METABOLISM OF CORTISOL IN THE HUMAN NEWBORN

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Abstract

^{14}C-cortisol was administered to two newborn infants and one adult, and the pattern of urinary metabolites analyzed and compared. Seventy to 86% of the radioactivity extracted in the unconjugated fraction and 86 to 100% of that extracted in the glucuronide fraction were accounted for in terms of known steroids. In the case of the infants, 6β-hydroxycortisol represented 64% of the radioactivity extracted in the unconjugated fraction, while tetrahydrocortisone (67 and 73%), cortolone (10 and 13%) and 6β-hydroxycortisol (16 and 13%) were the sole significant components of the glucuronide fraction. A small amount of radioactivity was extracted in the steroid sulfate fraction, but there was no detectable incorporation of ^{14}C into cortisol sulfate. Substantial additional amounts of radioactivity became extractable by either butanol extraction (18%) or amberlite XAD-2 chromatography (28%) of the urine. The nature of these metabolites remains to be defined. In the adult control the distribution of the radioactivity in the unconjugated, glucuronide and sulfate fractions represented 8.7, 84.4 and 2.1% of the total radioactivity excreted. Following the

simultaneous administration of ^{14}C-cortisol and ^{3}H-6β-hydroxy-cortisol to a newborn infant, the total production (173 μg/day), peripheral production (131 μg/day) of 6β-hydroxycortisol from ^{14}C-cortisol and the secretion rate of 6β-hydroxycortisol (42 μg/day) were established. The data do not support the view that the excretion of 6β-hydroxycortisol, or the formation of cortisol sulfate, are of sufficient quantitative significance to qualify as compensatory mechanisms for the decrease in steroid glucuronide excretion during early infancy. They suggest the existence of an alternate pathway of cortisol metabolism involving the excretion of highly water soluble metabolites - the nature of which remains to be defined.

Abstrait

Après l'administration intraveineuse de ^{14}C-cortisol à 2 enfants nouveau-nés et à un homme adult, les métabolites urinaires de ce stéroid ont été étudiés et comparés. De 70 à 86% de la radioactivité extraite dans la fraction nonconjuguée et de 86 à 100% de la radioactivité extraite dans la fraction glucuronide, était associée à des métabolites qui furent characterisés. Chez les nouveau-nés, le 6β-hydroxycortisol représentait 64% de la radioactivité présente dans la fraction nonconjuguée. Tetrahydrocortisone (67 et 73%), cortolone (10 et 13%), et 6β-hydroxycortisol (16 et 13%) étaient les métabolites principaux de la fraction glucuronide. La fraction sulfate contenait une quantité mesurable de radioactivité mais le cortisol sulfate urinaire ne contenait pas de quantité détectable de ^{14}C. Une quantité additionnelle de radioactivité fut récupérée après extraction de l'urine avec du butanol (18%) ou après chromatographie de l'urine sur colonne d'amberlite XAD-2 (28%).

La nature de ces métabolites n'a pas été définie. Chez l'adult, la distribution de la radioactivité dans la fraction nonconjguée, glucuronide et sulfate représentait 8.7, 84.4 et 2.1% de la totalité de la radioactivité excrétée. Dans un seconde étude, après l'administration simultanée de ^{14}C-cortisol et de ^{3}H-6 β-hydroxycortisol à un enfant nouveau né, le taux de production (173 µg/jour) la production périphérique de 6 β-hydroxycortisol à partir de cortisol (131µ g/jour) et le taux de sécrétion du 6 β-hydroxycortisol (42µ g/jour) furent établis. Les résultats de ces études ne supportent pas le point de vue selon lequel l'excrétion de 6β -hydroxycortisol ou la formation de cortisol sulfate représentent des méchanismes métaboliques, qui compenseraient une formation insuffisante de stéroids glucuronides après la naissance. Par ailleurs, ils suggérent l'existence de métabolites de cortisol hautement polaires et probablement nonconjugués. Toutefois leur nature précise reste à définir.

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INDEX

LIST OF TABLES

List of Abbreviations Used

gm	gram
Rf	mobility relative to the solvent front
mg	milligram
NADPH	reduced nicotinamide adenine dinucleotide phosphate
mμ	millimicron
mm	millimeter
cm	centimeter
ml	milliliter
Δ 4	double bond between C-4 and C-5
C	centigrade
μg	microgram
M	molar
U	Units
dpm	disintegration per minute
cpm	count per minute
N	normal
Rs	mobility relative to the reference substance
wt	weight
kg	kilogram
Cpd	compound
S.A	specific activity

corticosterone sulfate: 11β-hydroxy-3, 20-diketopregn-4-ene-21-yl sulfate
cortisol (F): 11β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione
cortisol sulfate: 11β, 17α-dihydroxy-3, 20-diketopregn-4-ene-21-yl sulfate
cortisone (E): 17α, 21-dihydroxypregn-4-ene-3, 11, 20-trione
cortisone sulfate: 17α-hydroxy-3, 11, 20-triketopregn-4-ene-21-yl sulfate
cortol: 5β-pregnane-3α, 11β, 17α, 20α, 21-pentol
αcortolone: 3α, 17α, 20α, 21-tetrahydroxy-5β-pregnan-11-one
βcortolone: 3α, 17α, 20β, 21-tetrahydroxy-5β-pregnan-11-one
2α-hydroxycortisol (2αOHF): 2α, 11β, 17α, 21-tetrahydroxypregn-4-ene-3, 20-dione
6β-hydroxycortisol (6β-OHF): 6β, 11β, 17α, 21-tetrahydroxypregn-4-ene, 3, 20-dione

6β-hydroxycortisone (6β-OHE): 6β,17α,21-trihydroxypregn-4-ene, 3,11,20-trione

6β-hydroxy,11-deoxycortisol: 6β,17α,21-trihydroxypregn-4-ene, 3,20-dione

tetrahydrocortisone glucuronide: 17α,21-dihydroxy-11,20-diketo-5β-pregnane-3α-yl-β-D-glucopyronosiduronic acid

tetrahydrocortisol (THF): 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one

tetrahydrocortisone (THE): 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione

6β-hydroxy,tetrahydrocortisol (6β-OHTHF): pregnane-3α,6β,11β,17α,21-pentol-20-one

REVIEW OF LITERATURE

In spite of numerous, valuable investigations (1-7), following administration of either trace amounts or pharmacological doses of cortisol, the metabolism of this steroid by the newborn remains poorly documented. This is in contrast with investigations in the adult where it has been possible to establish a well defined metabolism of cortisol (8,9). The one point which has been made clear, is that a striking difference exists between the metabolism of cortisol in newborns and adults.

The metabolism of endogenous cortisol in normal adults is quite complete in the sense that little unaltered cortisol is excreted in the urine (10,11). Following administration of ^{14}C-cortisol to adult man about 90% of the radioactivity is accounted for in terms of known metabolites and only small amounts remain unidentified (8). The major part of cortisol metabolites (about 80%), are excreted as glucuronide esters, whereas the unconjugated metabolites represent a minor fraction (about 5%). Enzymatic reduction of the α, β unsaturated 3-ketone and the subsequent conjugation of the 3 α-hydroxyl group with glucuronic acid represents the main pathway of cortisol metabolism in the adult. The reaction involves first, the saturation of the Δ 4,5 double bond by Δ4-5 reductase which results in the

formation of a dihydro derivative of cortisol; then the conversion of the C-3 carbonyl group by 3α-dehydrogenase to the corresponding alcohol. The hydrogen donor of the above reactions is $NADPH_2$. Tomkins found that in the rat this two-step reduction of cortisol to a tetrahydroderivative is catalyzed by enzymes present in the soluble fraction of liver homogenate (12).

Brown-Grant (13) working in vitro with liver of rats, mice, hamsters, rabbits, and guinea pigs, demonstrated the presence of Δ 4-5 reductase activity in both the microsomal and the 105,000 g supernatant fractions of liver homogenates. It was observed that 11-keto steroids were, in general, better substrates than 11 β-hydroxy steroids with respect to Δ4-5 reductases.

After the reduction of ring A, the cortisol molecule undergoes conjugation with glucuronic acid at the level of the C-3 hydroxyl group. The active form of glucuronic acid which is involved in glucuronide ester synthesis is uridine diphosphate glucuronic acid (UDPGA). The enzyme system which catalyses the transfer of glucuronic acid from UDPGA to the alcohol acceptor is glucuronyl transferase which was located in the microsomal fraction of liver homogenates (14).

Corticosteroids possessing an α, β unsaturated 3-ketone structure may be conjugated with glucuronic acid at the level of the C-21 hydroxyl group. Indeed, Pasqualini (15) and Mattox (16) reported that cortisol and cortisone are excreted to a small extent as conjugates of glucuronic acid.

The two main tetrahydroderivatives formed following ring A

reduction of cortisol are tetrahydrocortisol, and tetrahydrocortisone. Allo-tetrahydrocortisol, a stereoisomer at C-5 is also a metabolite of appreciable significance. Little interconversion takes place between tetrahydrocortisone and tetrahydrocortisol, probably because of the rapid conjugation of these steroids with glucuronic acid, which may represent a limiting step with respect to that reaction (17,18).

It seems that the conversion of the 11-hydroxyl to the 11-ketone, takes place in the instance of cortisone and cortisol, that is before ring A reduction. 11β -hydroxydehydrogenase which is present in the liver, is the enzyme which catalyses this reaction, NADP being the required co-factor (19). This enzyme was also studied in placenta homogenates where it has been located in the microsomal fraction (20).

Mahesh and Ulrick studied the oxidation of cortisol to cortisone in rat kidney homogenates. When the whole homogenate was fractionated by ultracentrifugation, the enzymatic activity was found mainly in the nuclear particles and in the microsomal fraction (21).

The in vitro reduction of the 11-ketone group of cortisone to cortisol was demonstrated in porcine, bovine, and rat liver homogenates (22).

Further reduction of the glycerol side chain occurs, resulting in the formation of cortols and cortolones; this results from the reduction of the C-20 ketone by 20 α - and 20 β -dehydrogenases, which represents an important step in cortisol metabolism (23).

The supernatant (17,500 g) of rat liver homogenate contains both the 20 α - and 20 β -enzymes. Tetrahydrocortisol and cortisol are reduced primarily to 20 β -hydroxy derivatives, while tetrahydrocortisone and 11-deoxycortisol are reduced to 20 α -hydroxy derivatives (23). A sex difference in the reduction of the C-20 ketone of cortisone was reported by Troop (24, 25). Homogenates prepared from male rat livers were from three to six times more active than similar preparations from females. The activity of the enzyme in male rats was dependent upon the presence of androgens and showed a tendency to be depressed by estrogens.

According to Fukushima (8) the cortols and cortolones account for 18 and 33% respectively of the metabolites of cortisol.

Following reduction of ring A, the cleavage of the glycerol side chain by a 17 α, 20-C_{21}-desmolase with consequent formation of the corresponding 17-ketosteroids represents another pathway of cortisol metabolism in adult man. The predominant 17-ketosteroids are of the 5β form such as 11-keto and 11 β - hydroxyetiocholanolone. The formation of the 5_{α} stereoisomers, (11 β -hydroxyandrosterone and 11-ketoandrosterone) is small as indicated in earlier studies of Burstein (26, 27).

After ^{14}C-cortisol administration to normal adults, Fukushima isolated 5% of the radioactivity as 11 β -hydroxy-etiocholanolone and 13% as 11-ketoetiocholanolone, the corresponding 5 α epimers representing very low values (8).

It is pertinent to quote here the values obtained by this author (8): quantitatively the most important metabolite is tetrahydrocortisone - 23% of the injected dose, followed by tetrahydrocortisol - 17%, and allo-tetrahydrocortisol - 8.5%. Among the C-20 reduced metabolites, α -cortolone represents 11.3%, β -cortolone 10.3%, β -cortol 9.8% and α -cortol 2%. Of the 17-keto steroids, 11-ketoetiocholanolone represents 12.8%, 11-hydroxy etiocholanolone 5% and 11-hydroxy androsterone 0.8%.

When ^{14}C-cortisol is administered to human newborns one observes quite a different pattern of cortisol metabolites.

In normal full-term and premature infants, the total recovery of urinary radioactivity is low. The mean recovery is 76% and the lowest values are observed in premature infants (3). The unconjugated fraction represents quite an important fraction of the total radioactivity recovered. When the unconjugated fraction is sequentially extracted with solvents of different polarity (such as methylene chloride followed by ethyl acetate), the mean recovery values for premature and full-term infants are 24 and 20% respectively, compared with 11% in adults.

Whereas approximately equal amounts of radioactivity are extracted by methylene chloride and by ethylacetate in the instance of adults, a much higher proportion is extracted by ethyl acetate in the instance of new-borns. This is in keeping with the presence of polar metabolites in the unconjugated fraction of new-born infant urine (4).

However, the most striking difference in cortisol metabolism between the newborn and the adult, is the finding of relatively small amounts of metabolites conjugated with glucuronic acid following the administration of either trace amounts or pharmacological doses of cortisol during the neonatal period (1, 3, 6, 28).

There exist differences of opinions with regard to the mechanism underlying the decreased formation of glucuronide metabolites. Thus following administration of tetrahydrocortisone, Bongiovanni (29) noted that the disappearance rate of this steroid (34 - 48 minutes) in two infants, one-day-old, was comparable to the disappearance rate observed in adults. When 25 mg of cortisol hemisuccinate was administered to new-born infants of 1 day of life, the same author found that the mean half-life of plasma 17-hydroxycorticosteroids was 997 minutes while in normal adults the corresponding figure was 112 minutes.

Based on the observation of this rapid disappearance rate of tetrahydrocortisone and of the prolonged half-life of "cortisol"

Bongiovanni postulated the existence of a relative deficiency of the enzyme(s) involved in the reduction of the α,β unsaturated ketone of cortisol during early life.

The validity of Bongiovanni's conclusions may be open to question, however, since the administration of such a large dose of cortisol may result in a prolonged half-life of the steroid.

This seems to be supported by the finding of Grumbach (30) who observed a mean half-life of 220 minutes after infusion of 7 - 10 mg/kg of cortisol hemisuccinate in 7 full-term infants less than 24 hours of age. After infusion of 4 mg of cortisol alcohol per kg, in 5 infants less than 1 day old, the mean half-life was 126 minutes.

When 5 mg/kg of cortisol was administered to newborns 53 - 84 hours of age, Reynolds (31) found a half-life of 244 minutes for plasma 17-hydroxycorticosteroids, and of 204 minutes for cortisol.

On the other hand, Cranny (32) infusing 1 mg/kg of cortisol to newborn infants, found a mean half-life comparable to that obtained in adults (94 minutes). When 2 mg of cortisol per kg were infused in newborns, half-life values longer than those resulting from the administration of 1 mg/kg were obtained (mean: 168 minutes).

When a tracer dose of ^{14}C-cortisol was administered to a neonate, Seely (33) found that the half-life of the total radioactivity was 245 minutes, whereas the half-life of cortisol was 44 minutes.

Migeon administered tetrahydrocortisone - 2 mg per kg of body weight - to newborns and measured the 17-hydroxycorticosteroids excreted in the following 24 hour urine. He found that in the newborn only 10 to 20% of the administered tetrahydrocortisone was recovered in the urine as glucuronide compared to a recovery of 60% in the adult subjects. The very small yield of glucuronide conjugates in newborn urine, when compared to those in the adult, suggested a glucuronyl transferase deficiency during the neonatal period (1).

The increased values of serum bilirubin observed in the first days of life(34, 35), may support the concept of a decreased capacity of the newborn to form glucuronide conjugates.

With respect to this, when the microsomal fraction of adult guinea pig liver was used as a source of glucuronyl transferase, bilirubin, in the presence of UDPGA, was conjugated as bilirubin-glucuronide. No bilirubin-glucuronide formation could be demonstrated when the reaction mixture was incubated with microsomes from fetal or newborn guinea pig liver (36). If a similar observation were made in humans, the concept that the

newborn infant has an inefficient mechanism for conjugation with glucuronic acid would be greatly strengthened.

On the other hand, an increased excretion of steroid glucuronides was observed in newborn infants under stress or following ACTH stimulation (37). This fact, which does not seem to support the idea of a deficiency of glucuronyl transferase in the neonate, would suggest that glucuroconjugation may not be maximal in the resting state but can be increased under conditions of stress.

As a partial compensation for these restrictions, the newborn infant seems to make use of alternate pathways of cortisol metabolism. In neonatal life it has been found that appreciable quantities of cortisol are converted to 6β-hydroxycortisol (4). Increased amounts of 6β-hydroxycortisol have also been demonstrated during normal pregnancy and especially in toxemia of pregnancy (38), in exogenous hyperestrogenism (39), and in adrenocortical hyperfunction (40).

Hydroxylation of steroids on position C-6 in vivo was first indicated in human urine by Lieberman, Fukushima, and Dobriner in 1950 (41).

Axelrod and Miller demonstrated the presence of a 6β-hydroxylase in rat liver slices (42). Hayano found 6β-hydroxylase in the corpus luteum of cow ovaries (43) and Haines reported that

hog adrenal tissue contains the enzyme (44).

Burstein, Dorfman and Nadel in 1954 were the first to identify 6β-hydroxycortisol in guinea pig urine and then in normal human urine (45).

6β-hydroxycortisol appears to occupy a unique status among urinary steroids since it is quantitatively the most important unconjugated steroid in human urine (46).

A significant part of this polar metabolite of cortisol was found conjugated with glucuronic or sulfuric acid in the urine of human premature neonates (37). In the adult, on the other hand, Frantz reported that 6β-hydroxycortisol was recovered almost exclusively in the unconjugated form, but did not exclude the possibility that it may be conjugated to some extent (46).

6β-hydroxylation of cortisol seems to take place largely at the periphery (4), although it may occur to some extent within the adrenals (47).

The large amounts of this steroid present in the urine of infants receiving large doses of cortisol and cortisone make it seem likely that the liver or some organ other than the adrenals is involved in 6β-hydroxylation (4).

However, Touchstone did isolate 6β-hydroxycortisol from adult human adrenal tissue, and demonstrated <u>in vitro</u> the synthesis of small amounts of 6β-hydroxycortisol by this tissue (47).

Lipman et al confirmed Touchstone's observation that the human adrenal possesses a 6 β -hydroxylating system, but found the human liver to be about five times as active as the adrenal in this respect (48).

Burstein et al (49) arrived at the conclusion that an adrenal secretion of 6 β -hydroxycortisol occurs in addition to its peripheral production. Their conclusions were based on the fact that following simultaneous administration of cortisol and 6 β -hydroxycortisol each bearing a different radioactive label, they found in all subjects investigated that the cumulative specific activity of urinary 6 β -hydroxycortisol was considerably lower than that of tetrahydrocortisone. By calculation, they established that of the total production of 6 β -hydroxycortisol, about 50% was secreted by the adrenal and the remaining, formed during the peripheral metabolism of cortisol.

Fukushima et al (50) reinvestigated the problem of the adrenal secretion of 6 β -hydroxycortisol. They found in three subjects that the secretion rate of 6 β -hydroxycortisol was almost negligible. However, in view of the reported adrenal biosynthesis of 6 β -hydroxycortisol in vitro, they did not rule out the possibility of adrenocortical secretion of this steroid.

The presence of increased amounts of polar steroids in neonatal urine may reflect the effect of high levels of maternal estrogens on cortisol metabolism in the newborn. Indeed, it is well documented that estrogens may profoundly affect cortisol metabolism. The

mechanism by which estrogens exert these effects on cortisol metabolism is not clear, but it is known that natural as well as synthetic estrogens influence the binding of cortisol by increasing the plasma level of corticosteroid binding globulin (51). As a consequence, the half-life of cortisol is prolonged, and its hepatic clearance probably decreased.

The finding that estrone depresses the conversion of cortisol to tetrahydrocortisone and tetrahydrocortisol by liver slices, while stimulating 6 β -hydroxylation, supports the concept that 6 β -hydroxycortisol formation represents a compensatory route of cortisol metabolism (48).

Estradiol-17β administered to adult subjects results in a decreased excretion of steroid glucuronides (52).

The inhibition of glucose-6-phosphate dehydrogenase in liver cells could be the mechanism involved in this process (53), with a consequent decreased availability of reduced triphosphopyridine nucleotide, which is the necessary factor for Δ 4-5 steroid reductase (12).

According to this view, estrogens would not interfere with the conjugation of the reduced metabolites of cortisol with glucuronic acid, but rather would impair the reduction of steroids bearing a Δ 4-5 ketone to their corresponding 3 α -hydroxy, 5 α or β metabolites.

Lipman et al (48) studied the in vitro conversion of radioactive cortisol to 6β-hydroxycortisol by tissues such as the liver, adrenal, placenta, and skeletal muscle. He found that all of the tissues studied

were capable of 6 β -hydroxylation of cortisol; among these, the liver and adrenal possessed far greater activity than the kidney, skeletal muscle or placenta. The liver appeared to be about four to five times as active as the adrenal. These authors tested the effect of estrone on the ability of various tissues to form 6β - hydroxycortisol from cortisol. The only tissue which showed some response to the presence of estrone was the liver, in which 6 β - hydroxylation was increased by 50 to 100 % over control values.

6 α -hydroxycortisol has also been identified in human urine (46). It was found upon purification of 6 β -hydroxycortisol that this steroid was contaminated with small amounts of 6α -hydroxycortisol, representing not more than 5-10 percent of the total 6 β -hydroxycortisol.

The finding of a low concentration of 6 β -hydroxycortisone in relation to 6 β -hydroxycortisol is interesting in view of the rough parity that exists between many of the 11-keto and 11-hydroxy metabolites of cortisol. These low values of 6 β -hydroxycortisone suggest among other possible explanations that 6β -hydroxylation may inhibit oxidation of the 11-hydroxyl group (46).

Ulstrom et al (4) found a more polar compound than 6 β -hydroxy- cortisol in both the unconjugated and the β -glucuronidase hydrolyzed fractions of newborn infants' urine. This compound which failed to show soda fluorescence but stained with blue tetrazolium and gave a positive Porter Silber (54) reaction was postulated to be tetrahydro 6 β -hydroxy- cortisol. This was confirmed by infrared spectroscopy.

Whether 6 β -hydroxycortisol represents an alteration of the peripheral metabolism of cortisol due to the impairment of ring A reduction as suggested by Katz (39) and Werk (55), or whether other factors come into play in its formation, such as enzyme induction (56) or transitional conditions away from the maternal environment, this steroid is now considered as reflecting a significant pathway for the disposal of cortisol (4, 46).

Another suggested pathway of corticosteroid metabolism during the perinatal period, is that involving sulfoconjugation.

Pasqualini and Jayle (57) after administration of large doses of cortisol and corticosterone to normal adults were the first to characterize their respective sulfates in the urine. Under their experimental conditions about one-third of the cortisol excreted and the major part of the corticosterone was present in the urine as sulfates.

Cortisol and cortisone sulfates were also found in premature and full-term newborns' urine by Drayer and Giroud (58), and measured in significant amounts compared to tetrahydrocortisone glucuronides in newborn infants' urine (43 - 114%) (59).

A marked increase in urinary excretion of cortisol and cortisone sulfate was observed in the last trimester of pregnancy. These values fall to normal levels after parturition, suggesting that these conjugates originate from the fetus (60).

Previously Diczfalusy (61) had demonstrated the capacity of fetal tissue especially the fetal lung, to conjugate estrogens as sulfate esters and Wengle had reported that the supernatant fraction of human fetal tissue homogenates has the capacity to sulfurylate some C-21 steroids, the adrenal homogenates were the most active in this respect (62).

Incubation of human newborn adrenal slices with cortisol, corticosterone, 11-deoxycortisol and 11-deoxycorticosterone lead to the formation of the corresponding sulfates (63).

The findings of cortisol sulfate in human cord plasma (64) together with results of adrenal incubation studies, support the hypothesis that corticosteroid sulfates are secreted by the fetal adrenal, but they do not exclude the possibility that liver steroid sulfokinases are in part responsible for the formation of these conjugates. Consequently it was proposed that the formation of sulfate esters could represent an alternate pathway for the disposal of cortisol in the newborn, thereby compensating for the deficiency of glucuronide conjugation observed postnataly.

Further investigations, however, following the simultaneous administration of cortisol and its sulfate, each bearing a different radioactive label, strongly indicated that cortisol sulfate was mainly a product of adrenocortical secretion (28, 65).

In view of the abrupt change of environment which the newborn infant experiences, it is not surprising that, among other metabolic events the mechanism for disposal of cortisol differs from that of the adult. These alterations in cortisol metabolism may reflect a transitional period of adaptation by the newborn to autonomous life. To what extent or for what length of time these particular pathways of metabolism are operating has not yet been fully defined.

GENERAL METHODS

In this chapter the description of the general methods used in this study will be presented.

1. Reagents

All reagents used for extraction and chromatography were purified and redistilled according to the procedure recommended by Bush (66).

Ethanol was used freshly redistilled.

Phenyl hydrazine HCl was recrystallized three times from ethanol.

Reagents used for acetylation such as pyridine and acetic anhydride were distilled according to Kliman and Peterson (67) and stored in a vacuum desiccator over a layer of calcium chloride.

Scintillation mixture was prepared by dissolving 4 gms of 2,5-diphenyloxazole and 0.1 gm of 1,4-bis-2-(5-phenyloxazolyl) benzene (Packard Instrument Co., La Grange, Illinois, USA) in 1 liter of toluene. Toluene spectroanalyzed was used non redistilled.

2. Reference Steroids

Non radioactive steroids were obtained from Steraloids Inc.

Tritiated and carbon 14 labelled steroids were purchased from New England Nuclear Corp. Their purity was verified by paper chromatography in appropriate solvent systems (63). The chromatograms were scanned to ensure the presence of a single symmetrical peak of radioactivity of the expected Rf value.

Preparation of Reference 1,2 ^{3}H-Steroid Sulfates

The sulfates of 1,2 ^{3}H-cortisol and 1,2 ^{3}H-cortisone were prepared by Dr. Hall by an adaptation of the method described by Duza (68). The labelled steroid was dissolved in the minimum amount of pyridine and 10 mg of triethylamine sulphur trioxide was added. The tube was shaken for 2 hours, following which the sulfates were redissolved in water and extracted with n-butanol.

The radiochemical homogenity of these steroid sulfates was assessed by thin layer and paper chromatography and, following enzyme hydrolysis of an aliquot (63), by the recovery of one single radioactive product possessing the same chromatographic mobility as that of the corresponding ^{3}H-steroid alcohol.

^{3}H-6β-hydroxycortisol was prepared by Dr. C. Branchaud by exposure of ^{3}H-6β-hydroxy, 11-deoxycortisol, kindly provided by Dr. Seghel of Ayerst Laboratories, Montreal, Canada, to a beef adrenal mitochrondrial fraction in the presence of an NADPH-generating system (69). Radiochemical homogenity of the compound was achieved after extensive paper and thin layer chromatography.

3. Chromatography

a) Paper Chromatography

In table I, the paper chromatographic systems used in this study have been assigned a code number by which they will be

Table I

Paper Chromatography Systems

System No.	Solvent composition		Reference No.
I	Ethyl acetate, chloroform, methanol, water	(25:75:50:50)	46
II	Isooctane, tertiary butanol, water	(500:250:450)	71
III	Benzene, n-butanol, methanol, water	(1000:100:300:300)	4
IV	Benzene, ethyl acetate, methanol, water	(100:20:50:50)	70
V	Light petroleum, benzene, methanol, water	(667:333:800:200)	72
VI	Benzene, methanol, water	(1000:440:360)	72
VII	Isooctane, toluene, methanol, water	(250:250:350:150)	71
VIII	Iso-amyl alcohol, hexane, conc.NH_4OH, water	(40:10:28:22)	73
IX *	Benzene, ethylacetate, methanol, water	(30:20:10:20)	

* This system represents a slight modification of the original Burstein system in which the phases are in the proportion of 30:10:10:20. (49)

referred to in the text.

Steroids extracts were applied and eluted from paper with ethanol. In the instance of very polar steroids the elution was done with ethanol water 9:1.

Whatman No. 2 chromatography paper (W. and R. Balston Ltd., London, England) was used unwashed after scanning under ultraviolet light (250 mμ) in order to eliminate those sheets showing fluorescent or ultraviolet absorbing impurities.

All paper chromatography systems were developed by descending chromatography at room temperature.

Radioactive steroid fractions were detected by scanning the paper chromatograms in a Packard Radiochromatogram Scanner (Model 7200).

b) Column Chromatography

Column chromatography was performed according to the method of Bradlow (74) on Amberlite XAD-2 obtained from the Rohm and Haas Co., Toronto, Ontario in a form of hydrated beads with an effective size of 0.3 - 0.45 mm. Column specifications were: internal diameter 2.5 cm, height 35 cm. The column was filled with a flurry of amberlite (100 gm) in H_2O, and eluted with 500 ml methanol.

Steroids bearing a dihydroxy acetone side chain were detected by the blue tetrazolium reaction (75).

Δ^4-3-ketosteroids were located on chromatograms by their absorption of ultraviolet light (250 mμ).

Cortol and cortolone were located on paper by spraying with phosphomolibdic acid (10% in absolute ethanol)(76). The strip was subsequently heated for several minutes at 80 - 90°C. The steroids appeared as dark blue spots against a yellow background.

4) Hydrolysis of Steroid Conjugates

a) Glucuronides

The urine brought to pH 5 with glacial acetic acid was buffered with 1/10 volume of 0.5 M acetate buffer pH 5.0 and hydrolyzed with β-glucuronidase (Ketodase, Warner-Chilcott) at a concentration of 500 Fishman U /ml urine. The hydrolysis was allowed to take place for 5 days at 37°C.

b) Sulfates

The urine brought to pH 5.2 with glacial acetic acid was buffered with 1/10 volume of 0.5 M acetate buffer pH 5.2 and hydrolyzed with Glusulase (Endo Labs, Garden City, N. Y.) which possesses β-glucuronidase and sulfatase activity in the

ratio of 3.8. A concentration of 1000 U sulfatase/ml urine was used. The hydrolysis was allowed to take place for 5 days at 37°C. Correction for experimental losses was made by means of radiochemically pure ^{3}H reference steroids added to the urine in amounts varying between 8.8×10^{4} dpm and 1.8×10^{5} dpm. The steroids used were ^{3}H-6β-hydroxycortisol, ^{3}H-tetrahydrocortisol, ^{3}H-tetrahydrocortisone, ^{3}H-cortisol, ^{3}H-cortisone, ^{3}H-cortisol sulfate and ^{3}H-cortisone sulfate.

When the appropriate ^{3}H reference steroid was not available, correction for experimental losses was made by establishing the loss of a ^{3}H standard of related polarity, for instance that of ^{3}H-tetrahydrocortisol in the instance of cortolone.

5) Calculation of Steroid Production Rate

The production rates of cortisol and 6β-hydroxycortisol were calculated according to this equation (70)

$$\text{Production rates per day} = \frac{S.W.XR}{C \times d} - W$$

in which:

S = specific activity of steroid administered in dpm/μg

W = μg amount of steroid administered

R = $\frac{\text{M.Wt. of steroid administered}}{\text{M·Wt. of urinary metabolite}}$

C = specific activity of urinary metabolite in dpm/μg

d = number of days of urine collection

When calculating the production rate of cortisol, C stands for the specific activity of tetrahydrocortisone. When calculating the production of 6β-hydroxycortisol, C stands for the specific activity of urinary nonconjugated 6β-hydroxycortisol.

The conversion factor of cortisol to 6β-hydroxycortisol was obtained by dividing the $^{14}C/^{3}H$ ratio obtained for 6β-hydroxycortisol once constant specific activity had been achieved by the $^{14}C/^{3}H$ ratio of the injected solution (49,77):

$$pF \rightarrow 6\beta\text{-OHF} = \frac{^{14}C/^{3}H \text{ in urinary } 6\beta\text{-OHF}}{^{14}C/^{3}H \text{ in injection solution}}$$

The peripheral production of 6β-hydroxycortisol was established by multiplying the cortisol secretion rate value by the conversion factor of cortisol to 6β-hydroxycortisol (49,77).

The secretory rate of 6β-hydroxycortisol is the total production of 6β-hydroxycortisol minus the peripheral production (77).

6) Counting and Calculations

Individual or simultaneous measurement of ^{3}H and/or ^{14}C counts were performed in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 4322 set at a single voltage of 1700 volts. Counts were obtained according to the discriminator method of Okita et al (78), as modified by Stachenko et al (79). Under these

conditions the ^{3}H c.p.m. appearing in the ^{14}C channel was 0.6%, that of ^{14}C in the ^{3}H channel 13%. The counting efficiencies for ^{3}H and ^{14}C were 18% and 52.5% respectively.

Steroid aliquots were transferred in glass vials, dried under infrared lamps, and counted in 15 ml of scintillation solution.

Steroid sulfates were dissolved in 0.2 ml of methanol prior to addition of the scintillation solution.

Each sample was counted for 10 minutes.

If a = contribution of ^{14}C count to the first channel:

$$a = \frac{\text{first channel cpm - background}}{\text{second channel cpm - background}}$$

b = contribution of ^{3}H count to the second channel

$$b = \frac{\text{second channel cpm - background}}{\text{first channel cpm - background}}$$

N_1 = cpm first channel - background

N_2 = cpm second channel - background

The ^{3}H and ^{14}C counts in the sample are calculated according to the formula:

$$^{3}H = \frac{N_1 - aN_2}{1 - ab}$$

$$^{14}C = \frac{N_2 - bN_1}{1 - ab}$$

$$^{14}C/^{3}H = \frac{N_2 - bN_1}{N_1 - aN_2}$$

Correction for quenching was made by recounting samples following addition of known amounts of ^{3}H and ^{14}C-toluene (69).

CHAPTER I

CHAPTER I

Metabolism of Cortisol in the Human Newborn

Experimental

Biological Material

Urine was collected from 2 newborn male infants aged 5 days (subject R) and 8 days (subject B) respectively, in a plastic, adhesive collection bag, and from an adult male volunteer aged 24 years (subject L).

The infants were born with anomalies incompatible with longterm survival, namely: meningomyelocele with hydrocephalus, for which neurosurgery was not indicated. Parental consent was obtained to undertake the study. Each subject received an intravenous injection of a tracer dose of $^{4\text{-}14}$C-cortisol which, in the instance of subject B, was diluted with 5 mg of chemically pure cortisol. The radioactive steroid was injected in 5-7 ml of 10% ethanol in 0.9% NaCl over a period of a few minutes. Urine was collected for 48 to 72 hours following the injection.

Table II presents data on the age and body weight of these subjects, as well as on the specific activity of the cortisol administered and amount of radioactivity voided during the 2 days following the injection.

Table II

Amount of ^{14}C-Cortisol Administered to Individual Subjects and Urinary Excretion of the Radioactivity

Subject	Age	Body wt. kg.	^{14}C-Cortisol injected dpm x 10^6	mg	Recovery: % injected dose* Day 1	Day 2	Total
Infant B	8 days	3.3	9.97	5	52.10	1.60	53.70
Infant R	5 days	2.8	7.03	0.02	65.00	1.39	66.39
Adult L	23 years	75.8	4.79	0.01	81.34	2.50	83.84

* A negligible amount of radioactivity was excreted during the third day of urine collection.

Processing of Urine

Six hours of urine from each subject was processed. The difficulty of extracting polyhydroxylated steroids (such as 6β-hydroxycortisol) from aqueous media indicated the need for a particularly exhaustive method of extraction. Accordingly, the urine was first extracted 3 times with 3 volumes of methylene chloride, then in portions of 40 ml by shaking successively in a set of 3 extraction funnels, each containing 5 volumes of ethyl acetate (70). Washings (x2) with 1 N NaOH were reduced to 1/20 volume of the organic phase. The organic phase was then shaken with 1/20 volume of H_2O acidified with a few drops of 10% glacial acetic acid. Despite these precautions, some radioactivity was lost in the washings, amounting to about 6.0% of the total radioactivity of the urinary aliquot processed.

In the following text the term "glucuronide" fraction refers to those steroids extractable by the organic solvents following β-glucuronidase hydrolysis; the "sulfate" fraction to those extractable by the same solvents following a second hydrolysis with Glusulase. "Polar" fraction refers to radioactivity extracted with ethyl acetate; "non polar" fraction to methylene chloride extracts.

Purification of Individual Steroids

The separation and purification of urinary metabolites by chromatography was performed according to methods described in detail in previous publications from this laboratory (28, 70).

Accordingly, a brief description of the chromatography sequence used will be limited to those metabolites which are quantitatively the most important.

The purification of 6β -hydroxycortisol was achieved by paper chromatography using systems IV, III and I in sequence, where its Rf was identical to that of authentic 6β -hydroxycortisol. The steroid gave a positive soda fluorescence, reduced blue tetrazolium, and formed a phenylhydrazone absorbing maximally at 410 mμ upon reaction with phenylhydrazine hydrochloride in sulfuric acid. Following acetylation with acetic anhydride in pyridine (28) the steroid diacetate was chromatographed in system II, where its mobility was the same as 6β -hydroxycortisol diacetate. The data presented for this steroid were corrected for experimental loss by use of a known amount of ^{3}H-6β -hydroxycortisol. (See General Methods).

Tetrahydrocortisone was purified in systems VI and II in sequence, acetylated, and further purified in system VII. It gave a positive Porter Silber reaction.

The purification of cortolone was undertaken using paper chromatography systems VI, II, and IV, following which the mobility of the radioactive metabolite was identical to that of reference cortolone. In the last paper chromatography system, where

separation of α- and β-cortolone is achieved, the mobility of the radioactive metabolite was that of α-cortolone. Acetylation of cortolone led to the formation of a triacetate, which proved to have the same mobility in system V as the acetylation product of reference cortolone.

RESULTS

Recovery of Urinary Radioactivity by Extraction Procedures

Of the radioactivity injected, 54 and 66% respectively was recovered in the urine voided 48 hours after injection in the instance of the infants, compared to 84% in the urine of the control adult subject (Table II). In all instances a negligible amount was excreted during the 3rd day of urine collection.

The amounts of radioactivity extractable in different urinary extracts by methylene chloride and ethyl acetate are presented in Table III, where they are expressed as percentage of the total content of radioactivity of the urine.

While in the adult 84% of the urinary radioactivity was extracted into the glucuronide fraction, and this mainly by methylene chloride (70%), only 27% was extracted from the urine of both infants in the same fraction. In the unconjugated fraction of the infants, steroids of high polarity were more abundant than steroids of low polarity, but this was not the case for the control. The total amount

Table III

Radioactivity Extracted in Various Urinary Fractions Following the Administration of 4-^{14}C-cortisol to Three Subjects: Percentage of Total Radioactivity of the Urinary Aliquot Processed.

Subject	Unconjugated		Glucuronide		Sulfate		Butanol	Amberlite column	Total	Unaccounted for
	LP*	HP*	LP	HP	LP	HP				
Infant B	3.70	13.70	17.30	10.10	0.90	1.70	18.00	--	65.40	34.60
	17.40**		27.40		2.60					
Infant R	4.30	7.75	20.40	7.00	5.69	3.49	--	28.40	77.03	22.97
	12.05		27.40		9.18					
Adult male L	5.40	3.30	69.90	14.50	1.34	0.76	--	--	95.20	4.80
	8.70		84.40		2.10					

* Steroids extractable with methylene chloride (LP) and ethyl acetate (HP)
**Sum of LP + HP

Correction for experimental losses were made by use of appropriate labelled reference steroids (see general methods),

of radioactivity accounted for in the unconjugated compared to the glucuronide fraction was 17 versus 27% and 12 versus 27% in the case of the infants, contrasting sharply with the results obtained in the control (8 versus 84%). Little additional radioactivity became extractable in the sulfate fraction except in the instance of infant R, where it amounted to 9%.

Following this extensive extraction procedure and hydrolysis, 52.6 and 51.4% of the radioactivity remained in the urine of the two infants. After extraction with an equal volume of butanol (infant B) or chromatography of the urine on amberlite XAD-2 column according to the method of Bradlaw (74) (infant R), additional amounts of 18 and 28% of the radioactivity were extracted. Thus the total amount of urinary radioactivity extractable following these different procedures was 65 and 77% for the infants - figures to be compared with 95% for the control subject (Table III).

Quantification of urinary Metabolites after Chromatography

The isotope content of individual metabolites purified from the unconjugated and glucuronide fractions is presented in Tables IV and V where the radioactivity of each metabolite is also expressed as percentage: a) of the total radioactivity of the urinary aliquot processed, and b) of the total radioactivity extracted in the corresponding fraction. From 71 to 87% of the latter figure could be

Table IV

Isotope Content of Metabolites Purified from the Unconjugated Fraction Following Administration of ^{14}C-Cortisol to Two Newborn Infants and One Adult Male

Urinary metabolite	^{14}C content of metabolite: dpm			% of total dpm in urine aliquot processed*			% of radioactivity extracted from unconjugated fraction		
	B*	R*	L*	B	R	L	B	R	L
6β-OHF	87,700	148,760	24,190	7.27	7.77	1.21	64.95	64.59	13.96
6β-OHE	13,140	10,890	--	1.09	0.57	--	9.70	4.73	--
F	5,600	--	9,830	0.47	--	0.49	4.16	--	5.67
THE	4,130	11,300	12,950	0.34	0.59	0.65	3.06	4.92	7.50
Cortolone	3,330	5,500	21,330	0.28	0.28	1.07	2.47	2.37	12.31
Cortol	3,160	--	--	0.26	--	--	2.34	--	--
E	--	12,050	5,050	--	0.63	0.25	--	5.20	2.91
2α-OHF	--	--	12,340	--	--	0.62	--	--	7.12
THE	--	--	8,570	--	--	0.43	--	--	4.94
Unknown	--	--	27,940	--	--	1.40	--	--	16.09
Total	117,100	188,500	122,150	9.71	9.84	6.12	86.68	81.81	70.50

* Total ^{14}C dpm in aliquot of urine processed: Subject B: 1,205,900 dpm
R: 1,913,140 dpm
L: 1,994,860 dpm

The letters B, R, and L stand respectively for: Infant B, infant R and adult male L.

Correction for experimental losses were made by use of appropriate labelled reference steroids. (see general methods).

Table V

Isotope Content of Metabolites Purified from the Glucuronide Fraction Following Administration of ^{14}C-Cortisol to Two Newborn Infants and One Adult Male

Urinary metabolite	^{14}C content of metabolite: dpm			% of total dpm in urine aliquot processed			% of radioactivity extracted from glucuronide fraction		
	B*	R*	L*	B	R	L	B	R	L
THE	221,140	383,330	698,100	18.34	20.04	35.00	67.10	73.10	41.50
THF	--	--	369,330	--	--	18.50	--	--	21.90
Cortolone	32,950	71,390	336,060	2.73	3.73	16.85	10.00	13.60	20.00
6β-OHF	51,800	67,430	--	4.30	3.52	--	15.70	12.86	--
Cortol	--	--	25,770	--	--	1.29	--	--	1.50
E	--	--	9,620	--	--	0.48	--	--	0.57
F	--	--	7,770	--	--	0.39	--	--	0.46
Total	305,900	522,150	1,446,300	25.37	27.29	72.51	92.80	99.56	85.93

*The letters B, R, and L stand respectively for: Infant B, infant R and adult male L.

Correction for experimental losses were made by use of appropriate labelled reference steroids (see general methods).

accounted for in the unconjugated fraction, and from 86 to 100% in the glucuronide fraction.

6 β -hydroxycortisol represented about 65% of the radioactivity which was extracted in the unconjugated fraction of the two infants, compared to 14% for the control subject. Besides this steroid the newborn excreted, as components of the unconjugated fraction, several other metabolites, but in markedly lower amounts -- which are in the range of the various steroids quantified in the corresponding fraction of the control subject.

In all three subjects the major metabolite of ^{14}C-cortisol in the glucuronide fraction was tetrahydrocortisone. This steroid represented up to 67 and 73% of the radioactivity present in this fraction in the case of the 2 infants. In the control, tetrahydrocortisone, tetrahydrocortisol (which is not found in infant urine in any significant amount) and cortolone amounted to 83% of the radioactivity extracted in the glucuronide fraction. In the infant urine cortolone was present in significant concentrations, together with 6β-hydroxycortisol, the sum of tetrahydrocortisone, cortolone and 6β-hydroxycortisol practically accounting for the whole radioactivity extractable in the glucuronide fraction. 6β-hydroxycortisol, although looked for, was not found in the glucuronide fraction of the adult, while cortisol and cortisone

were characterized in this fraction, but in amounts representing about 0.5% of the total radioactivity extracted.

The amount of radioactivity extracted following Glusulase hydrolysis (sulfate fraction) was small -- about 2% of the total urinary radioactivity of the aliquots processed -- except for infant R, where it amounted to about 9%. 6β-hydroxycortisol was tentatively characterized as a minor component of this fraction, amounting to 0.33% of the radioactivity extracted. The major component of the fraction, however, possessed in system VIII a greater polarity than 6β-hydroxycortisol. No attempt was made to characterize this material.

The processing of the sulfate fraction of the control revealed the presence of an ultraviolet absorbing area (240 mμ) which, in systems IV and II, had the same mobility as cortisol. The material eluted from this area, when subjected to the Porter Silber reagent, gave the absorption curve in visible light characteristic of a dihydroxyacetone side chain. The radioactivity associated with this fraction, however, was of little quantitative significance. Radioactive areas corresponding to the mobility of cortisol and cortisone were not detected upon chromatography of the sulfate fraction of infant urine.

As previously pointed out, a very significant amount of radioactivity, 18 and 28% respectively, was extracted from the urine of the

infants following butanol extraction or amberlite column chromatography. An attempt to purify this material indicated the presence of one main fraction -- which amounted in both infants to 30 - 35% of the radioactivity extracted. It gave negative soda fluorescence, blue tetrazolium and Porter Silber reactions, and possessed a much greater polarity than 6β-hydroxycortisol in system I, with an Rs of 5.2 with respect to corticosterone sulfate.

Discussion

The poor recovery of ^{14}C-cortisol in the urine of the newborn infant in terms of known metabolites, the distribution of the radioactivity in the unconjugated and glucuronide fractions in often comparable amounts, together with a large proportion which remains unaccounted for, has long been recognized (1-3, 6).

Such findings have led to speculations regarding the "immaturity" (1, 2, 29, 39) of steroid metabolizing enzymes and the existence of alternate pathways of cortisol metabolism involving 6β-hydroxylation of cortisol (4) and the formation of the sulfates of cortisol and cortisone (59). However, while in adult subjects the metabolism of cortisol appears thoroughly documented (8), in the newborn there is a lack of quantitative studies of cortisol metabolites -- which could provide a proper background to these hypotheses.

Previous studies following the simultaneous administration of ^{3}H-cortisol and ^{14}C-cortisol sulfate to the human newborn had already indicated that sulfoconjugation is of little significance during ^{3}H-cortisol metabolism, and that the incorporation of this steroid into 6 β-hydroxycortisol was of lesser quantitative importance than expected (7, 28). Such conclusions were tentative, however, because of the low recovery of radioactivity in the various urinary fractions studied.

An almost unanimous finding with respect to cortisol metabolism during early life remains the observation of the small amount of radioactivity released following extensive β -glucuronidase hydrolysis (1-3, 6, 28, 58).

In the present investigation an attempt at a quantitative definition of the pathways of metabolism of ^{14}C-cortisol during the neonatal period was undertaken with the aim of eventually assessing their comparative significance.

The relative distribution of radioactivity in the unconjugated, glucuronide and sulfate fractions, as well as in the fraction unaccounted for, prior to butanol extraction or amberlite column chromatography, compares well with the results of authors who used similar procedures of exhaustive extraction of urine with methylene chloride and ethyl acetate (3). 6 β-hydroxycortisol was the major component of the unconjugated fraction in the newborn infants, and tetrahydrocortisone that of the glucuronide fraction in both the adult control and the infants.

The results indicate that the greater ability of the adult to metabolize cortisol in the glucuronide fraction is mainly due to the presence of tetrahydrocortisol and the greater amount of tetrahydrocortisone and cortolone in this fraction.

The patterns of cortisol metabolites in the unconjugated and glucuronide fractions of the infants do not suggest, by themselves, however, that alternate pathways of cortisol metabolism come into play, although a large proportion of the radioactivity extracted in the unconjugated fraction (82-87%) and following β-glucuronidase hydrolysis (93-100%) was accounted for in terms of known metabolites. The presence of 6β-hydroxycortisol as, by far, the main unconjugated metabolite of cortisol in newborn infant urine, supports the original finding of Ulstrom et al (4), but the data can hardly be interpreted as indicating that 6β-hydroxycortisol formation compensates for a decreased excretion of cortisol metabolites in the glucuronide fraction of the newborn. Along the same lines, the small percentage of radioactivity recovered in the sulfate fraction, even in the instance of infant R, and the low incorporation of ^{14}C-cortisol in compounds tentatively identified as 6β-hydroxycortisol and cortisol indicate, as previously suggested (28), that sulfoconjugation plays little part in cortisol metabolism. Such a statement would remain valid even if the radioactivity extracted in the sulfate fraction of subject R (about 9%) were totally accounted for. This conclusion is not in contradistinction

to previous reports from this laboratory (59) that cortisol sulfate is present in newborn infant urine in significant concentrations compared to tetrahydrocortisone glucuronide. It merely emphasizes that this conjugate does not reflect to any significant extent the metabolism of cortisol, and adds further support to the finding (28) that its urinary concentration is closely related to its secretion rate value.

The additional amount of radioactivity extracted either by butanol or eluted from amberlite column with methanol is quantitatively more important than that extractable in the sulfate fraction. Indeed the total amount of radioactivity in the unconjugated, glucuronide and either butanol or amberlite fractions, represents 62% (infant B) and 68% (infant R) of the urinary radioactivity extracted, compared to total extractable amounts of 65 and 77%. However, the appraisal of the significance of this finding with respect to cortisol metabolism during the neonatal period has to await the proper identification of the components of the butanol and amberlite fractions.

The relevance of these data to the assessment of adrenocortical function during the neonatal period relates to the presence of tetrahydrocortisone as a major metabolite in the glucuronide fraction. The fact that the greater incorporation of cortisol occurs in this metabolite should make its quantitative and specific measurement a simple but quite reliable index of adrenocortical activity postnatally.

CHAPTER II

CHAPTER II

Simultaneous Metabolism of Cortisol and 6β-Hydroxycortisol by Human Newborn

Experimental

Biological Material

Infant P., a male, was born with meningomyelocele accompanied by severe hydrocephalus, for which neurosurgery was not indicated.

At the time of the study the infant was 2 days old and weighed 3 kg. Having obtained parental consent, 4-^{14}C-cortisol (2.42×10^6 dpm) and 1,2-^{3}H-6β-hydroxycortisol (2.48×10^6 dpm) were administered intravenously in a total volume of 5 ml of 10% ethanol in 0.9%NaCl. Urine was collected in adhesive bags during the 3 days which followed the injection; within this period of time 62% of the ^{3}H and 41% of the ^{14}C were recovered in the urine. It was not possible to establish with any degree of certainty whether these low recoveries were in part due to urine spillage at the time of changing the collection bags.

Processing of Urine

1) Extraction

The urine collected during the first 2 days, which contained practically the whole radioactivity recovered following the injection, was pooled and extensively extracted with ethyl acetate prior to and following sequential enzyme hydrolysis. Briefly, the procedure involves

the extraction of the urine in 20 ml portions in each of 3 successive separatory funnels, each containing 100 ml of ethyl acetate. After pooling the organic phases, the washings with 1N NaOH and water were reduced to a minimum in order to avoid losses of highly water soluble metabolites. After extraction of the unconjugated fraction, the urine was subjected to two enzyme hydrolyses -- the first with Ketodase at a concentration of 500 units of β-glucuronidase/ml, the second with Glusulase at a concentration of 1000 units of sulfatase/ml.

The hydrolysates were extracted with ethyl acetate according to the procedure described above, the extract of the first hydrolysate being referred to as the glucuronide fraction, the extract of the second as the sulfate fraction. Hydrolysis conditions have been described in general methods.

Since, at this point, substantial amounts of radioactivity remained in the urine, it was subjected to one additional extraction with n-butanol, using a procedure similar to that employed for ethyl acetate extraction.

2) Paper Chromatography

Separation of individual steroids by paper chromatography was achieved according to methods previously described (28). Therefore chromatography sequences will be outlined only briefly for those steroids of particular quantitative significance.

a) The unconjugated fraction was first subjected to chromatography on system IV, in which 6β -hydroxycortisone separated from the bulk of the radioactivity which remained close to the line of application.

This latter fraction was chromatographed in system I, following which two main areas of radioactivity were separated: one possessing the mobility of standard 6β-hydroxycortisol, the other slightly more polar. Upon further purification of the 6β-hydroxycortisol fraction on system IX, two main fractions were separated: one corresponding to the mobility of 6β-hydroxycortisol and the other, which will be referred to as compound II, had an Rs of 0.5 with respect to that steroid. The $^{14}C/^{3}H$ ratio of 6β-hydroxycortisol was 0.02, indicating a very substantial loss of ^{14}C from the previous chromatography in system I (following which the ratio was 0.08).

The specific activity of the 6β-hydroxycortisol fraction was established with respect to its ^{3}H content by counting and subjecting triplicate aliquots to the Porter Silber reaction. One additional chromatography in system III provided evidence that constant specific activity had been achieved (Table VIII).

The 6β-hydroxycortisone fraction, separated after the first chromatography, was further purified in systems IX and I. It gave a positive Porter Silber reaction, and its $^{3}H/^{14}C$ ratio after this last chromatography was 0.07. There was not enough radioactivity to allow further purification.

The fraction slightly more polar than 6β-hydroxycortisol in

system I (see above) was purified in systems III, IX, and I. On the grounds that it reduced blue tetrazolium chloride, gave a positive Porter Silber reaction, did not absorb UV (260 mμ), and was, in system III, more polar than 6 β-hydroxycortisol, it will be referred to as 6 β-hydroxy, tetrahydrocortisol (4).

Compound II (the metabolite of highest polarity in the unconjugated fraction) was subjected to chromatography in systems III and IX, following which its $^{14}C/^{3}H$ ratio was 0.7 and 0.6 respectively. This metabolite gave a positive blue tetrazolium chloride and Porter Silber reaction. No attempt was made at further characterization.

b) The glucuronide fraction was first chromatographed on system VI for 16 hours. Two areas of radioactivity were detected: one had the same mobility as tetrahydrocortisone and gave an intense blue tetrazolium chloride reaction; the other remained on the line of application. Tetrahydrocortisone was further purified on systems II and VI, following which constant specific activity of the metabolite was achieved, as established by ^{14}C count and by the Porter Silber reaction (Table VIII).

The polar fraction was chromatographed on system I, where it separated into several components. The major component possessed the mobility expected in this system for 6 β-hydroxytetrahydrocortisol, and gave positive blue tetrazolium chloride and Porter Silber reactions. This fraction was further purified as described in the instance of

unconjugated 6 β-hydroxytetrahydrocortisol (see above). Chromatographed simultaneously in system III, the 6β-hydroxytetrahydrocortisol from the unconjugated and conjugated fractions had the same mobility and the same Rs with respect to 6β-hydroxycortisol. 6β-hydroxytetrahydrocortisol was present in the unconjugated fraction in greater amounts than in the glucuronide fraction.

c) The sulfate fraction was purified on system VI. Small amounts of radioactivity were detected corresponding to the chromatographic mobility of cortisol and tetrahydrocortisone. Both fractions gave a positive Porter Silber reaction. A more polar fraction which had not migrated from the line of application was rechromatographed in system I, where three radioactive areas were detected by scanning. All were more polar than 6β-hydroxycortisol, with respect to which their Rs' were 0.28, 0.71, and 0.88 respectively. Their $^{14}C/^{3}H$ ratios were 1.27, 0.84 and 1.19 in order of decreasing polarity.

d) The butanol extract was dried under air, redissolved in ethanol, and stored at -20° C for 24 hours. The ethanol, which contained most of the radioactivity, was decanted. Large amounts of impurities which were not ethanol soluble were thus eliminated. The ethanol extract was applied on system VIII, where two large areas of radioactivity were detected by scanning: one well-defined peak possessing an Rf of 0.73 (Rs with respect to cortisol sulfate: 1.4) and one less well-defined area, even more polar, with an Rf of 0.15.

The less polar compound was subjected to further purification in systems III, IX and I. In the last system its Rs with respect to corticosterone sulfate was 5.1.

The more polar area was rechromatographed in system VIII for 72 hours, following which it still remained close to the line of application.

RESULTS

Distribution of ^{14}C and ^{3}H in Various Urinary Extracts

The amounts of radioactivity extracted in different urinary fractions (namely: the unconjugated, glucuronide, sulfate and butanol fractions) are expressed in Table VI as percentages of the total radioactivity present in the urine, prior to extraction. 51% of the ^{14}C and 60% of the tritium was accounted for in these various fractions, 41% of the tritium being extracted in the unconjugated fraction. Significant amounts of ^{3}H were also extracted into the glucuronide (8.4%), butanol (6.8%) and sulfate (3.1%) fractions in order of decreasing importance, quantitatively. The distribution of ^{14}C metabolites of cortisol in the unconjugated (13%), glucuronide (17%) and sulfate (3%) fractions is within the range expected for infants of this age. An additional 18% of the ^{14}C and about 7% of the tritium was extracted with butanol. A second butanol extraction removed less than 3% of the radioactivity still present in the urine; 12.7% of

Table VI

Radioactivity Extracted in Various Urinary Fractions Following the Administration of ^{14}C-Cortisol and ^{3}H-6β-Hydroxycortisol to One Newborn Infant. Percentage of Total Urinary Radioactivity.*

Unconjugated			Glucuronide			Sulfate			Butanol			Total		Unextracted	
^{14}C	^{3}H	$^{14}C/^{3}H$	^{14}C	^{3}H	$^{14}C/^{3}H$	^{14}C	^{3}H	$^{14}C/^{3}H$	^{14}C	^{3}H	$^{14}C/^{3}H$	^{14}C	^{3}H	^{14}C	^{3H}
12.6	41.0	0.66	17.0	8.4	4.36	2.9	3.1	2.0	17.9	6.8	5.7	51.4	60.4	12.7	21.0

*These figures do not include losses incurred during extraction and washings.

the ^{14}C and 21% of the tritium remained unextractable.

Pattern of Metabolites

The principal metabolites of 6β-hydroxycortisol and ^{14}C-cortisol purified from the above fractions by chromatography are presented in Table VII. The values are expressed in terms of either ^{14}C or ^{3}H as percentage of the total ^{14}C or ^{3}H radioactivity extracted in the respective urinary fractions. In the instance of the major steroids, constant specific activity was reached; in the case of metabolites of lesser quantitative significance, two to three chromatographies and some tests of identification were carried out. These values represent minimum percentages, since correction for experimental losses was not applicable under the present experimental conditions.

6β-hydroxycortisol and 6β-hydroxycortisone were the main metabolites of 6β-hydroxycortisol in the unconjugated fraction. They contained relatively small amounts of ^{14}C, as reflected by their $^{14}C/^{3}H$ ratios. The amount of ^{3}H in these two metabolites accounted for practically all of the tritium extractable in the unconjugated fraction. In contrast, the incorporation of ^{14}C into 6β-hydroxycortisol and 6β-hydroxycortisone was not much greater than that observed in the instance of such minor metabolites as cortisol, cortisone and tetrahydrocortisone. While the three last-mentioned steroids were

Table VII

^{14}C-Cortisol and ^{3}H-6β-Hydroxycortisol Metabolites in Various Urinary Fractions

Fraction	Metabolite	% of Radioactivity Excreted in Corresponding Fraction		Final $^{14}C/^{3}H$ of Metabolite
		^{14}C	^{3}H	
Unconjugated	6β-OHF	2.0	18.0	0.02
	6β-OHE	2.7	7.0	0.07
	6β-OH THF	2.2	0.2	2.60
	Cpd II	2.5	0.8	0.64
	Cortisol	2.6	-	-
	Cortisone	2.0	-	-
	THE	2.0	-	-
Glucuronide	THE	20.0	-	-
	Cortolone	3.0	-	-
	6β-OHF	0.01	0.6	0.02
	6β-OH THF	0.30	0.06	6.00
Sulfate	Cortisol	4.0	-	-
	THE	4.0	-	-
Butanol	Less Polar	4.0	1.6	4.20
	More Polar	2.6	1.6	2.70

devoid of ^{3}H, it should be pointed out that the isotope ratio of 6β-hydroxytetrahydrocortisol was 2.6, indicating a very significant incorporation of ^{14}C into this metabolite.

Of all the metabolites purified, tetrahydrocortisone was the predominant one in the glucuronide fraction, followed by cortolone, which was present in amounts representing about 10% of the tetrahydrocortisone value. Neither steroid contained ^{3}H. From the specific activity of tetrahydrocortisone, the secretion rate of cortisol was established as being 5.7 mg/day. The only metabolites extractable in the glucuronide fraction which contained ^{3}H were all more polar than 6β-hydroxycortisone. In general, their content of radioactivity was too low to permit further purification and attempts at identification. This did not apply to the major metabolites, namely: 6β-hydroxycortisol and 6β-hydroxytetrahydrocortisol, which were further purified to a final $^{14}C/^{3}H$ isotope ratio of 0.02 and 6.00 respectively.

The values for the total production rate, the peripheral production and the secretion of 6β-hydroxycortisol are presented in Table IX. The results indicate that the total production and the peripheral production of 6β-hydroxycortisol are low, and that the secretion rate of the steroid (which represents the difference between these two values) (77) is in the order of 42μg/day, a small figure indeed compared to that of cortisol (5.7 mg/day) (Tables VIII and IX).

Table VIII

Production Rate of Cortisol and 6β -Hydroxycortisol*

Urinary metabolite	Specific activity dpm/μg after chromatography:		Secretion rate of cortisol	Production rate of 6β -hydroxycortisol
	No. 3	No. 4	μg/day*	μg/day*
^{14}C-THE	370	380	5,690	--
^{3}H-6β -OHF	12,900	13,000	--	173

* Calculated from the formula:

$$\frac{S \times W \times R}{C \times d} - W$$

in which:
S = specific activity of steroid injected (cpm/μg)
W = amount in μg of steroid injected
R = mol.wt.of steroid injected / mol.wt.urinary metabolite
C = specific activity of urinary metabolite (cpm/μg)
d = number of days of urine collection

Table IX

Peripheral Production and Secretion Rate of 6β-Hydroxycortisol*

6β-Hydroxycortisol µg/24 hr			pF→ 6β-Hydroxycortisol**	Cortisol µg/24 hr
Total production	Peripheral production	Secretion		Secretion rate
173	131	42	0.023	5690

*Peripheral production was calculated from the production rate of cortisol and the conversion factor of cortisol to 6β-hydroxycortisol (pF→ 6β-hydroxycortisol) (49)

**The conversion factor of cortisol to 6β-hydroxycortisol was calculated from the formula

$$pF \rightarrow 6\beta\text{-OHF} = \frac{^{14}C/^{3}H \text{ in urinary } 6\beta\text{-OHF}}{^{14}C/^{3}H \text{ in injection solution}}$$

Cortisol and tetrahydrocortisone were characterized in the sulfate fraction in low yields. Both were devoid of tritium.

Of the two main components of the butanol extract, the one referred to as the less polar fraction was purified by repeated chromatography (systems VIII, IX, III, I) to an isotope ratio of 4.2. This material did not react with phenylhydrazine hydrochloride in sulfuric acid, did not reduce tetrazolium chloride, and showed a negative soda fluorescence reaction, suggesting a polyhydroxylated, saturated compound. This hypothesis would be consistent with its high polarity with respect to solvent extraction and chromatographic mobility. The isotope ratio of the more polar fraction was 2.7. In view of the small degree of purification to which this fraction was subjected however, the significance of this ratio is open to question.

DISCUSSION

It should be pointed out that speculations or conclusions on the data presented above must take into consideration two main facts: the first is that this data results from a single observation on an abnormal infant -- although there is no reason to believe that his disease affected adrenocortical function and steroid metabolism; the second is related to the low recoveries of radioactivity following the injection, and their influence upon the validity of the

calculations of the metabolic parameters considered.

However, one is justified in considering that meaningful observations are supplied by: a) the relative distribution of the radioactivity into different urinary fractions; b) the conversion of cortisol to various metabolites, including 6β-hydroxylated steroids, and c) the isotope ratio of these urinary metabolites.

Evidence that 6β-hydroxycortisol is mainly excreted as such and as 6β-hydroxycortisone was obtained by the observation that 41% of the ^{3}H administered was recovered in the unconjugated fraction, a value -- interestingly enough -- comparable to those published by Burstein on two adult subjects aged 24 and 29 years (49). Of this total amount, at least 18% was characterized as 6β-hydroxycortisol and 6% as 6β-hydroxycortisone.

The very low incorporation of ^{14}C-cortisol into these metabolites, as reflected by a $^{14}C/^{3}H$ ratio lower than 0.1 compared to the ratio of the injection solution of 1.0, is in keeping with observations reported from this laboratory following administration of labelled cortisol to such infants (28). The low peripheral conversion value of cortisol to 6β-hydroxycortisol obtained in the present study is consistent with these findings.

The study does not support the view that the excretion of 6β-hydroxycortisol, could reflect an alternate pathway of cortisol metabolism during early life, even if, as observed by previous

investigators (4), this steroid is the predominant component of the unconjugated fraction. This does not negate the possible significance of 6β-hydroxylation with respect to other aspects of cortisol metabolism in the neonate, as will be discussed later.

With respect to the metabolism of cortisol, the present investigation confirms observations which are now fairly well established in subjects of this age group (28), namely: the particular distribution of the ^{14}C radioactivity within various urinary fractions; the relatively low recovery of metabolites in the glucuronide fraction; the predominance of tetrahydrocortisone in this same fraction; the small incorporation of cortisol into the sulfates of cortisol and cortisone; and the large proportion of radioactivity remaining in the urine, part of which was recovered following butanol extraction.

Of interest is the sharp contrast between the $^{14}C/^{3}H$ ratios of 6β-hydroxylated non reduced metabolites of cortisol (as exemplified by 6β-hydroxycortisol with a ratio of 0.02) and that of 6 β-tetrahydrocortisol with a ratio of 2.6 in the unconjugated fraction and of 6.0 in the glucuronide fraction. This suggests that 6β-hydroxylation of certain metabolites of cortisol could take place more readily than 6β-hydroxylation of cortisol or than the reduction of 6β-hydroxycortisol to 6β-tetrahydrocortisol. This could be due in part to the rapid excretion of 6β-hydroxycortisol prior to any extensive metabolism,

as suggested by the large amount of ^{3}H excreted into the unconjugated fraction. The 6β-hydroxylation of tetrahydrocortisol may represent one step in the metabolic degradation of the steroid prior to conjugation or, alternatively, prior to further hydroxylation (e.g., at the 20-ketone level), which would result in the formation of highly water soluble steroids.

The validity of this hypothesis could be greatly strengthened if future investigations were to include better criteria for the identification of 6β-hydroxytetrahydrocortisol than those presented here, although the occurrence of this metabolite in infant urine has been previously documented, following identification by infrared spectroscopy (4). Studies of the metabolic fate of tetrahydrocortisol in infants of this age group aimed at demonstrating that 6β-hydroxylation of this steroid occurs during its metabolism would also be valuable.

The present investigation added little to the characterization of the very significant fraction of cortisol (and 6β-hydroxycortisol) metabolites which are either extractable by butanol or remain unextractable under our experimental conditions. Speculation on the nature of the butanol extractable material of "low polarity" is made difficult because of the absence of positive reaction to usual qualitative screening tests such as ultraviolet absorption, soda fluorescence, blue tetrazolium chloride and Porter Silber reactions.

These negative reactions in themselves suggest a metabolite bearing both a saturated ring A and a reduced α ketol sidechain. It is of interest that in the search for a steroid of similar properties, we observed that the $NaBH_4$ reduction product of ^3H-6β-hydroxy-cortisol possesses closely related mobilities in three different paper chromatography systems (I, IX, VIII). Such a product should be expected to be closely related to pregn-4-ene-6β, 11β, 17α, 3, 20, 21 hexol. The ^3H content of the two steroid fractions separated by chromatography of the butanol extract positively indicate that some of their components are 6β-hydroxysteroids, but their isotope ratios suggest again that they originate from cortisol to a much larger extent than from 6β-hydroxycortisol, once more implying the possibility of 6β-hydroxylation of reduced metabolites of cortisol. To this extent the butanol fraction represents mainly a parameter of cortisol metabolism.

SUMMARY

Summary

Following ^{14}C-cortisol administration to 2 newborn infants and 1 adult, the pattern of urinary metabolism was analyzed and compared.

- 54 and 66% of the radioactivity injected was recovered in the urine voided within 48 hours after ^{14}C-cortisol administration in the instance of the newborns, whereas 84% of the radioactivity injected was recovered in the urine of the adult subject. In the three instances, negligible amounts of radioactivity were voided during the 3rd day of urine collection.

- The total amount of urinary radioactivity recovered following extensive extractions of unconjugated and conjugated steroids was 65 and 77%, in the instance of the newborns, figures to be compared with 95% in the instance of the adult subject.

- 17 and 12% of the radioactivity was extracted in the unconjugated fraction in the instance of the newborns and 8.7 % in the unconjugated fraction of the adult control.

- Small amount of radioactivity was extracted from the urine of newborns following β-glucuronidase hydrolysis in both cases - 27% as compared to a value of 84% for the adult.

- Following glusulase (sulfatase) hydrolysis, low amounts of radioactivity were extracted from both infant and adult urine.

- 6β-hydroxycortisol is the most important metabolite in the unconjugated fraction of the infants (65% of the radioactivity appearing in this fraction compared to 14% for the adult subject).

- Tetrahydrocortisone was the major metabolite in the glucuronide fraction of both the infants and the adult control.

Following the simultaneous administration of ^{14}C-cortisol and ^{3}H 6β-hydroxycortisol to a newborn infant, low values for total production (173 µg/day) and peripheral production (131 µg/day) of 6β-hydroxycortisol from ^{14}C-cortisol were observed.

The secretion rate of 6β-hydroxycortisol was established as being 42 µg/day, an almost negligible value compared to that of cortisol (5,700 µg/day).

Although 6β-hydroxycortisol represents the predominant component of the unconjugated fraction, the data does not support the view that this metabolite is produced in sufficient amount to be considered as reflecting an alternate pathway of cortisol metabolism during the postnatal period. This also applies to cortisol sulfate since this urinary steroid contained very small amounts of radioactivity, indicating that it is formed to a very small extent during the peripheral metabolism of cortisol.

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