concentrations attained 1 mg. of depot-tetracosactrin was approximately equivalent to 80 units of corticotrophin-gel. Mean plasma cortisol levels had returned to normal by 24 hours after the corticotrophin-gel, and by 48 hours after both doses of the depot tetracosactrin. To maintain a continuously elevated plasma cortisol corticotrophin-gel in this dose must be given twice daily and 1 mg. of the depot-tetracosactrin once daily; after 2 mg. alternate daily administration might be sufficient. All three preparations gave maximal adrenal cortical stimulation over the first four hours. The depot-tetracosactrin was simpler to give than the corticotrophin-gel. Local discomfort was more common after the synthetic preparation.

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Simple Screening Test for Cushing's Syndrome

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Cushing's syndrome is now generally recognized to be due to the prolonged excessive secretion of cortisol (hydrocortisone), and all of the main clinical features of this disorder have been accidentally reproduced at one time or another in patients on corticosteroid therapy. Gross cases may present no difficulty in diagnosis, but in recent years it has become apparent that these are in a minority, since adrenal hyperfunction is often present in patients with few signs and symptoms suggestive of classical Cushing's syndrome (Ross *et al.*, 1966). Obesity is almost invariably present, but is of little diagnostic value because of its high incidence in the population as a whole, particularly in women between the ages of 20 and 50 years, who are the most likely to be suffering from Cushing's syndrome. Nevertheless, obesity is of some help in drawing attention to the possibility of this disease, and the suspicion of adrenocortical overactivity may be reinforced if the obesity is of central distribution and accompanied by hypertension, menstrual irregularities, hirsutism, or diabetes mellitus.

Clinical acumen, however, is insufficient to establish the diagnosis of this disease, and confirmation depends on showing that cortisol production is in excess of normal. The most reliable means of doing this is to measure the cortisol secretion rate by isotope dilution (Cope and Black, 1958; Brooks *et al.*, 1963; Cope and Pearson, 1965; Ross *et al.*, 1966). This technique has proved invaluable in equivocal cases, but is too highly specialized for routine clinical use. The same objection applies to the estimation of urinary cortisol excretion by chromatography, another procedure which has high diagnostic value (Cope and Black, 1959; Brooks *et al.*, 1963; Rosner *et al.*, 1963). At present most laboratories are only equipped to estimate urinary 17-oxogenic steroids or 17-hydroxycorticoids.

It is now clear, however, that these levels often fall within the accepted normal range in patients with undoubted Cushing's syndrome, and so are an unreliable index of cortisol production in this disease. Indeed, Cope and Pearson (1965) have shown that there is no correlation between the cortisol secretion rate and 17-oxogenic steroid excretion over the normal range and the region just above it where so many of the equivocal values lie.

In order to recognize Cushing's syndrome in patients who are not yet grossly diseased, a test is needed the results of which more accurately reflect the cortisol secretion rate, and which is simple enough to be carried out routinely for screening purposes. In 1964 a simple fluorimetric method was described for estimating unconjugated 11-hydroxycorticoids in urine (Mattingly *et al.*, 1964). These urinary 11-hydroxycorticoid estimations showed a better correlation with the cortisol secretion rate over the required range than did the 17-oxogenic steroids. Elevated levels were found in a number of patients with Cushing's syndrome, and it was suggested that this method might be of value in screening patients for this disease. The present paper extends these observations to include 34 patients with Cushing's syndrome, and compares their urinary 11-hydroxycorticoid excretion with that of control subjects and patients with "simple" obesity.

Materials and Methods

The ages of the subjects ranged from 13 to 72 years. They were divided into three groups:

(1) A control group consisting of 50 females and 59 males made up of normal subjects and of convalescent hospital patients. The latter were not acutely ill and were under no obvious stress at the

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time of urine collection. Patients with endocrine, liver, or renal diseases were excluded.

(2) A group of 62 female and 13 male patients who were obviously obese but had no other manifestation of Cushing's syndrome.

(3) A group of 27 female and 7 male patients with Cushing's syndrome. The series consisted of 25 patients with adrenal hyperplasia, 4 with adrenal tumours, and 5 with adrenal hyperplasia associated with "non-endocrine" tumours. The diagnosis was confirmed independently in 20 cases by estimation of the cortisol secretion rate (Mattingly *et al.*, 1964) and by inspection of the adrenal glands at operation or necropsy in the remainder. In all cases plasma 11-hydroxycorticoid levels were measured by fluorimetry (Mattingly, 1962). The normal diurnal variation of these levels was absent or greatly reduced in all 34 patients, and in 33 cases the plasma 11-hydroxycorticoid levels and urinary corticosteroids failed to suppress normally during dexamethasone administration (Mattingly, 1963; Cope, 1966).

Urine Collections.—Collections of urine over 24-hour periods from normal subjects and outpatients were made with no interruption of their daily lives. In ward patients a normal ward routine was followed, but care was taken to ensure that the patients were not subjected to any stressful procedure during the collection period. Subjects were instructed to empty their bladders before defaecation to avoid losses at this time. Urine was collected in Winchester bottles without any added preservative. The bottles had been washed with either chromic acid or the detergent Decon 75 (Medical-Pharmaceutical Developments Ltd.). This latter substance, unlike most other detergents, does not fluoresce and so does not interfere with the steroid estimations (V. H. T. James, personal communication). Since it is more convenient to use than chromic acid it was used exclusively in the latter stages of the work. If the analyses were not undertaken immediately a 20-ml.

aliquot of the urine was deep-frozen within a few hours of the completion of collection.

Determination of 11-Hydroxycorticoids.—The fluorimetric method of Mattingly *et al.* (1964) was used throughout, using 2 ml. samples of urine. Latterly, the detergent Decon 75 was used to wash glassware in place of the less convenient chromic acid. In all cases the alkali wash consisted of 2 ml. of 1N sodium hydroxide. The fluorescence was read on a direct-reading fluorimeter (model 27A, Electronic Instruments Ltd.), with excitation at 430 m μ and emission at 540 m μ . The precision and accuracy of the method were good. The coefficient of variation over the normal range was 8%, and the mean recovery of added cortisol in amounts ranging from 0.1 to 5 μ g. was 97.2%. Results are expressed throughout in terms of cortisol.

Determination of 17-Oxogenic Steroids.—These were measured in the routine biochemistry laboratory by the method of Gibson and Norymberski (1954) or of Appleby *et al.* (1955). The same 24-hour urine samples were used as those for the determination of 11-hydroxycorticoids, but 17-oxogenic steroid estimations were not carried out on all of them.

Results

Control Group.—In 50 females between the ages of 14 and 67 the urinary excretion of 11-hydroxycorticoids ranged from 78 to 311 μ g./24 hours, with a mean of 174 ± 53 (S.D.) μ g./24 hours (Fig. 1). In 59 males between the ages of 17 and 70 years the figures were somewhat higher, and ranged from 108 to 396 μ g./24 hours, with a mean of 229 ± 66 (S.D.)

FIG. 1.—Levels of free 11-hydroxycorticoids in the urine of female subjects. Each dot represents one estimation, and in some cases of Cushing's syndrome more than one estimation was done on each patient. In the tumour section the open circles indicate patients with adenoma and the solid circles those with carcinoma. FIG. 2.—Levels of free 11-hydroxycorticoids in the urine of male subjects. Each dot represents one estimation, and in some cases of Cushing's syndrome more than one estimation was done on each patient. FIG. 3.—Levels of 17-oxogenic steroids in the urine of female subjects. Each dot represents one estimation, and in some cases of Cushing's syndrome more than one estimation was done on each patient. In the tumour section the open circles indicate patients with adenoma and the solid circles those with carcinoma. The vertical broken lines show the normal range. FIG. 4.—Levels of 17-oxogenic steroids in the urine of male subjects. Each dot represents one estimation, and in some cases of Cushing's syndrome more than one estimation was done on each patient. The vertical broken lines show the normal range.

$\mu\text{g.}/24$ hours (Fig. 2). In this small series the level of urinary 11-hydroxycorticoids did not appear to be related to the age of the subject.

Obese Group.—The excretion of 11-hydroxycorticoids was similar to that in the control group, and the ages of the subjects were comparable to those of the controls. In females the amounts ranged from 74 to 294 $\mu\text{g.}/24$ hours, with a mean of 166 ± 53 (S.D.) $\mu\text{g.}/24$ hours (Fig. 1), and in males the range was from 92 to 352 $\mu\text{g.}/24$ hours, with a mean of 245 ± 77 (S.D.) $\mu\text{g.}/24$ hours (Fig. 2). The urinary excretion of 17-oxogenic steroids was measured in most of these patients. In females it ranged from 3.4 to 24.8 mg./24 hours; 6 of the 41 estimations were above the accepted upper normal limit of 18 mg./24 hours (Fig. 3), as quoted by Brooks *et al.* (1963). One estimation fell below the accepted lower normal limit of 5 mg./24 hours, but the urine volume was low, and this result may have been due to the collection being incomplete. In the relatively small number of males the values all fell within the normal range of 6 to 22 mg./24 hours. The actual range found was from 7.0 to 18.4 mg./24 hours (Fig. 4).

Patients with Cushing's Syndrome.—The 11-hydroxycorticoid excretion was raised above normal levels in all 34 patients. In the 27 female patients the excretion ranged from 399 to 5,570 $\mu\text{g.}/24$ hours, while in the seven male patients the levels ranged from 611 to 7,000 $\mu\text{g.}/24$ hours (Figs. 1 and 2). Particularly high values were found in patients with an adrenal carcinoma and in patients with hyperplasia associated with a non-endocrine tumour. There was a considerable day-to-day fluctuation in the urinary 11-hydroxycorticoid excretion in patients with adrenal hyperplasia. For example, one female patient showed levels on four separate occasions of 1,440, 638, 815, and 3,700 $\mu\text{g.}/24$ hours. The excretion of 17-oxogenic steroids in both female and male patients showed a considerable overlap with the normal ranges. In a number of patients more than one estimation was made on different 24-hour urine collections. Only 29 out of the total of 51 estimations were above the upper limits of normal (Figs. 3 and 4). High levels were invariably found, however, in patients with an adrenal carcinoma and in patients with hyperplasia associated with a non-endocrine tumour.

Discussion

These results show that by measuring the urinary output of free 11-hydroxycorticoids a clear distinction can be made between patients with established Cushing's syndrome and those with simple obesity. This method therefore provides a relatively simple screening test, which can be used to decide whether patients with suspected Cushing's syndrome should be further investigated. Our results confirm the findings of previous workers that the urinary 17-oxogenic steroid excretion is often within the normal range in patients with Cushing's syndrome (Cope and Black, 1959; Brooks *et al.*, 1963; Cope and Pearson, 1965; Ernest, 1966; Ross *et al.*, 1966), and that, in addition, the levels in some obese patients are elevated (Cohen, 1958; Simkin, 1961; Baird, 1963; Gogate and Prunty, 1963).

The incidence of Cushing's syndrome and obesity is higher in women than in men, and this is reflected in the relative proportions of the sexes in this study. Furthermore, the normal ranges of urinary steroids differ between men and women so that it is necessary to consider the data separately.

In the control group the mean value for the free 11-hydroxycorticoids in women was 174 $\mu\text{g.}/24$ hours, ranging from 78 to 311 $\mu\text{g.}/24$ hours. In men the mean value was 229 $\mu\text{g.}/24$ hours, the range being 108 to 396 $\mu\text{g.}/24$ hours. These levels are very similar to those found by De Moor *et al.* (1962), who, using a comparable method of estimation, found a mean value for females of 179 $\mu\text{g.}/24$ hours, and for males of 201 $\mu\text{g.}/24$ hours. On the other hand, Gantt *et al.* (1964) have reported levels that are substantially lower than those quoted above.

Their mean level was 86.8 $\mu\text{g.}/24$ hours in 17 healthy adults between the ages of 20 and 40 years, with a range of 31 to 150 $\mu\text{g.}/24$ hours. Their method involved a more complex schedule for washing the methylene dichloride extracts, and in our hands led to a reduction in the recovery of added cortisol from 95% to 80%, and to a 40% loss in the fluorescence of a urine specimen estimated by both methods. Presumably some of the more polar steroids which are estimated with cortisol in our method are removed by this technique.

The reliance which can be placed on the results of any scientific work depends mainly on the validity of the methods used to produce those results. Criticisms can be levelled against this fluorescence method for the estimation of 11-hydroxycorticoids, but its virtues far outweigh its disadvantages. It is accurate and precise, and is quick and easy to carry out. Six estimations can be completed in less than two hours. In contrast to the 17-oxogenic steroid estimation the presence of glucose in the urine does not interfere with this method.

The justification for its use as a measure of adrenocortical activity lies in the good correlation between this method and the isotopic estimation of the cortisol secretion rate, but it should be emphasized that this fluorescence method was originally proposed as a measure of free 11-hydroxycorticoids in the urine, and not of cortisol alone (Mattingly *et al.*, 1964). There is little doubt that other steroids are measured besides cortisol, and these include corticosterone and 20-hydroxycortisol. Indeed, cortisol may comprise only a small proportion of the total fluorescence in some urines. Espiner (1965), using paper chromatography and an internal isotope standard to correct for losses, found that the amount of free cortisol accounted for only 8 to 73% of the fluorescence measured in different urine samples. In addition there is a small amount of nonspecific substances included in the estimation. The total fluorescence increases with time if the urine is not deep-frozen within 24 hours of completing the collection, and the increase is greatest in infected urines.

Drugs administered to patients do not appear to interfere with these estimations, with the exception of spironolactone and mepacrine. Mepacrine may continue to be excreted for many days after cessation of administration (Freitas e Costa *et al.*, 1965); we found the same phenomenon in one patient on spironolactone who is not included in this series. It is well known that oestrogens raise the plasma levels of 11-hydroxycorticoids, and that high levels are found in women taking oral contraceptives (Metcalf and Beaven, 1963). It might be expected that this would affect the amount of free 11-hydroxycorticoids found in the urine, but the increase of plasma 11-hydroxycorticoids is due to an increase in the protein-bound cortisol which is not filtered through the renal glomeruli. In a small number of healthy women taking contraceptive pills, who are not included in our control group, the urinary excretion of 11-hydroxycorticoids at the end of the three-week cycle fell within the normal range at a time when their plasma 11-hydroxycorticoids were elevated. Nevertheless, if high urinary levels are found in patients taking contraceptive pills it is recommended that the treatment be stopped and a repeat estimation done a month later. Katz *et al.* (1962) found that an unconjugated 11-hydroxycorticoid—namely, 6- β -hydroxycortisol—was increased in the urine after oestrogen administration. This particular steroid is not measured by the fluorimetric technique used here, but their findings suggest that oestrogens produce an alteration of cortisol metabolism, and it is possible that this might result in an increased excretion of other unconjugated 11-hydroxycorticoids in the urine.

It is easy when introducing a new diagnostic test to use an elliptical argument in deciding its value; a suspected case shows a positive result in the new test, therefore the diagnosis is confirmed in that case, and therefore the test is useful in diagnosing such cases. This pitfall has, we hope, been avoided here. In all the cases of Cushing's syndrome the diagnosis was

confirmed by independent means. A raised midnight plasma 11-hydroxycorticoid level was found in all cases, and in 33 out of the 34 patients the morning plasma 11-hydroxycorticoid levels failed to suppress normally during the administration of 2 mg. of dexamethasone daily for 48 hours (Mattingly, 1963; Cope, 1966). Raised cortisol secretion rates were found in the 20 patients in whom this investigation was carried out, including the one patient who suppressed normally on dexamethasone. In the remaining 14 patients the diagnosis was ultimately confirmed at operation or necropsy. It was not practicable to carry out these investigations on all the patients with "simple" obesity, but a random sample of 20 obese patients showed normal midnight plasma 11-hydroxycorticoid levels and normal suppression during dexamethasone administration.

There is considerable controversy over whether adrenocortical activity is significantly increased in "simple" obesity. It has been shown in numerous studies that urinary corticosteroid excretion may be raised and that the mean cortisol secretion rate may be higher than normal, a few patients showing grossly elevated levels (Gogate and Prunty, 1963; Migeon *et al.*, 1963; Schteingart *et al.*, 1963; Dunkelman *et al.*, 1964; Copinschi *et al.*, 1966). It is possible, however, that the latter cases were atypical cases of Cushing's syndrome which could not be recognized from the clinical findings. Our results do not support the view that there is a marked deviation of cortisol secretion from normal in obese subjects, since their urinary 11-hydroxycorticoid levels are very similar to those in the control group.

The control group consisted of healthy adults and convalescent hospital patients not suffering from endocrine, liver, or renal disorders that might affect the excretion of steroids in the urine. Care was taken to ensure that these subjects were not stressed during the urine collection period, since both emotional and physical stress will temporarily increase adrenocortical activity and result in a raised urinary 11-hydroxycorticoid excretion. It should be remembered that this study has been based on the urinary 11-hydroxycorticoid excretion in adults and teenagers, and it is probable that the normal ranges will be lower in younger children. For practical purposes, however, this is of little importance, since Cushing's syndrome is rare in this age group.

Summary

Urinary 11-hydroxycorticoids were measured in 34 patients with established Cushing's syndrome, and the results compared with those found in 75 obese patients and in a control group of 109 subjects made up of healthy volunteers and convalescent hospital patients. Raised levels were found in all

the cases of Cushing's syndrome while normal levels were found in the obese group. Urinary 17-oxogenic steroids, on the other hand, showed a considerable overlap between these groups, and normal levels were not infrequently found in patients with Cushing's syndrome.

The fluorimetric estimation of urinary 11-hydroxycorticoids is a relatively simple and rapid procedure, suitable for routine clinical use. It provides a reliable screening test for patients with suspected Cushing's syndrome.

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