KINETIC ASPECTS OF CORTISOL-4-C^{14} METABOLISM IN A PATIENT AFTER SUBTOTAL ADRENALECTOMY FOR CUSHING'S SYNDROME ASSOCIATED WITH BILATERAL ADRENAL HYPERPLASIA

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Research Article

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Although the major urinary metabolites of cortisol [1] have been identified (1–9) and quantitatively estimated (1, 10–13) in man, kinetic aspects related to the formation of these metabolites have been less clearly defined. In a previous publication (14) the pathways of formation, metabolite pool sizes, and the rates of formation of the metabolites of cortisol in two normal humans have been reported. The general method was the administration of a small quantity of cortisol-4-C14 during a steady-state condition (with reference to cortisol turnover) and the determination of the specific activities of cortisol and its metabolites in consecutive 15-minute urine samples. Equations expressing turnover of cortisol and rates of metabolite formation (15) were subsequently applied to these data.

[1] The compounds referred to are: cortisol or F, $11\beta,17\alpha,21$-trihydroxy-4-pregnene-3,20-dione; THF, $3\alpha,11\beta,17\alpha,21$-tetrahydroxy-$5\beta$-pregnane-20-one; allo-THF, $3\alpha,11\beta,17\alpha,21$-tetrahydroxy-$5\alpha$-pregnane-20-one; THE, $3\alpha,17\alpha,21$-trihydroxy-5β-pregnane-11,20-dione; cortol, $3\alpha,11\beta,17\alpha,20\alpha$ or $\beta,21$-pentahydroxy-5β-pregnane; cortolone, $3\alpha,17\alpha,20\alpha$ or $\beta,21$-tetrahydroxy-5β-pregnane-11-one; cortisone or E, $17\alpha,21$-dihydroxy-4-pregnene-3,11,20-trione; 6β-hydroxycortisol or 6β-OH-F, $6\beta,11\beta,17\alpha,21$-tetrahydroxy-4-pregnene-3,20-dione; 6β-hydroxycortisone or 6β-OH-E, $6\beta,17\alpha,21$-trihydroxy-4-pregnene-3,11,20-trione; 20α-DHF, $11\beta,17\alpha,20\alpha,21$-tetrahydroxy-4-pregnene-3-one; 20β-DHF, $11\beta,17\alpha,20\beta,21$-tetrahydroxy-4-pregnene-3-one; 9α-fluorocortisol, 9α-fluoro-$11\beta,17\alpha,21$-trihydroxy-4-pregnen-3,20-dione; 11β-OH-Etio or 11β-hydroxyetiocholanolone, $3\alpha,11\beta$-dihydroxy-5β-androstane-17-one; and 11-O-Etio or 11-ketoetiocholanolone, 3α-hydroxy-5β-androstane-11,17-dione.

To aid in the interpretation and applicability of such kinetic measurements, it seemed advisable to carry out further studies in man using similar techniques in which cortisol turnover was fixed by administration of a continuous infusion so that the calculated kinetic parameters could be compared with the known turnover rate. The patient chosen for study was a 23-year-old woman who, 6 weeks before the initial study, had had a subtotal adrenalectomy as treatment for Cushing's syndrome associated with adrenal hyperplasia. From the time of subtotal adrenalectomy to the time of the studies reported, the patient required cortisone as adrenal maintenance therapy. The initial observations of cortisol metabolism were obtained 24 hours after a surgical procedure, cholecystectomy. A similar study was repeated 4 months later when the patient appeared totally recovered from the cholecystectomy. In this paper, a comparison of kinetic parameters is made with reference to the low recovery of urinary cortisol metabolites, and attention is called to the low excretion of the 5α isomer, allo-THF, which we have reported to occur in Cushing's syndrome associated with adrenal hyperplasia (16), and to the formation of 6β-hydroxycortisol-4-C14 from cortisol-4-C14.

EXPERIMENTAL PROCEDURE

Subject. B.R., a woman who developed symptoms of Cushing's syndrome at 18 years of age, was treated on two occasions, 4 years apart, by single doses of pituitary irradiation equivalent, totally, to 1,600 roentgens. Because of recurrence of symptoms at age 23, two years after the last pituitary irradiation, a right total and a left subtotal adrenalectomy were performed at the Peter Bent Brigham Hospital, Boston, on January 12, 1959. After adrenalectomy, inasmuch as no urinary metabolites of cortisol were measurable even with administration of exogenous ACTH, the patient was maintained with 50 mg cortisone and 0.1 mg 9α-fluorocortisol per day by oral administration. Six weeks after adrenalectomy she was

FIG. 1. PLASMA CORTISOL CONCENTRATION DURING THE EXPERIMENTAL PERIODS. Cortisol infusion is cortisol hemisuccinate administered at the rate of 200 mg cortisol per 24 hours. Interruption along the abscissa indicates a 4-month period of convalescence.

re-admitted to the Peter Bent Brigham Hospital for a cholecystectomy and appendectomy. Immediately preceding surgery, a constant intravenous infusion of cortisol hemisuccinate [2] equivalent to 200 mg cortisol per 24 hours was begun, and was maintained throughout both surgery and the ensuing 24-hour period (Figure 1).

The initial study was carried out on the day after surgery (post-operative period). A small dose of cortisol-4-C^{14} (9.39 × 10^5 cpm, approximately 1 μc, 234 μg) was administered intravenously over approximately 3 minutes to the patient while she was receiving the constant infusion of cortisol. Urine volume was maintained with an intravenous saline drip, and samples were collected at 15-minute intervals by means of an indwelling catheter. Three plasma samples were taken during the kinetic study to check on the steady-state condition (see Figure 1). Specific activities, in counts per minute per microgram, of cortisol and its metabolites were determined in each urine sample and expressed as a function of time, corresponding to the mid-point of the period of urine collection. Pool sizes and pathways were determined by isotope dilution procedures after the intravenous administration of small amounts of unlabeled cortisol metabolites in a manner previously described (14).

The second study was carried out 4 months later when the patient had totally recovered from the cholecystectomy (control period). During the intervening 4 months it was found necessary to maintain the patient on a daily oral dose of 37.5 mg cortisone and 0.1 mg 9α-fluorocortisol. The procedure followed was identical to that carried out during the post-operative period. The cortisol-4-C^{14} administered was 1.56 × 10^6 cpm, approximately 1.5 μc and 375 μg.

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[2] Solu-Cortef, sodium salt of cortisol hemisuccinate, Upjohn Co., Kalamazoo, Mich.

Steroids administered.[3] The techniques employed in purifying and preparing the cortisol-4-C^{14} and the metabolites THF, THE, and β-cortolone administered intravenously in these studies have been described previously (14).

Analytic methods. Plasma and urine samples were kept frozen until used. Extractions were performed as previously described (14) with the following exceptions. a) After an initial triple chloroform extraction to yield "free" metabolites, urines were further extracted 3 times with double volumes of ethyl acetate to obtain the free 6β-hydroxycortisol (6). b) The residual urine was adjusted to pH 5 and incubated overnight at 47° C with β-glucuronidase[4] added to a final concentration of 1,000 Fishman units per ml. The glucuronide fraction containing THF, allo-THF, THE, and cortolone was prepared by extracting the incubated urine 3 times with double volumes of chloroform and washing quickly twice with 0.05 volumes 0.01 N sodium hydroxide and once with 0.05 volume water. c) In those instances in which cortol was determined, the urine was extracted as above, but the alkaline and water washes were omitted, since such treatment decreased the yield. Instead, purification was achieved by passing the chloroform extract through a Florisil column as described by Nelson and Samuels (17). The 25 per cent methanol-in-chloroform eluate from the Florisil column was chromatographed on paper for cortol.

Extracts representing the glucuronide fractions were chromatographed in a toluene: 75 per cent methanol system for 39 hours at 32° C on unwashed Whatman no. 1 paper, used throughout this study. The THF, allo-THF, and THE areas, located by blue tetrazolium reduction on a 2-mm test strip, were eluted and aliquots taken for counts per minute and microgram content with the phenylhydrazine reagent (18) by using the appropriate standards. Allo-THF was estimated with THF as a quantitative standard. Where contamination due to chromatographic overlap was observed, areas were eluted and re-chromatographed for complete resolution.

Metabolites possessing a C_{17}, C_{20}-dihydroxyl configuration (20α-DHF and 20β-DHF in the free extracts, and cortol and cortolone in the glucuronide extracts) were located on the chromatographic channels by placing 1-cm strips of paper directly in planchets and determining the radioactivity in a windowless gas-flow counter (see *Radioactive determinations*). Ultraviolet scanning also delineated the two epimers of 20-DHF. The radioactive peak areas were eluted, oxidized with periodic acid (19), and chromatographed, alongside corresponding 11-oxy-C_{19} standard reference steroids, in the system 67 per cent ligroin in toluene: 75 per cent methanol in water for 8 hours at 32° C. The radioactive 11-oxy-C_{19} areas were located once again by determining the radioactivity in 1-cm

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[3] Cortisol-4-C^{14} obtained from the Endocrinology Study Section of the National Institutes of Health, U. S. Public Health Service.

[4] Ketodase, Warner-Chilcott Laboratories, Warner-Lambert Pharmaceutical Co., N. Y.

strips of the chromatographic channels. The peak radio-active areas were eluted. Micro-Zimmermann and radioactivity determinations were performed on aliquots of the eluate and specific activities calculated.

6β-hydroxycortisol. The free ethyl acetate extract, after a quick washing with 0.01 N sodium hydroxide and water, was evaporated under reduced pressure to dryness. The residue was chromatographed overnight at 32° C in the descending system, 9 per cent butanol in benzene: 50 per cent aqueous methanol. The 6β-hydroxycortisol area, readily located by scanning in the ultraviolet (235 mμ), was subsequently eluted and estimated with the phenyl-hydrazine reagent, with cortisol as a quantitative standard. Further characterization of the 6β-hydroxycortisol-4-C¹⁴ was achieved, with methods similar to those described previously (20), by combining eluted with authentic unlabeled 6β-hydroxycortisol and re-chromato-graphing in a single narrow channel. Each centimeter of chromatogram was subsequently eluted into clean nickel-plated steel planchets, dried, counted, and transferred, with filtration through coarse micro sintered-glass funnels, to 12 × 75 mm culture tubes for the phenylhydra-zine reaction. The coincidence of radioactivity and steroid content for 6β-hydroxycortisol is shown in Figure 8.

Plasma cortisol concentrations were determined by a chromatographic procedure employing isotope dilution with cortisol-4-C¹⁴. To a known volume of plasma (approximately 10 ml), 0.1 ml of an ethanolic solution of cortisol-4-C¹⁴ containing 3.4 μg cortisol and 1.08×10^6 cpm was added. After thorough mixing, the plasma was extracted with chloroform in the same manner as the urine glucuronides. The residue was applied to Whatman no. 1 paper strips, which had been washed with ethanol in a Soxhlet extractor for 24 to 48 hours, while the paper was still moist with ethanol. The trips were placed in the chromatography tank as rapidly as possible to prevent drying the chromatography paper. Chromatography was performed in the toluene: 75 per cent methanol in water system, at 32° C, overnight. The cortisol areas were located by ultraviolet absorption as well as by adjacent standard cortisol migrations. These areas were eluted immediately to known volume with ethanol. Aliquots were taken for fluorometric estimation of cortisol by the procedures outlined by Sweat (21) and Peterson and Wyngaarden (22). Aliquots also were taken for radioactivity determinations. Plasma concentrations of cortisol were calculated from the change in the specific activity of the cortisol thus determined.

Urinary cortisol specific activities were obtained by chromatographing the urine free chloroform extract in the same manner as the plasma cortisol extracts. Sufficient cortisol was present in the urine samples to permit the use of the phenylhydrazine reagent for quantitative estimation.[5]

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[5] Fukushima and co-workers (9), employing reverse isotope dilution techniques, with normal adult urine extracts, have stated that only half of the radioactivity present in the cortisol area on paper chromatograms was in this hormone. The data obtained in the current study

Radioactive determinations were made in a Nuclear-Chicago D-47 windowless flow counter at infinite thinness, with the exceptions of those instances in which 1-cm strips of chromatograms were counted directly and where recovery experiments from urine were performed. In the latter experiments appropriate corrections were made for C¹⁴-beta radiation self-absorption. For specific activity determinations, expressed as counts per minute per microgram, samples were counted in duplicate with a maximal probable error of 3 per cent. Background was 20 cpm.

RESULTS

Plasma cortisol concentration and the steady state. The plasma concentrations of cortisol at various times during the experimental periods are illustrated in Figure 1. With the exception of the single elevated level immediately after operation, the values are remarkably constant before and after surgery and during the control period. These data are consistent with steady-state conditions prevailing during the post-operative and control periods. The mean plasma cortisol concentrations are 26 μg per cent during the control period and 33 μg per cent during the post-operative period. Cortisol pool sizes and half-lives are shown in Figure 2.

Turnover of cortisol. Plots of the logarithm of the urinary cortisol specific activity as a function of time for the post-operative period and control period, both 8 hours long, are shown in Figure 2. Inasmuch as both curves exhibited first-order decay characteristics, half-lives, pool sizes, and turnovers were calculated for cortisol as described previously (14), and are listed in Figure 2. There is excellent agreement between the actual rate of infusion of cortisol and the turnover of cortisol calculated from the slope of the specific activity curve.

It is interesting to note in the plot of the data of the post-operative period in Figure 2 that if a

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suggest either that no such contamination of the cortisol occurred, or that if a contaminant was present, its properties and specific activity are similar to cortisol. It must be emphasized that in this study, urines were extracted *before* hydrolysis to obtain the cortisol fraction in contrast to extraction *after* hydrolysis as reported by Fukushima and co-workers (9). Metabolites existing as glucuronides and having the mobility of cortisol may be released by β-glucuronidase. On previous occasions (14) the specific activity of cortisol calculated with the Porter-Silber reaction gave identical results with that calculated by the more specific fluorescent technique of Sweat (20).
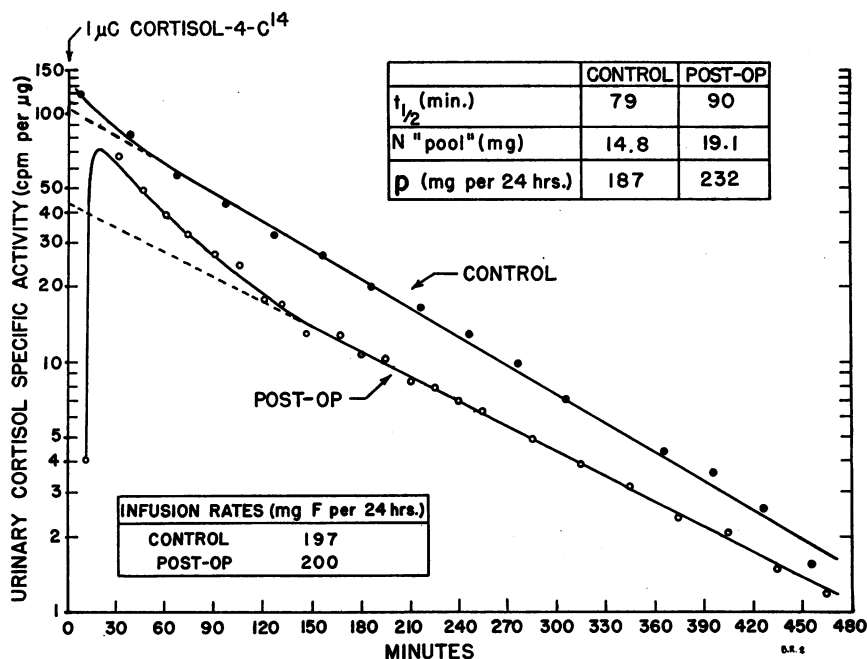
FIG. 2. URINARY CORTISOL SPECIFIC ACTIVITIES AS A FUNCTION OF TIME AFTER THE ADMINISTRATION OF A SMALL DOSE OF CORTISOL-4-C^{14}. p is turnover. Values correspond to the mid-point of the period of urine collection. Straight-line portions, plotted by the method of least squares, were log s.a. $= 2.023-3.79 \times 10^{-3} t$ (control) and log s.a. $= 1.649-3.35 \times 10^{-3} t$ (post-operative) where t $=$ minutes.

cortisol half-life $t_{\frac{1}{2}}$ were calculated from the slope during the first 2 to 3 hours of the study, it would have been markedly different (approximately 54 minutes) from that obtained using the slope after 2 hours. It is the half-life of 90 minutes that is characteristic of the steady state. The delay in apparent mixing of cortisol-4-C^{14} in the post-operative study is striking, and is accompanied by a somewhat longer cortisol half-life and larger cortisol pool than are observed in the control study.

It was possible further to confirm that the turnover of cortisol coincided with the infusion rate by applying a form of isotope dilution equation, $p = C/SA_m$, hereafter referred to as the integral specific activity equation, where p is the rate of turnover of cortisol, C is the counts per minute of cortisol administered at time zero, and SA_m is the specific activity of a metabolite derived exclusively from cortisol (23). SA_m is determined from a urine pool collected over a period sufficiently long to insure almost complete excretion of radioactivity. The specific activity of the allo-THF in the post-operative period is shown in Figure 3, and it can be seen that the major portion of the radio-

activity has already been excreted by 8 hours; therefore, only a small extrapolation of the curve (see shaded area) was necessary to compute the specific activity of allo-THF for a 24-hour urine

CORTISOL TURNOVER
CALCULATED FROM ALLO-THF SPECIFIC ACTIVITIES
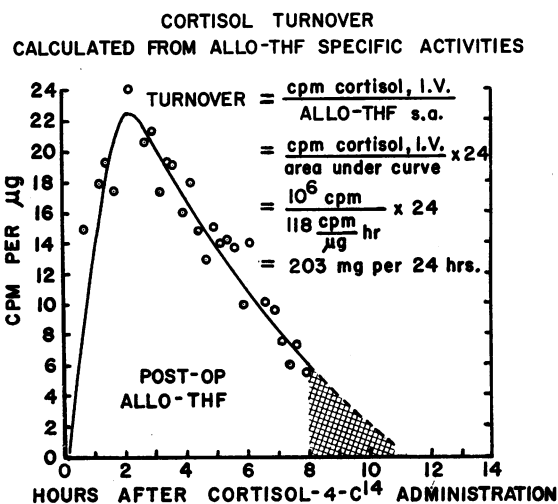
FIG. 3. ALLO-THF SPECIFIC ACTIVITIES (POST-OP) AS A FUNCTION OF TIME FOR THE CALCULATION OF CORTISOL TURNOVER BY THE INTEGRAL SPECIFIC ACTIVITY METHOD (23). See text for details.

pool. Under these steady-state conditions of the experiment, the calculations shown in Figure 3 are permissible. The 24-hour turnover rate of 203 mg agrees well (a 15 per cent difference not considered significant) with both the amount of cortisol infused per 24 hours, 200 mg, and the turnover rate, 232 mg, calculated from the slope of the cortisol curve of Figure 2. Similarly, the 24-hour turnover of cortisol in the control period calculated by the integral specific activity method applied to 6β-hydroxycortisol was 177 mg (see Figure 9). This agreed well with the amount of cortisol infused per 24 hours, 200 mg, and the value of 187 mg calculated from the slope of the cortisol curve of Figure 2.

Metabolite specific activities. The chromatographic separation of the major cortisol metabolites is illustrated in Figure 4 for the 15-minute urine sample of the control period collected 300 minutes after cortisol-4-C¹⁴ administration. There is a close correspondence for the values of radioactivity and amount in micrograms for THF (III), allo-THF (IV), and THE (V). Portions of peaks III and V were oxidized with sodium bismuthate (24) and the products chromatographed as shown in Figure 5. The values

for radioactivity and amount in micrograms again coincided, the specific activity was essentially the same as that of the C_{21} parent steroid, and the products formed from THF and THE migrated with 11β-hydroxyetiocholanolone and 11-keto-etiocholanolone, respectively. A small amount of 11-keto-etiocholanolone is discernible in chromatogram A of Figure 5 and is due to some oxidation of the 11-hydroxyl group by bismuthate.

The specific activities of the four cortisol metabolites, THF, allo-THF, THE, and cortolone are presented in Figure 6, a–d. Both the control and post-operative curves for each metabolite, together with a segment of the respective cortisol specific activity curves, are shown in each of the four graphs comprising Figure 6. The times of administration of the unlabeled metabolites used in the isotope dilution calculations in both the post-operative and control periods are indicated by the vertical lines at 2, 4, and 6 hours. A striking feature is the similarity in the over-all appearance of the post-operative and control curves for each metabolite. The resemblance [6] of these curves to

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[6] That the specific activity-time curves as such may not clearly reveal changes in rates of formation of metabolites is emphasized by the fact that under these steady-
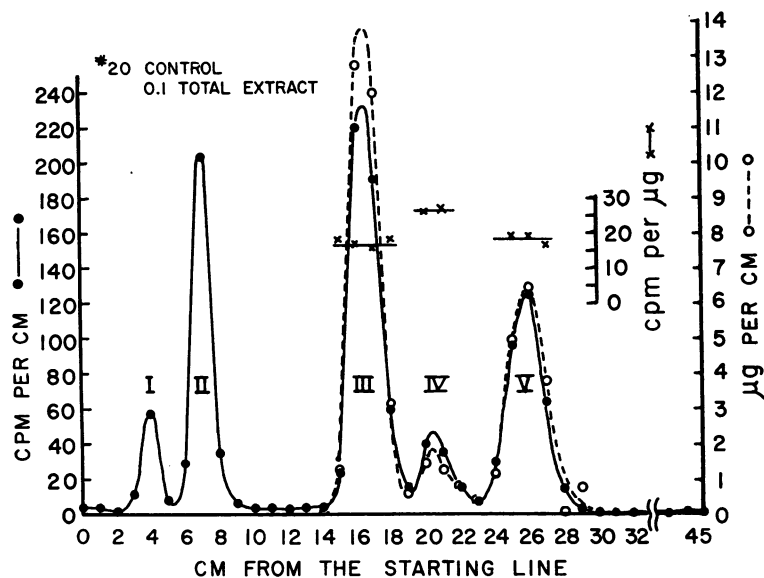
Fig. 4. PAPER CHROMATOGRAPHIC SEPARATION OF METABOLITES FROM ONE 15-MINUTE SAMPLE OF URINE. Each centimeter of paper eluted for analysis. Peaks III, IV, and V estimated by the acid phenylhydrazine reaction and identified further in Figure 5. I, cortols; II, cortolones; III, THF; IV, allo-THF; V, THE.[1] Sample is that analyzed in Table II. Chromatography was in the toluene-methanol system.
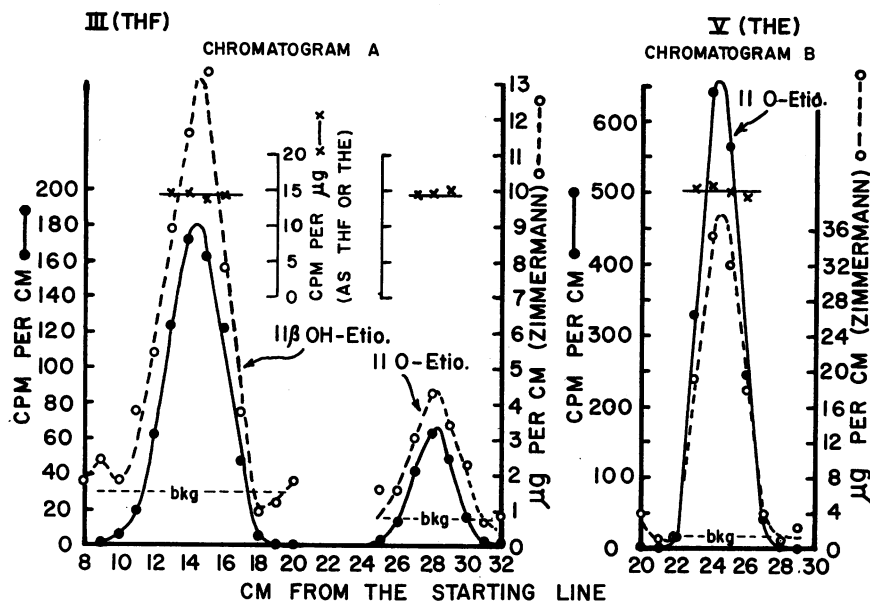
FIG. 5. BISMUTHATE OXIDATION PRODUCTS OF COMPONENTS III (THF) AND V (THE) OF FIGURE 4. Chromatography was in the ligroin-toluene-methanol system, overnight at 32° C. Each centimeter strip eluted for radioactivity and micro-Zimmermann analyses. Specific activities of the oxidation products are equivalent to those of the parent compounds. The small amount of 11-O-Etio [1] formed from THF is due to a small amount of oxidation of the 11-hydroxyl to ketone by the bismuthate. bkg refers to Zimmermann background.

those previously published for a number of normal adults (14) includes: no alteration of the allo-THF specific activity upon administration of unlabeled THF, THE, or β-cortolone (see Figure 6b) ; a decrease in cortolone specific activity upon addition of unlabeled THF, suggesting some conversion of THF to THE and cortolone (see Figure 6d) ; a decrease in THF and cortolone specific activity upon addition of unlabeled THE, suggesting some conversion of THE to both THF and cortolone (see Figure 6a,d) ; small decreases in THF and THE specific activity upon administration of unlabeled β-cortolone, suggesting some conversion of β-cortolone to THE and THF (see Figure 6a,c) ; and the suggestion that the cortisol specific activity curve intersects the metabolite (THF, allo-THF, or THE) curve approximately

state conditions the calculated rate of formation of a metabolite, $p = Nm/A$ (14, 15), is directly proportional to its pool size N as well as to the specific activity-time functions m/A. For example, the very low rate of allo-THF formation (see Table I) is not evident from the specific activity-time curves for allo-THF, but is apparent when the low allo-THF pool size is properly included in the calculated rate of formation.

at the latter's peak (see Figure 6a-c). Because unlabeled THF (see Figure 6a, control) was administered before the maximum in THF specific activity had been reached, the intersection of the cortisol curve with the undiluted THF specific activity curve is not evident. The close similarity however, of the control allo-THF specific activity curve (see Figure 6b) to the THF specific activity curve during the first 2 hours suggested that the control cortisol curve would intersect the theoretical control THF specific activity curve, obtained by extrapolation of the THF curve along the allo-THF curve, at its approximate maximum. A conversion of THF to THE is not made apparent by this experiment, i.e., it is difficult to discern a fall in THE specific activity after the administration of unlabeled THF. The fall in cortolone specific activity after the administration of unlabeled THE is not so great as that observed in a previous study (14), presumably because of the smaller quantity of unlabeled diluent administered in the current work. The pathway from THF to cortol is evident in Figure 7 by the plateau in cortol specific activity after the ad-

SPECIFIC ACTIVITY OF THF

(a)

SPECIFIC ACTIVITY OF ALLO-THF

(b)

SPECIFIC ACTIVITY OF THE

(c)
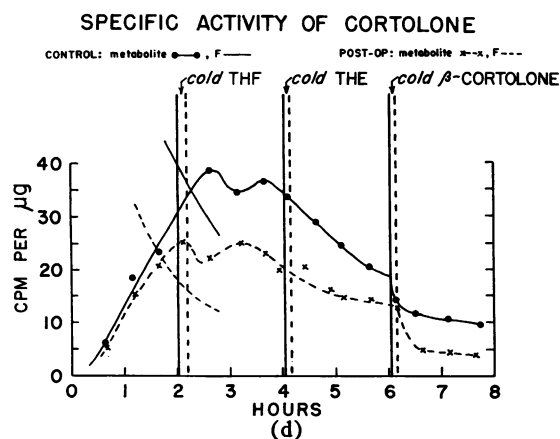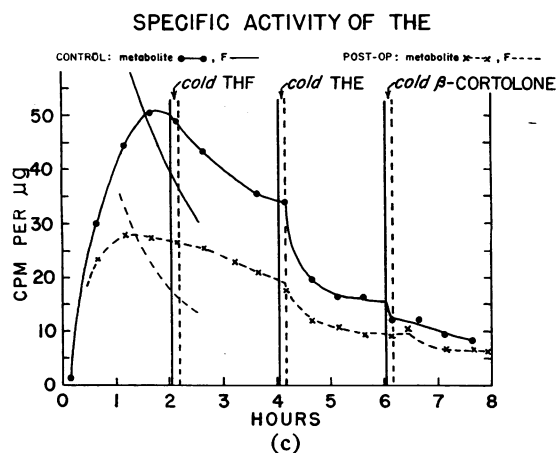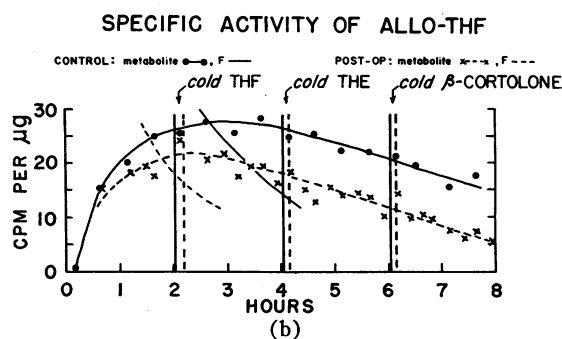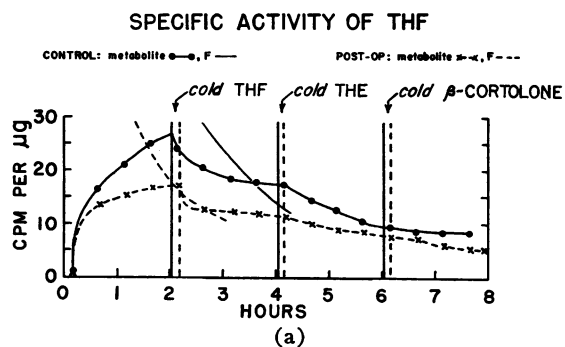
SPECIFIC ACTIVITY OF CORTOLONE

(d)
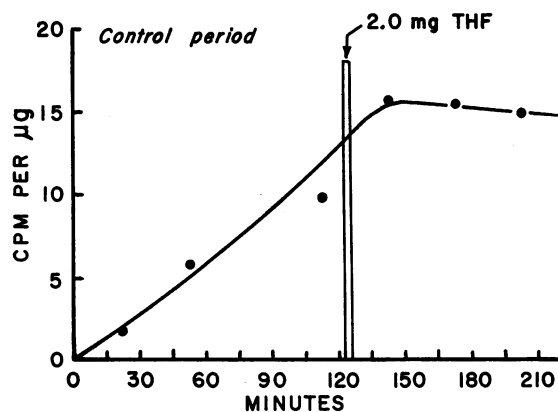
Fig. 7. Specific activities of cortol as a function of time for a portion of the control period. The conversion of THF to cortol is evident. Values for the entire curve are not available.

ministration of unlabeled THF. Unfortunately, the entire cortol curves were not available. As in the previous study (14), the slopes of the cortisol specific activity curves and hence, it may be inferred, the metabolism of cortisol, as such, were not noticeably altered by the administration of the unlabeled metabolites (see Figure 2). A similar inference can be made about 6β-hydroxycortisol (see Figure 9).

Amounts excreted, pool sizes, and rates of formation. The data shown in Figure 6 were used to calculate pool sizes and rates of formation of metabolites. Table I presents the measured major urinary metabolites and the pool sizes calculated by isotope dilution (14). The fall in specific activity of a metabolite after dilution with unlabeled metabolite was employed for the isotope dilution calculation. Correction was made for the conversion of unlabeled diluent to another metabolite. For example, after the administration of unlabeled THF, the decrease in THE specific ac-

Fig. 6. Specific activities as functions of time for 4 metabolites of cortisol. Portions of cortisol curves are included to indicate product-precursor relationship.[8] Note that unlabeled, or cold, THF, allo-THF and THF control curves are almost superimposable. This suggests that although unlabeled THF was added before the control THF maximum was reached, the F curve would intersect the undiluted THF control curve appromately at its maximum. The unlabeled metabolites were administered as follows: Control period: 2.0 mg THF, 2.4 mg THE, and 2.3 mg β-cortolone. Post-op period: 1.9 mg THF, 2.0 mg THE, and 2.3 mg β-cortolone.

tivity was used to determine the amount of un-labeled THF that remained to dilute the THF pool.

Allo-THF excretion. The paucity of allo-THF formation in this patient, in both the post-operative and control periods, is evident in the calculation of pool size (see Table I, b) and is confirmed by the ratio of THF to allo-THF in the excretion of metabolite glucuronides (see Table I, f). Only 2 to 4 per cent of the total THF, allo-THF, and THE excreted is represented by allo-THF in both the control and post-operative periods. This is in marked contrast to the 15 to 30 per cent proportion of allo-THF reported for normal adults (8, 9, 12, 25).

THF: THE. In Table I, f, the ratios of THF : THE, 3.1 in the post-operative and 2.1 in the control period, are both highly elevated when compared with the values (≤ 1.0) in normal adults (8, 9, 12, 25) and those (approximately 1.0) in adults stimulated with ACTH (11, 12, 26). The higher value of the THF : THE ratio

in the post-operative as compared with the control period of Table I is consistent with previous findings (12) that a variety of "stress" situations, surgery included, produce elevations in the THF : THE ratio and that this phenomenon involves alterations in the metabolism of cortisol.

Correlation of pool size with excretion data. It is evident from the data presented that the ratios of the calculated pool sizes (see Table I, e) are equal to the relative proportions of the metabolites excreted as glucuronide (see Table I, f). These observations are consistent with the assumptions that, if the various steroid-glucuronides are distributed in the same space or compartment, then the rate of excretion of each steroid-glucuronide is proportional to its pool size. For the metabolites THF and THE, this can be expressed in the equation $THF_g/THE_g = THF_u/THE_u$, where g indicates the amount in the glucuronide pool and u the amount excreted per unit of time as glucuronide. This observation has been emphasized in a previous report (14).

TABLE I

Readily miscible pool sizes and rates of formation of cortisol metabolites *

Compound a	Calculated pool size† b	Rate of formation c	Metabolite ratio d	Ratio from calculated pools e	Ratio from urinary excretion‡ f
	mg	*mg per 24 hours*			
Post-operative period					
THF	3.6	42	THF :THE	3.3	3.1
Allo-THF	0.15§	2.5	THF :allo-THF	26**	26
THE	1.1	60	THF :cortolone	6	§§
cortolone	0.6‖				
		Sum 104			
F	19¶	232			
F		203**			
Control period					
THF	3.2	49	THF :THE	2.1	2.1
Allo-THF	0.19§	1.2	THF :allo-THF	17**	17
THE	1.5	49	THF :cortolone	5	§§
cortolone	0.6‖				
		Sum 99			
F	15¶	187			
F		177††			

* Details as in footnote 1, Methods, and Results.
† Calculated by isotope dilution, unless otherwise indicated, using the decrease in specific activity of the glucuronide fraction (see Figure 6) after intravenous administration of unlabeled metabolite.
‡ Computed before administration of unlabeled metabolite
§ Calculated from N = ka where N is pool size, a is amount of metabolite excreted per unit time, and k is a proportionality factor calculated from the excretion of THF and THE. This calculation has been discussed elsewhere (14).
‖ Cortolone includes both 20α and 20β epimers.
¶ Total radioactivity injected divided by cortisol specific activity extrapolated to time zero.
** Calculated from the integral of the allo-THF specific activities (see Figure 3).
†† Calculated from the integral of the 6β-hydroxycortisol specific activities (see Figure 9).
‡‡ Since the allo-THF pool size is calculated from urinary excretion data, the pool size ratio equals the urinary ratio.
§§ Not available.

Recovery and partition of radioactivity in one 15-minute urine sample *

Urine fraction†		Preliminary column fractionation‡			
		2 per cent	25 per cent	Methanol	Total
	cpm	*cpm*			
I Non-conjugated					
a soluble in chloroform	2,510 (7.7)	128 (0.4)	2,260 (.69)	89 (0.3)	2,477 (7.6)
b soluble in ethyl acetate (after chloroform)	600 (1.8)	40 (0.1)	524 (1.6)	30 (0.1)	594 (1.8)
Subtotal	3,110 (9.5)				
II Glucuronide§					
a soluble in carbon tetrachloride	1,350 (4.1)	795 (2.4)	444 (1.4)	‖	1,239 (3.8)
b soluble in chloroform	22,000 (68)	165 (0.7)	19,400 (60)	1,790 (5.5)	21,400 (66)
c soluble in ethyl acetate	3,060 (9.4)	‖	2,240 (6.8)	429 (1.3)	2,669 (8.2)
Subtotal	26,400 (81)			Total column recovery	25,300 (86)
III Residue	3,200¶ (9.8)				
IV Total radioactivity	32,710				

* Sample no. 20, *control* period, 300 minutes after i.v. cortisol-4-C¹⁴. Per cent of total radioactivity in parentheses.
† Urine extracted 3 times with double volumes of the solvents indicated. No alkaline washing procedures were employed.
‡ Florisil column as referred to by Nelson and Samuels (18). Per cent refers to the amount of methanol in the chloroform employed to elute the column. Methanol was used to strip the column.
§ Hydrolysis with Ketodase 72 hours at pH 5, 37° C, 200 Fishman units per ml.
‖ Negligible.
¶ Corrected for self-absorption with cortisol-4-C¹⁴.

Rates of formation of metabolites. The rates of formation (see Table I, c) of THF, allo-THF, and THE calculated according to Zilversmit, Entenman, and Fishler (15) accounted for only 50 per cent of the turnover of cortisol in both the post-operative and control periods. It is assumed in such a calculation that the remaining 50 per cent of the turnover of cortisol would be compounds not metabolized by way of THF, allo-THF, and THE. These would include such compounds as unmetabolized cortisol, 6β-hydroxy-cortisol, 20α-DHF, 20β-DHF, and perhaps others as yet unidentified.

An attempt to account for the 2 mg of cortisol and its metabolites excreted during a 15-minute interval is shown in Table II.[7] Sample no. 20,

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[7] Under the steady-state conditions, calculated from the turn-over data of Figure 2 and the infusion rate of 200 mg per 24 hours, metabolites equivalent to 2 mg of corti-

the 15-minute urine sample obtained 300 minutes after the i.v. administration of the cortisol-4-C¹⁴, was fractionated as shown. It can be seen that of the total radioactivity present in the sample, 9.5 per cent was extractable with organic solvents prior to β-glucuronidase hydrolysis, whereas 81 per cent of the radioactivity was extractable with organic solvent after treatment of the urine with β-glucuronidase, and 9.8 per cent remained in the urine as a residue. Preliminary column fractiona-

sol are excreted per 15 minutes. The amount of metabolites excreted in sample no. 20, 1 hour after the administration of unlabeled THE, did not appear to be significantly augmented by the administration of the unlabeled diluents. For example, the 15 minute excretions of THF and THE (control) during the first 2 hours of the experiment, before pool size determinations, were approximately 400 μg and 190 μg, respectively, whereas in sample no. 20 the excretions were 397 μg and 226 μg, respectively (see Table III).

tion of the non-conjugated and glucuronide fractions on Florisil yielded maximum radioactivity in the 25 per cent methanol-chloroform eluate of the chloroform extract. Paper chromatography (Table III) of the glucuronide fraction (see Table II, b) demonstrated that 89 per cent of the radioactivity was accounted for by the compounds indicated. These metabolites, 843 μg, approximate the sum of the calculated rates of formation of THF, allo-THF, and THE (see Table I, c), i.e., 99 mg per 24 hours or 1,030 μg per 15 minutes. Hence, it would appear that about 50 per cent of the infused cortisol was excreted as metabolites whose formation proceeded through either THF, allo-THF, or THE. The non-conjugated urine fractions, the glucuronide fractions soluble in carbon tetrachloride and in ethyl acetate, designated I a, b and II a, c in Table II, were exhaustively studied, and a number of highly polar substances, several corresponding in chromatographic mobility to 6β-hydroxycortisol, 6β-hydroxycortisone, 20α-DHF, and 20β-DHF, were observed. Because losses for these metabolites were not ascertainable, however, it was not possible to determine by summation of weights whether these numerous metabolites were equivalent to 1 mg per 15-minute period. It was possible, however, to establish 6β-hydroxycortisol as the major component of the non-conjugated fraction soluble in ethyl acetate, after chloroform (Ib of Table II). This extract, when chromatographed in the benzene-butanol system, displayed a radioactive peak whose mobility was identical with that of authentic 6β-hydroxycortisol. When a portion of

TABLE III

Paper chromatography of glucuronide fraction from one 15-minute urine sample *

Compound	Radio-activity	Amount
	cpm	*μg*
THF	6,450	397
allo-THF	1,370	55
THE	4,400	226
cortols	994	†
cortolones	3,260	165
"overflow"	435	†
Total	17,269	843

* 19,400 cpm, representing glucuronide fraction soluble in chloroform, 25 per cent Florisil column cut (fraction II b of Table II). Toluene: 75 per cent methanol system for chromatography. 89 per cent recovery of radioactivity.
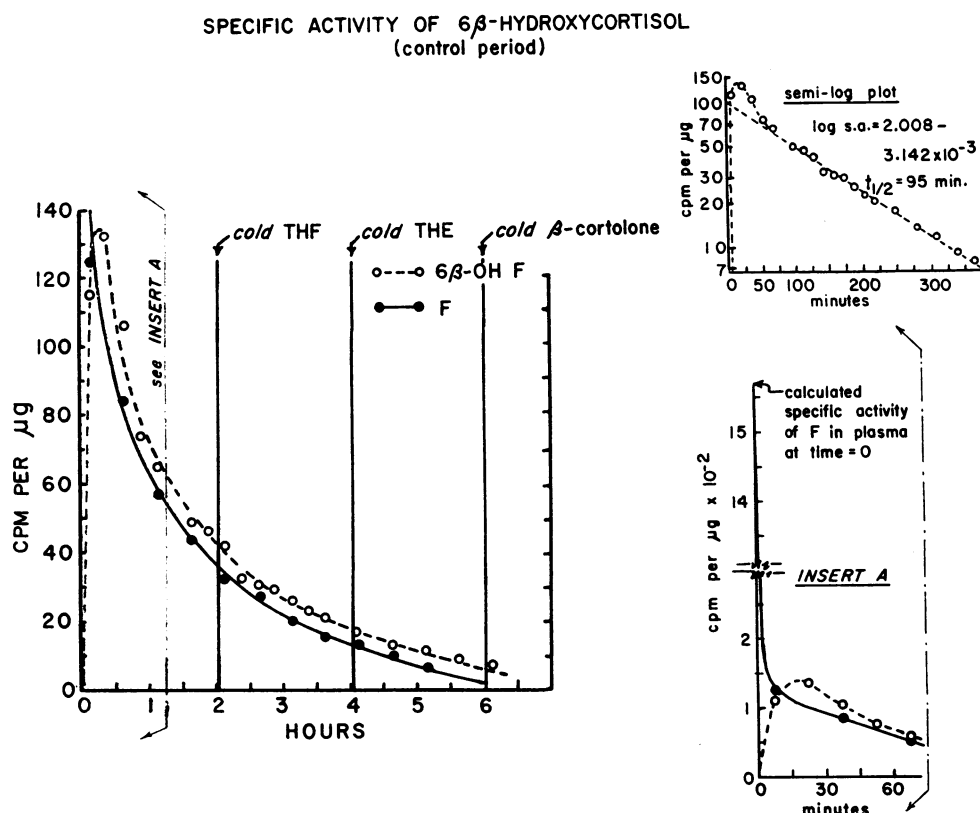

FIG. 8. PAPER CHROMATOGRAPHY OF A MIXTURE OF URINARY 6β-HYDROXYCORTISOL-4-C[14] AND AUTHENTIC UNLABELED 6β-HYDROXYCORTISOL. Chromatographed in 90 per cent butanol in benzene: 50 per cent aqueous methanol overnight at 32° C. Each centimeter strip was eluted for radioactivity and acid phenylhydrazine analyses.

this radioactive peak was mixed with unlabeled 6β-hydroxycortisol re-chromatographed, radioactivity and weight values coincided as shown in Figure 8. Sufficient 6β-hydroxycortisol was excreted to determine the specific activity-time curve for this metabolite, and this is shown in Figure 9 for the control period. The curves of Figure 9 are not incompatible with a product-precursor relationship,[8] and hence suggest that in man a major portion of 6β-hydroxycortisol is derived from cortisol rather than being synthesized directly in the adrenal gland. These suggestions are supported by the observations of Cohn, Upton, and Bondy

[8] In steady-state conditions the specific activity curve of the precursor will intersect that of the metabolite at the latter's maximum if theoretical product-precursor relationships hold (15). Because the slopes of the specific activity curves of Figure 9 change so rapidly, the product-precursor relationship should be considered approximate only.

FIG. 9. SPECIFIC ACTIVITY OF 6β-HYDROXYCORTISOL AS A FUNCTION OF TIME FOR THE CONTROL PERIOD. Insert A suggests a product-precursor relationship. Specific activity of F in plasma at time zero is total radioactivity administered/calculated plasma F content. Integral specific activity was determined from the area beneath the semi-log plot (see Figure 3), and from this the cortisol turnover was 177 mg per 24 hours. Turnover rate for 6β-hydroxycortisol $K = 0.693/t_{\frac{1}{2}} = 10.5$ per 24 hours. Turnover is 10.5 N mg per 24 hours, where N (not determined) is pool size of 6β-hydroxycortisol. Note maintenance of specific activities of cortisol and 6β-hydroxycortisol after administration of unlabeled metablites. 6β-hydrxycortisol determined by acid phenylhydrazine reaction.

(27) that after intravenous administration of cortisol-4-C¹⁴ to patients with cirrhosis of the liver, radioactive 6β-OH cortisol was clearly present in hepatic vein plasma, but barely detectable in peripheral venous blood.

DISCUSSION

Peterson (28) has reviewed certain aspects of the turnover of cortisol, calculated both by determination of the slope of the cortisol specific activity curve and by the principle of dilution of isotopically labeled cortisol by one of its urinary metabolites (23), in normal volunteers and in individuals in the hyperthyroid and myxedematous states. The application reported herein of similar and additional kinetic parameters to studies of the

metabolism of cortisol in a single patient with Cushing's syndrome has revealed several distinct features.[9] Inasmuch as the turnover of cortisol calculated from the slope of the log specific activity of cortisol as a function of time after the administration of a trace dose of cortisol-4-C¹⁴ has been frequently employed to characterize adrenal secretion rates of cortisol (28), it was considered important to demonstrate that this calculated rate is indeed equivalent to the actual turnover of cor-

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[9] Although the source of excess cortisol has been removed by subtotal adrenalectomy, the patient is spoken of as having Cushing's syndrome not only for easy reference but because she still exhibits certain characteristics of cortisol metabolism associated with Cushing's syndrome and adrenal hyperplasia (16).

tisol. Under the steady-state conditions of continuous cortisol infusion reported here, agreement between the turnover of cortisol computed from the specific activity of cortisol curves and the actual rates of infusion of cortisol (see Figure 2) was demonstrated.[10] Fortunately, it was possible to calculate the turnover of cortisol in the postoperative period and control periods by applying the equation $p = C/SA_m$ (see Figures 3, 9, and Results) and thus to demonstrate that these calculated values of 203 and 177 mg per 24 hours, respectively, accorded both with the values calculated from the slope of the cortisol specific activity curves and with the actual rates of infusion of cortisol.

Inasmuch as two normal young adults, also turning over cortisol at a rate of 200 mg per 24 hours, were previously (14) shown to have cortisol half-lives of 77 and 88 minutes, it was considered that the half-lives, as such, of 79 and 90 minutes in the control and post-operative periods, respectively, were in no way indicative of an alteration in the metabolism of cortisol in this patient with Cushing's syndrome.[9]

The calculated pool sizes in Table I show marked differences from those of two normal individuals studied previously (14) in that the pool sizes of THE and allo-THF are both low, the latter extremely so. These two diminished pool sizes result in high ratios of THF:THE and THF:allo-THF in the urine (see Table I, f). The heightened values of THF:THE are due presumably to factors other than the high turnover of 200 mg cortisol per 24 hours because the normal adults turning over cortisol at the same rate reached a THF:THE ratio of only 1:1 (14). This suggests a partial inhibition in the rate of transformation of cortisol to cortisone in the Cushing's patient.[9] The relatively small THE pool sizes, however, found in the face of rates of THE formation almost equivalent to those in the two normal subjects, with rates of 72 and 81 mg

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[10] Correlation of the infusion rate of cortisol-hemisuccinate with the turnover of cortisol calculated from the miscible cortisol pool demonstrates not only the rapid rate of hydrolysis of the hemisuccinate to "free" cortisol but also the lack of transformation of any significant amount of the steroid nucleus while the compound was still in the hemisuccinate form. Had the latter occurred to a measurable extent, the rate of dilution of the cortisol pool would have been diminished accordingly.

per 24 hours, suggest that a large portion of the THE formed may be further metabolized to compounds other than cortolone. To substantiate such conjecture, transformations of THE into such metabolites must be directly observed in future work. The decrease in the ability to form the 5α isomer, allo-THF, is clear. It results not only in a small allo-THF pool size, but also in a diminished rate of formation of this metabolite (see Table I, c). A diminished excretion of allo-THF appears to be a feature associated with Cushing's syndrome due to adrenal hyperplasia and has been documented by Guignard, Crigler, and Gold (16).

The application of the Zilversmit equations (15) to the calculation of the rates of formation of the metabolites THF, allo-THF, and THE represented as much as 80 per cent of the turnover of cortisol in two normal adults (14), whereas in the Cushing's patient (see Table I) only 50 per cent of the turnover of cortisol was calculated to proceed through these same 3 metabolites. The normal range for the per cent of the turnover of cortisol proceeding through pathways involving these 3 metabolites is not available, although it is interesting to note the variations in recovery of the metabolites of cortisol-4-C^{14} obtained in a recent thorough quantitative study carried out by Fukushima and co-workers (9) in 5 normal men in 6 experiments. The recovery of radioactivity in the total neutral steroid fraction from the first 24-hour urine samples after the administration of a tracer dose of cortisol-4-C^{14} was less than 65 per cent of the total radioactivity in the 24-hour urine in 3 of the 6 experiments and as high as 92 per cent in the 3 others. About 50 per cent of the radioactivity in the neutral fractions was located in THF, alo-THF, and THE, with the remainder of the radioactivity existing almost entirely as glycerol-type C_{21} metabolites. Romanoff and co-workers (25) have also found, after cortisol-4-C^{14} administration to 16 normal men, that even though 80 to 96 per cent of the administered radioactivity appeared in the urine, only 50 per cent of the total urine radioactivity was extractable with ethyl acetate after β-glucuronidase hydrolysis. It has not been shown, as yet, whether in such urines with considerable metabolite-conjugates non-hydrolyzable by β-glucuronidase the remaining urinary radioactivity resides in metabolite-conjugates

with essentially the same quantitative and qualitative content of metabolites as that in the β-glucuronidase-hydrolyzable fraction. Certainly the possibility that the β-glucuronidase-resistant fractions may contain a high percentage of compounds that are not metabolized through pathways involving THF, allo-THF, and THE must remain open for consideration until proven otherwise.

In the present study, calculations of the rates of formation of metabolites by means of the Zilversmit equations (15) were carried out only after it was ascertained that steady-state conditions prevailed and that theoretical product-precursor relationships [8, 11] existed for the specific activity curves. It was desirable to determine whether calculations thus made were consistent with the steady-state conditions imposed. Consequently, recovery of radioactivity and amounts in micrograms was performed on the 15-minute urine sample taken 5 hours after cortisol-4-C[14] administration in the control period (see Table II). Since the cortisol turnover of 200 mg per 24 hours was equivalent to 2 mg per 15 minutes, then the sum of all the cortisol metabolites present in the urine sample must be at least 2 mg.[7]

It is clear from Tables II and III that the large group of identifiable metabolites represented by fraction IIb was equivalent, at 850 μg, 19,400 cpm, to about half of the turnover of cortisol. It is this fraction that contains the metabolites of cortisol whose pathways of formation are through THF, allo-THF, and THE.[12] This observation is consistent with the Zilversmit calculations, in that the rates of formation of THF, allo-THF, and THF also only account for 50 per cent of the cortisol turnover. In addition, this observation indicates that the remaining 10,000 cpm [13] must be associated with the metabolites equivalent to the remaining 1 mg of cortisol turning over during the 15-minute interval. If there are a number of metabolites of cortisol formed by pathways not involving THF, allo-THF, or THE, then,

from the nature of the product-precursor curves, each of these metabolites will have specific activities greater than that of the cortisol in this sample (15), where it is (see Figure 2) 7.5 cpm per μg. Hence it is possible to have a maximum of 10,000/7.5, or 1.3 mg of metabolite present in the remaining 10,000 cpm.[14] The specific activity of 6β-hydroxycortisol in this sample at 300 minutes was 12 cpm per μg (see Figure 9). If the other remaining metabolites had mean specific activities in a similar range, then the turnover of cortisol would be accounted for. It remains, however, for further studies to demonstrate conclusively the rates of formation of the more polar metabolites of cortisol and to determine the role played by conjugates other than glucuronides.

SUMMARY

Several kinetic parameters of cortisol metabolism, such as half-life, turnover, metabolite pool sizes, and rates of formation of metabolites were obtained on two occasions in a 23-year-old woman who had recently undergone subtotal adrenalectomy for Cushing's syndrome associated with adrenal hyperplasia and who thereafter required steroid therapy for maintenance. The first study, postoperative, was performed 24 hours after a cholecystectomy and the second, control study, 4 months later. In each study, cortisol turnover was fixed by administration of a continuous infusion, as the hemisuccinate, at 200 mg per 24 hours so that the calculated turnovers could be compared with the known infusion rates. A small amount of cortisol-4-C[14] was rapidly administered intravenously during the steady-state condition, and from urine samples collected every 15 minutes for 8 hours, the specific activities of cortisol, 6β-hydroxycortisol, THF, allo-THF, THE, cortolone, and cortol were determined. At 2-hour intervals small known amounts of unlabeled metabolites were administered intravenously, and from the change in specific activity of the diluted metabolite, pool sizes of the respective metabolites were calculated by isotope dilution.

Turnover of cortisol. From the linear portion of the log specific activity of cortisol as a function of time and from the radioactivity administered,

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[11] Inferred for THF as previously described in Results.

[12] The remaining fractions of Table II, with the exception of the unknown residual fraction III, were shown to contain negligible quantities of THF, allo-THF, and THE.

[13] In Table II, total radioactivity in cpm $-$ II b cpm $=$ 10,000 cpm.

[14] If this calculated maximum were less than 1 mg, it would indicate that the residual radioactivity could not contain the remaining 1 mg of metabolite.

the following cortisol parameters were calculated. For the post-operative period: half-life, 90 minutes; pool size, 19.1 mg; and turnover, 232 mg per 24 hours. For the control period: half-life, 79 min; pool size, 14.8 mg; and turnover 187 mg per 24 hours. Cortisol turnover calculated by the integral specific activity method for the two metabolites allo-THF and 6β-hydroxycortisol—by the equation $p = C/SA_m$, where p is the rate of cortisol turnover, C the counts per minute of cortisol administered at time zero, and SA_m the specific activity of a metabolite derived exclusively from cortisol—agreed with the fixed infusion rates in both studies. The amounts of THF and THE excreted were directly proportional to their pool sizes calculated by isotope dilution. From this relationship, allo-THF pool sizes were calculated and were found to be very small.

Rates of formation of metabolites. The rates of formation of THF, allo-THF, and THE in mg per 24 hours were computed from the kinetic data and were found to approximate only 50 per cent of the turnover of cortisol. Recovery of the group of cortisol metabolites cortol, cortolone, THF, allo-THF, and THE also approximated only 50 per cent of the cortisol turnover and agreed with the kinetic calculations. These data suggest the possibility of a large portion of cortisol metabolized by pathways other than THF, allo-THF, and THE.

6β-hydroxycortisol was shown to be a metabolite of cortisol by the demonstration of a product-precursor relationship between 6β-hydroxycortisol and cortisol in a plot of the specific activities of the two steroids as functions of time.

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