A METHOD FOR DETECTING CORTISONE IN BODY FLUIDS

BY

C. L. COPE, M.D., F.R.C.P.*Physician and Senior Lecturer, Postgraduate
Medical School of London*

The pioneer work of Venning *et al.* (1946) in studying the urinary excretion of cortisone-like steroids in the urine in various disease states has been followed by relatively little research confirming and extending their observations. This has been largely due to the laborious and time-consuming methods of assay which they had to employ. With the increasing recognition of the widespread influence of cortisone in so many disease processes, and the growing interest in the behaviour of the adrenal gland in these diseases, the need for simpler methods for detecting and assaying such substances in body fluids has become great.

There are several chemical methods for estimating the urinary products of adrenal metabolism, based on their chemical properties, particularly their reducing power and their ability to liberate formaldehyde. In the course of investigations using these chemical methods we formed the impression that the variations in chemically determined steroid in the urine do not parallel the changes in cortisone content (Cope, 1951). The latter, indeed, represents only a small fraction of the reducing steroid found to be present by chemical means.

A method has therefore been developed for detecting the compounds cortisone and 17-hydroxycorticosterone in body fluids which is based on the specific ability of these compounds to reduce the number of circulating eosinophil cells in the blood. Suitably prepared extracts of urine or other body fluids are injected into adrenalectomized mice and the subsequent percentage fall in blood eosinophil cells is measured. A roughly proportional relation can be shown between the extent of this fall and the amount of cortisone injected.

Preparation of Urine Extracts

A 24- or 48-hour collection of urine without preservative, preferably checked for completeness by determination of its creatinine content, is acidified to the extreme range of thymol blue (approximately pH 1) with concentrated sulphuric acid. It is stood at room temperature overnight and then extracted by shaking in separating funnels with four successive quantities of chloroform, each being equal to 15% of the urine being extracted. The resultant chloroform emulsions are centrifuged clear of aqueous phase in a large centrifuge. The clear chloroform phase is evaporated under reduced pressure to a volume of about 30 ml., the temperature of the water bath being kept below 50° C. throughout the distillation. The concentrated chloroform extract is transferred quantitatively to a small separating funnel and is washed three times with N/10 soda (6 ml.) and then three times with water (6 ml.), each washing being back-extracted with chloroform, which is added to the extract. The washed chloroform solution is then evaporated to dryness under reduced pressure at a temperature below 50° C.

Solvent for Injection.—The solvent found most suitable is propylene glycol: 0.2 ml. of this is added to the dried extract. The tube is gently warmed to about 45° C. and is rotated slowly until the extract is fully dissolved. Normal saline, 0.8 ml., is then added, and the tube is shaken to give a homogeneous mixture.

Some precipitation may occur on dilution, but this seems to be unimportant.

Adrenalectomized Mice.—Mice have been adrenalectomized by single-skin and bilateral-muscle incision. The best anaesthetic has been found to be 1.5 to 2 mg. of pentobarbitone sodium subcutaneously, supplemented by small quantities of open ether. Only mice from which both adrenals have been successfully removed without rupture are used for the tests. They are maintained on saline (1%) and full diet for at least four days before use. The same animals may be used for several tests on different days.

The Eosinophil Test

Blood is drawn from the tail vein to the 0.5 mark on a white-cell pipette. In a well-warmed room no difficulty is found in obtaining enough blood by a razor cut across one of the four tail veins. The blood is diluted to the top mark with Randolph's diluting fluid, and eosinophil cells are counted in duplicate in the Fuchs-Rosenthal chamber. Randolph's (1944) diluting fluid consists of 0.1% phloxine and 0.1% methylene blue, each dissolved separately in 50% propylene glycol. The two solutions are mixed in the proportion of two parts of phloxine solution to one part of methylene blue solution immediately before use. The eosinophils are very stable and are easily recognized in this diluent, but they require about 15 minutes to stain and to settle in the counting chamber.

The equivalent of 12 hours' urine extract in 20% propylene glycol is then injected subcutaneously and the animals are returned to their cages. The volume of the injection should not exceed 0.5 ml. Further eosinophil counts are done at four and again at six hours after injection. The original tail cut will usually provide blood for these two further samples.

Interpretation.—The significant response is the maximum drop in eosinophil cells expressed as a percentage of the initial value. This drop may be greatest at either four or six hours. Spontaneous rises above starting value may occur after injection of inert extracts, when they are interpreted as zero fall. Only a very small activity of the extract seems to be enough to prevent such spontaneous rises. It appears that the tendency of cortisone-like substances to drop the eosinophils is a much more potent stimulus than the tendency of minor trauma or other factors to produce a rise in eosinophils in the adrenalectomized mouse. Propylene glycol alone does not affect the eosinophil level, though it has been found an unsuitable solvent for assay of these steroids by the glycogen storage method. Speirs and Meyer (1949) have studied the behaviour of mouse eosinophils under laboratory conditions.

Results

Preliminary work with this method has been more concerned with general variations than with careful assay of individual urine specimens. An accurate dose-response curve has therefore not been attempted on large groups of animals. A small series of trials has, however, been made to determine the sensitivity of the test to cortisone-like substances. Five-microgram doses of cortisone acetate gave a mean depression of the eosinophil count of 45%, 10- μ g. doses a mean drop of 68%, and 20- μ g. doses a mean drop of 80.5%. Even with these small animal groups the tendency to a response proportional to the logarithm of the dose is apparent. Individual variations in response are indicated graphically in the accompanying Chart.

The response to extracts of normal urine is fairly wide, but the drop in eosinophil count, after the equivalent of 12 hours' urine from "normal" hospital patients, is usually between 20 and 60% under the conditions we have used, the mean for a small group being 46%. This is equivalent to about 10 μ g. of cortisone a day. Such a figure is considerably lower than has been found by other methods (Venning *et al.*, 1946), and a considerably higher amount has actually been isolated from

Mouse eosinophil drop (%) after injection of cortisone acetate, urine extracts, and blood extracts.

normal urine (Schneider, 1950). Our own low results may be due to the method of preparation of the extract and to other factors. At present, therefore, we do not attach significance to the absolute figure, but stress rather the value of the method for comparative purposes and for following changes under various conditions.

In contrast to the normal urines, ten extracts of urine from four cases of Addison's disease showed much lower average activity. Seven of these had no activity, and only two had activity as great as the normal. These two active extracts were both derived from a patient whose Addison's disease was clinically mild and who had active pulmonary tuberculosis. The mean drop for all ten extracts in this group was 10%. Five extracts from three cases of panhypopituitarism gave no significant drop in the eosinophil count. A pooled extract derived from five patients all eight months pregnant gave eosinophil depressions in six mice equivalent to about 25 μ g. of cortisone a day, a figure more than twice that found in normal urine. Another pooled extract of urine excreted during A.C.T.H. administration was tested at several dose levels and gave a response equivalent to 120 μ g. a day. Similarly, an extract from the urine of a woman with advanced adrenal cortical carcinoma with metastases, and associated with many features of Cushing's syndrome, was tried in 12-, 6-, and 3-hour doses, and indicated an excretion of about 40 μ g. a day, or about four times the normal average.

Several specimens of blood have been tested, a different method of extraction being necessary. Two 20-ml. samples of venous blood showed no activity, but in two of three 20-ml. samples of arterial blood slight activity was suggested. Much further trial is required before this difference can be regarded as significant.

Comment and Conclusions

The method proposed has been found a useful and practical one for detecting cortisone-like activity in urine extracts. Its applicability to blood is uncertain. Preliminary results with the method show activity in normal urine extracts and a raised excretion in late pregnancy, during A.C.T.H. administration, and in a case of adrenal cortical carcinoma. The cortisone-like activity was absent from the urine of most cases of Addison's disease and from three cases of hypopituitarism.

Further work is needed before the method can become established as a reliable one for quantitative assay. When expressed in terms of cortisone acetate the activity found in normal urine and in late pregnancy is lower than was found by Venning *et al.*, using the glycogen storage method. The cause of this discrepancy is being investigated.

Since this work has been completed observations on similar lines have been reported by Rosenberg and Lewis (1950).

My thanks are due to the Medical Research Council for a generous gift of cortisone acetate, and to Miss Xenia Boysen for technical assistance in the preparation of extracts and in the eosinophil counts.

REFERENCES

- Cope, C. L. (1951). *Clin. Sci.* In press.
 Randolph, T. G. (1944). *J. Allergy*, **15**, 89.
 Rosenberg, E., and Lewis, R. A. (1950). *J. appl. Physiol.*, **3**, 164.
 Schneider, J. J. (1950). *J. biol. Chem.*, **183**, 365.
 Speirs, R. S., and Meyer, R. K. (1949). *Endocrinology*, **45**, 403.
 Venning, E. H., Kazmin, V. E., and Bell, J. C. (1946). *Ibid.*, **38**, 79.

ADDENDUM.—Further work at present in progress indicates that the nature of the solvent has a large effect on the rate of absorption of cortisone and, therefore, on the eosinophil depression resulting from a given dose. The responses shown in the first three columns of the chart were obtained with cortisone acetate in saline suspension. When, however, the injected cortisone acetate is dissolved in a volume of 20% propylene glycol equal to that used for our urine extracts a much smaller eosinophil drop occurs. The responses to our normal urine extracts, when compared with cortisone acetate injected under identical conditions, are then equivalent to daily cortisone outputs averaging 50 μ g. The cortisone equivalent of other extracts is correspondingly increased, and these then become comparable to those found by Venning *et al.*

UNUSUAL AURAE IN TWO CASES OF EPILEPSY IN CHILDHOOD

BY

B. D. WYKE, M.B., B.S. Sydney

Beit Memorial Research Fellow; Formerly
Fellow of the Rockefeller Foundation

(From the Nuffield Department of Surgery, Radcliffe
Infirmary, Oxford)

The term "aura," in relation to seizures, was introduced into medicine in A.D. 100 by Pelops, the instructor of Galen, although the Corpus Hippocratica (see the book *De Morbo Sacro*) witnesses that premonitory subjective symptoms ushering in seizures were not unknown to earlier Greek physicians. The first attempt at systematic description of such seizure prodromata was that made by Aretaeus of Cappadocia early in the second century A.D. Since his account the varieties of epileptic aura that have been described are legion. In more recent times they have been the subject of detailed study and classical account by Hughlings Jackson (1931-2), Gowers (1901, 1907), Penfield and Gage (1933), Penfield and Erickson (1941), and Kinnier Wilson (1941).