersed to a depth of 1–2 mm. in the liquid benzene layer. This is observed through the window cut in the lining paper of the tank. It is essential that the paper must not reach down into the aqueous-methanol layer. At an average room temperature of 25°, 4 hr. is the usual length of time taken by the solvent front in climbing almost the full length of the free limbs of the paper. The paper is then removed and air-dried in a ventilated cabinet.

After chromatography, the individual steroid bands are located by ultraviolet contact photography (Bush, 1952; Haines, 1952). Depending upon the uniformity and cleanness of the background, as little as 1 μg. of steroid may be detected by this means. This amount, however, will seldom be obtained from 1 ml. of plasma, so that the positions of the desired zones on the plasma-extract strips must usually be obtained by analogy with the positions of the reference compounds. If the solvent fronts are uniform in all strips, and the reference compounds are at the same level, this procedure offers no difficulties. Repeated trials with amounts of hydrocortisone and corticosterone ranging from 1 to 10 μg./strip showed marked uniformity of R_F values on the various strips of any one chromatogram, regardless of sample size.

Elution and measurement

After the desired zones are located by comparison with the pure reference steroids, equal segments bearing the zones are cut from the chromatogram strips for elution. The corresponding segments from the blank strips serve as reagent blanks for the subsequent measurements. The segments from the reference strips serve as controls of the recovery from each chromatogram.

Capillary elution by the method of Hanes & Isherwood (1949), with absolute methanol as the eluent, resulted in considerable losses of steroid, which were greater the longer the period of elution. This suggested the possibility of progressive destruction of steroid by light (Savard, 1954) or by high surface exposure to air. When the elution was speeded by feeding the methanol on to the paper segment through a very fine glass capillary spout under a small head of pressure, this defect was remedied. However, this required the use of quantities of methanol as large as those used for elution by immersion of the paper segments, so that the latter procedure was adopted because of its simplicity. The methanolic eluates were finally made up to suitable volume with more methanol, and samples taken for measurement of the steroids by phosphoric acid- or sulphuric acid-fluorescence reactions as described elsewhere (Kalant, 1958).

RESULTS

Preliminary experiments indicated that continuous dialysis for 6 hr., with hourly changes of dichloromethane, was sufficient to extract completely 3–4 μg. of added hydrocortisone from 1 ml. of plasma. In practice, however, the following schedule was more convenient. The dialysis was begun at the end of the day, and the first period of extraction lasted overnight. After decantation of the dichloromethane next morning, a fresh quantity was added and dialysis was continued for 1 hr. while the first extract was being evaporated. This step was repeated once.

Recovery studies were made of the whole procedure, starting with elution of pure steroids from filter-paper strips, and then adding one stage at a time. In twelve trials recovery of 1–5 μg. samples of hydrocortisone, applied to filter-paper strips and then eluted immediately, averaged $97 \pm 2\%$ (S.E.). After chromatography and elution, recoveries averaged $97 \pm 10\%$ for ten samples of 1 μg., but only $75 \pm 8\%$ for eight samples of 5 μg. Recovery of added corticosterone and hydrocortisone, in amounts ranging from 0·5 to 1·0 μg. in 1 ml. of plasma, carried through the whole procedure of dialysis, chromatography and elution, averaged $89 \pm 11\%$ (twelve experiments).

The hydrocortisone concentration in femoral arterial plasma, as determined by this method in ten samples from five normal dogs under light Pentothal anaesthesia, was $0{\cdot}33 \pm 0{\cdot}06$ μg./ml. No constant or significant arteriovenous difference in concentration was found; this is in agreement with the findings of Nelson, Samuels, Willardson & Tyler (1951) in human subjects.

DISCUSSION

The procedure described above is not a new one in principle, as pointed out in the introduction. The present work demonstrates the possibility of applying the principle quantitatively to small samples of peripheral blood plasma, and offers convenient apparatus for the purpose. It is not offered as an alternative to the method of Nelson & Samuels (1952), but as a complementary procedure for use in different situations. In addition to permitting the measurement of steroids other than 17-hydroxycorticosteroids (if present in sufficient amount), it has the advantage of requiring only 1 ml. of plasma, an amount obtainable from a single rat or guinea pig. Therefore it lends itself to metabolic studies in small laboratory animals, for which suitable methods have not been available hitherto.

The reliability of this method, however, depends upon a series of precautions that must be observed scrupulously. Since the final techniques of measurement are not strictly specific, the cleaning of the apparatus and of the paper for chromatography, as well as the purification of reagents and solvents, are of the utmost importance in order to avoid contaminants which exceed the steroids in amount. Also the chromatography must give fully reproducible results if zones too faint to appear on ultraviolet contact photographs are to be successfully located by comparison with the positions of reference compounds. These requirements are so stringent that the method is not likely to be suitable for routine use on a large scale. However, where the means are available to control these factors, and to run simultaneous checks of the efficiency of each stage of the procedure in the manner described above [cf. study of the Nelson & Samuels (1952) procedure by Harwood & Mason (1956)], this procedure promises to be useful in experimental endocrinology.

SUMMARY

1. A method of simultaneous dialysis and extraction of relatively polar steroids from blood has been adapted for use with 1 ml. samples of peripheral blood plasma.

2. A special microdialysis cell has been devised for the purpose. Details of construction and use of the cell are given.

3. Corticosteroids, extracted from plasma by this method, are isolated by ascending chromatography on paper, located by ultraviolet contact photography, eluted and measured by a fluorophotometric procedure.

4. Recovery of hydrocortisone added to plasma in amounts of 1 μg./ml. averaged 89 %. Recovery of 5 μg. amounts was considerably less satisfactory. Hydrocortisone levels in ten samples of femoral arterial blood plasma obtained from dogs averaged 0·33 μg./ml.

5. The advantages and limitations of the method are discussed.

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The Free Amino Acids in Growing and Non-Growing Populations of *Escherichia coli*

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It was reported by Taylor (1947) that there were no free amino acids in *Escherichia coli* and other Gram-negative bacteria, and this was considered to represent a fundamental distinction between them and the Gram-positive organisms. Proom & Woiwod (1949), using the more sensitive method of paper chromatography, showed that extracts of coliform bacteria contained a large selection of amino acids. From the conditions of their experiments it was difficult to be sure that the free amino acids they found were not the result of contamination from the hydrolysed casein of the growth medium. More recently free amino acids have been demonstrated in Gram-negative bacteria grown in glucose and ammonium salts, where there is no possibility of contamination by the medium (see Mandelstam, 1955, 1956*a*; Britten, Roberts & French, 1955; Markovitz & Klein, 1955).

Although it seems likely that the free amino acids are on the pathway of protein synthesis (Britten *et al.* 1955), there has not yet been any detailed study of their behaviour in *E. coli*. In the present paper the level of the free amino acids during growth and during nitrogen or carbon starvation is described. Experiments were done on several coliform strains in an attempt to ensure that the conclusions should be generally valid and not due to the peculiarities of a single strain.

MATERIALS AND METHODS

Organisms. A coliform organism called *Bacterium cadaveris* by Gale & Epps (1944) (NCTC 6578) and the following strains of *E. coli* were used: ML30, K12, NCTC1433, ML328c (leucine-requiring), 160–37 (requiring arginine or ornithine), NCTC 4139 (proline-requiring). The organisms were grown with shaking at 35° in the following medium: NH_4Cl, 0·5 g.; $(NH_4)_2SO_4$, 0·5 g.; KH_2PO_4, 13·6 g.; $MgSO_4$, 20 mg.; $Fe(NH_4)_2(SO_4)_2,6H_2O$, 15·6 mg.; glucose, 20 g.; water to 1 l. The pH was adjusted to 7·2 with NaOH. For the amino acid-requiring strains the medium was supplemented with L-arginine HCl (100 μg./ml.) or DL-leucine (300 μg./ml.) or L-proline (150 μg./ml.). The bacteria were generally harvested towards the end of the exponential phase of growth, when the culture contained about 0·8mg.