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GENERAL REVIEW

CARBOXYLIC ACID METABOLITES OF STEROIDS

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INTRODUCTION

Between 1935 and 1965, the major part of our current knowledge of corticosteroid metabolism in the human, and to a lesser extent in other species, was obtained. The urinary metabolites of cortisol that were isolated and identified during this period were the neutral ones, resulting primarily from modifications occurring in the fused ring system. The ketol side chain was considered uninteresting and received little attention, because it underwent only one change of any quantitative consequence, which involved reduction at C-20. A few workers recognized that the neutral metabolites accounted for only a part of the total excretory products of cortisol in humans; estimates of the unidentified fraction ranged from 25% to 55% [1–6]. Qualitatively similar conclusions were drawn from studies with primates [7], rat [8, 9], cat and rabbit [10, 11]. The unidentified fractions could not be extracted into organic solvents from alkaline or neutral aqueous solution and appeared to be very polar. From their properties it was suggested that some of the metabolites could be acids [12, 13]. In this review we will summarize the current status of the acidic metabolites of the corticosteroids.

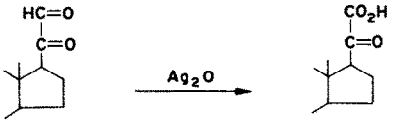
Terminology

The chemical terminology of the steroidal carboxylic acids has been defined in the Revised Tentative Rules for Nomenclature of Steroids proposed by the International Union of Pure and Applied Chemistry and the IUPAC–IUB Commission on Biochemical Nomenclature. The steroid acids are named by the rules which apply to other classes of steroids. In addition, the acid substitutent is indicated by the suffix "-oic acid" preceded by the number of the carbon atom which is the acid function. The suffix of the acid derived from carbon 20 is 17β-carboxylic acid. The androstane-17-carboxylic acids have been given the trivial name "etianic acids"; 4-androstene-17-carboxylic acids are called "etienic acids". The systematic terminology of all the steroids mentioned in this review is presented in table 1.

Chemical synthesis of steroid acids

(a) *C_{20} acids.* Etienic and etianic acids are readily prepared by treating corticosteroids with periodic acid in acid solution [14]. This reaction is usually quantitative. The acids are easily crystallized and provide suitable derivatives for structural determinations [14] and chromatographic analysis of corticosteroids [15–17]. They were used to confirm the identities of corticosteroids and their metabolites early in the history of steroid chemistry [14]. Methods for the synthesis of the C_{20} acids are summarized in table 2. The etienic acid derived from cortisol is highly fluorescent in sulfuric acid–ethanol reagent, and this property has been used to enhance the sensitivity of the fluorimetric analysis of cortisol [18, 19]. Recently, the reactivity of the carboxylic acid function has been used to prepare steroid derivatives for affinity chromatography on aminosepharose [20].

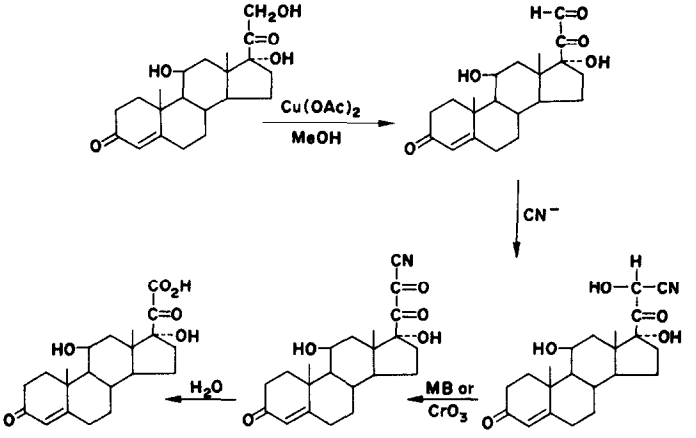
(b) *Steroidal oxo acids.* Direct conversion of 17-deoxy oxoaldehydes to oxo acids in good yield can be achieved by oxidation with silver oxide [32].



Oxidation of the 17-hydroxy-steroids by silver oxide gives a very poor yield of the oxo acid [33]. Unless special precautions are taken [34], prolonged exposure of these steroids to alkaline silver oxide leads to complete loss of the side chain and the generation of C_{19} steroids. A more generally applicable procedure is outlined in scheme 1. The oxoaldehyde is converted to a cyanohydrin which is readily oxidized to the oxo acid with methylene blue or chromium trioxide [35]. Direct syntheses of the 20-oxo acid methyl esters have been described. These procedures are applicable to the synthesis of 17-deoxy steroids but give poor yields with 17-hydroxy steroids as precursors. Lewbart and Mattox [36] oxidized 20-hydroxy-21-oic acid methyl esters to 20-oxo acids with chromic acid. Since chromic acid under the conditions used oxidizes all hydroxyl groups to carbonyl groups, the method is of limited applicability. Laurent *et al.* [37] used a modification of this procedure to achieve the same goal. An alternative procedure similar in principle to that described by Corey *et al.* [38] for the conversion of geranial to methyl geraniate was also used by Laurent *et al.* [37] for the same purpose.

Table 1.

Cortisol	11β,17,21-trihydroxy-4-pregnene-3,20-dione
etianic acid	3α-hydroxy-5β-androstane-17β-carboxylic acid
etienic acid	3-oxo-4-androstene-17β-carboxylic acid
DOC	21-hydroxy-4-pregnene-3,20-dione
fluocortolone	6β-fluoro-11β,21-dihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione
21-dehydrocortisol	11β,17-dihydroxy-3,20-dioxo-4-pregnen-21-al
cortolic acid	3α,11β,17,20α-tetrahydroxy-5β-pregnan-21-oic acid
β-cortolic acid	3α,11β,17,20β-tetrahydroxy-5β-pregnan-21-oic acid
cortolonic acid	3α,17,20α-trihydroxy-11-oxo-5β-pregnan-21-oic acid
β-cortolonic acid	3α,17,20β-trihydroxy-11-oxo-5β-pregnan-21-oic acid
cortienic acid	11β,17-dihydroxy-3-oxo-4-androstene-17β-carboxylic acid
cortoic acids	all 17α-hydroxy-21-oic acid metabolites of cortisol or cortisone
cortol	5β-pregnane-3α,11β,17,20α,21-pentol
β-cortol	5β-pregnane-3α,11β,17,20β,21-pentol
cortolone	3α,17β,20α,21-tetrahydroxy-5β-pregnan-11-one
β-cortolone	3α,17β,20β,21-tetrahydroxy-5β-pregnan-11-one
THF	3α,11β,17,21-tetrahydroxy-5β-pregnan-20-one
THE	3α,17,21-trihydroxy-5β-pregnane-11,20-dione
isocortisol	11β,17,20ξ-trihydroxy-3-oxo-4-pregnen-21-al
isocortisone	17,20ξ-dihydroxy-3-oxo-4-pregnen-21-al
isoTHE	3α,17,20ξ-trihydroxy-11-oxo-5β-pregnan-21-al
21-hydroxypregnanolone	3α,21-dihydroxy-5β-pregnan-20-one
pregnanetriol	5β-pregnane-3α,20,21-triol
Reichstein's Compound E	11β,17,20ξ,21-tetrahydroxy-4-pregnen-3-one
Reichstein's Compound U	17,20ξ,21-trihydroxy-4-pregnene-3,11-dione

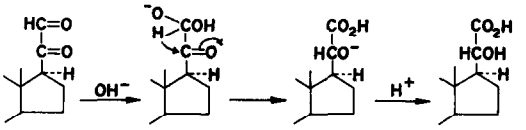


Scheme 1. Synthesis of 17-hydroxy-20-oxo-acids.

(c) *Steroidal 20-hydroxy acids.* The chemistry of the 20 hydroxy acids was first studied by Lewbart as an outgrowth of work directed to studies on the mechanism of the Porter–Silber reaction [39, 40] and to identify the artifacts caused by traces of cupric ion in solutions of corticosteroids [41]. In both cases, the common intermediates were steroidal 20-oxo-21-aldehydes. Extended treatment of the oxoaldehydes with cupric acetate resulted in the formation of the epimeric 20-hydroxy-21-oic acids [42, 43]. Alternatively, alkali catalyzed a rapid internal dismutation of oxoaldehydes [44, 45] (scheme 2).

Table 2. Chemical methods used for synthesis of 17β-carboxylic acids

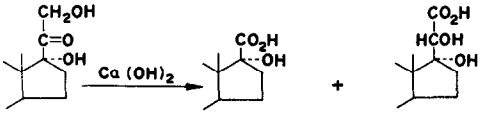
Procedure	Reference
Periodic acid oxidation of corticosteroids	[14, 21, 22]
Photolysis of 20-oxo-21-nitrites	[23]
From 4-ene-16-keto steroids	[24]
Chromic acid oxidation of 17-deoxycorticosteroids	[22, 25, 26]
Barbier–Wieland degradation of sterols	[27]
Chromate oxidation of pregnanes via 21-benzal derivatives	[28]
Base cleavage of 20-oxo-21-pyridinium iodide	[29]
Persulfate oxidation of allopregnane derivatives	[30]
Alkaline degradation of keto side chains	[31]



Scheme 2. Synthesis of 20 hydroxyacids.

Steroid acids as artifacts

It has been known since 1938 that the corticosteroid side chain is susceptible to degradation by dilute alkali [31, 46]. That this is so is not surprising, because the ketol and dihydroxyacetone side chains resemble the terminal groups of the ketol sugars, which are known to be converted to acids by this treatment. Among the degradation products are etienic acids and 20-hydroxy acids [47–50] as illustrated in scheme 3. The ketol side chain degradation is retarded but not eliminated when an atmosphere of nitrogen replaces oxygen [48, 50, 51]. Degradation of steroids during saponification of steroid esters in aqueous solution therefore may be a serious problem if access to air is not minimized and the basicity of the solution is not carefully controlled [52].



Scheme 3. Alkaline degradation of steroids.

Lability of the steroid is evident even when the pH is neutral, though the rate of breakdown is slow. Several days at 15°–35° are necessary to observe significant acid formation from cortisol [53]. Under even relatively mild conditions, cortisol can break down further to C_{19} compounds [54, 55]. The formation of acids under neutral conditions could be rationalized by postulating a saccharinic acid type transformation of a ketoaldehyde generated by the presence of trace amounts of cupric ion in the water or buffer [41, 54–57] as shown in scheme 4. Aldosterone is destroyed by acid and heat as well as by alkali [58].

Biosynthesis of etienic acids

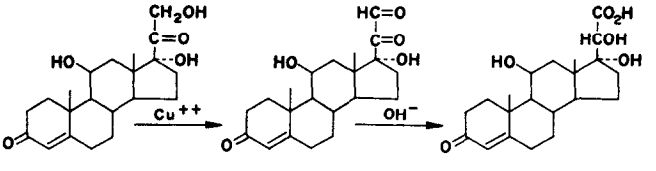
In the earliest report of acid formation, Picha *et al.* [59] in 1952 isolated 3-oxo-4-androstene-17-carboxylic acid following perfusion of 11-deoxycorticosterone (DOC) through rat liver. The *in vitro* synthesis of etienic acids by the livers and adrenals of a number of animal species are summarized in table 3. The recovery of minute amounts of C_{20} acids along with many neutral steroids from the hog adrenal reported by Neher and Wettstein [60] may have been the consequence of artifact formation during the course of a complex isolation procedure. It is unlikely that this is true for the other isolations reported, and it must be concluded that the etienic acids are minor products of the *in vitro* metabolism of 17-deoxycorticosteroids.

Biosynthesis of 20-oxo-21-oic acids

A. *From corticosteroids.* In 1964, Schneider [32] demonstrated that guinea pig slices oxidized DOC to acidic metabolites. The major product was the etienic acid, isolated as the ester glucuronide. The α-oxo acid, 3,20-dioxo-4-pregnen-21-oic acid was also isolated from the same incubation. Schneider suggested that the etienic acid was derived from the oxo acid. Gerhards *et al.* [67] isolated 6-fluoro-3,20-di oxo-11β-hydroxy-16-α-methyl-1,4-pregnadien-21-oic acid from a patient given fluocortolone, demonstrating for the first time that steroidal oxo acids are formed by humans. It is probable that the excretion of steroidal oxo acids in humans is a rare event. Neither cortisol, 11-deoxycorticosterone nor corticosterone administered to patients by intravenous infusion was converted to detectable amounts of the corresponding urinary oxo acids [68, 69]. There are three possible explanations that could account for these observations. First, corticosteroids may not be oxidized to oxo acids. Second, oxo acids are formed and rapidly oxidized to other products. Third, steroid acids, like the homologous bile acids, are excreted *via* the biliary route. The first possibility cannot be tested directly, for to do this requires proving a negative. If, however, oxo acids administered to subjects intravenously are not metabolized further and are excreted unchanged, then the lack of oxo acid accumulation would imply that they are not formed from corticosteroids.

Labeled 11,17-dihydroxy-3,20-dioxo-4-pregnen-21-oic acid was chemically synthesized from [1, 2 ^{3}H]-cortisol [35] and administered to two patients. The principal products isolated from the urine were a relatively non polar acid and a highly polar water soluble acid. Neither metabolite was an oxo acid. Other radioactive products of the administered oxo acid were isolated but none corresponded to any known metabolite derived from cortisol. This evidence, though inferential, is entirely in accord with the assertion that cortisol is probably not catabolically degraded to oxo acids by humans.

B. *From progesterone.* The first evidence that progesterone can be metabolically converted to acidic steroids was shown in the rabbit [71]. The urinary



Scheme 4. Artifactual formation of cortoic acids.

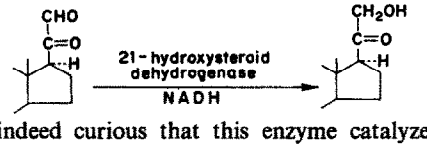
Table 3. *In vitro* biosynthesis of etienic acids

Acid	Parent steroid	Tissue	Experimental conditions	Reference
etienic acid	DOC	rat liver	perfusion	[59]
etienic acid	DOC	bovine adrenal		[61]
etienic acid	Unknown	hog adrenal	isolation	[60]
etienic acid	DOC	rat adrenal	incubate with quartered gland	[62]
etienic acid	DOC	hamster	liver homogenate	[66]
11β-hydroxy-etienic acid	DOC	guinea pig liver slices	*in vitro* incubation	[32]
11β,-18-dihydroxy-3-oxo-4-etienic acid	Unknown (probably 18 hydroxycorti-costerone)	hog adrenal	isolation	[63]
3-oxo-4-androstene-17β-carboxylic acid 20→18 lactone	DOC	bovine adrenal	perfusion	[64]
3-oxo-4-androstene-17β-carboxylic acid 20→18 lactone	Unknown	baboon	catheterization	[65]
11β-hydroxy-3-oxo-4-androstene-17β-carboxylic acid 20→18 lactone	DOC	bovine	perfusion	[64]

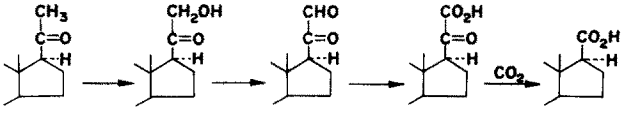
metabolites were tentatively identified as C-21-carboxy compounds [71, 72]. After chromic acid oxidation, 3,6,20-trioxo-5α-pregnan-21-oic acid was recovered [73]. It is likely that this is not the true metabolite, since oxidation with chromic acid could convert hydroxyl groups to carbonyl groups. Senciall and coworkers have suggested that progesterone is first hydroxylated to DOC which is the proximate intermediate [74]. In support of this proposition, they found that DOC as well as progesterone was converted to uncharacterized acids by rabbit liver fractions [75]. There were substantial species differences in the degree of conversion of progesterone to acid metabolites. Rabbit liver was most active, followed in decreasing order by guinea pig, hog, rat and human [76].

The suggestion of Schneider [32] that DOC is oxidized to 3-oxo-4-etienic acid through 3,20-dioxo-4-pregnen-21-oic acid has never been directly tested. There are observations in the older literature which could be explained by this sequence. When [21-^{14}C]-progesterone was administered to mice or rats, $^{14}CO_2$ was exhaled to the extent of 4–17% of the dose [77–80]. Unidentified acidic transformation products were isolated from the tissues [80]. The probable sequence of events is shown in scheme 5.

Hydroxylation of progesterone to DOC occurs *in vivo* in the adrenal and other tissues [81]. The conversion of the ketol side chain to the glyoxal side chain has never been directly demonstrated in living systems. Chemical oxidation of corticosteroids to keto aldehydes occurs easily and it might have been anticipated that a biological system catalyzing this oxidation would be present in tissues. This does not appear to be the case. Oxidation of corticosteroids to 21-dehydrocorticosteroids containing the ketoaldehyde side chain is catalyzed by cytochrome *c*, but the reaction occurs only above pH 8.5, under unfavorable concentrations of reactants, and at a rate too slow to be of physiological significance [82]. The corticosteroid–cytochrome *c* reaction may be a model for a more physiological system, but this possibility is entirely speculative. Evidence for formation of 21-dehydrocorticosteroids is lacking, perhaps because an NADH enzyme, 21-hydroxysteroid dehydrogenase [82–85], specifically reduces 21-dehydrocorticosteroids to corticosteroids. The reverse reaction does not occur to any significant extent:



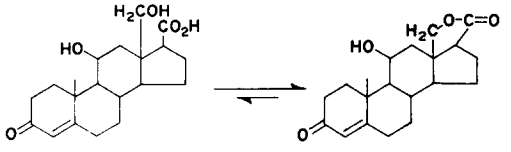
It is indeed curious that this enzyme catalyzes the reduction of a specific substrate which does not appear to be formed during corticosteroid metabolism. The limited substrate specificity of the enzyme suggests that it plays a role in steroid metabolism, but what that role is has yet to be demonstrated.



Scheme 5. Formation of 20-oxo acids from progesterone.

The oxidation of 21-dehydrocorticosteroids to 20-oxo, 21-oic acids proceeds readily, mediated by ketoaldehyde dehydrogenases of liver [86] and adrenal [87]. The adrenal enzyme is of broad specificity and oxidizes aldehydes from diverse origins. The liver enzyme is quite specific for ketoaldehydes, a fact which may reflect a specific function in the synthesis of keto acids from steroids as well as from methylglyoxal [88].

A. *Etienic acids.* A steroid resembling that isolated by Neher and Wettstein (table 3) was formed when DOC was incubated with quartered rat adrenals [62]. The favorable stereochemistry of the side chains permits the steroid acid to lactonize readily. The steroid acid was isolated in this form.



Levy *et al.* [63] perfused 11-deoxycorticosterone through bovine adrenal glands and isolated 3-oxo-4-androsten-17β-carboxylic acid 20 → 18 lactone and its 11β analogue, confirming that the cow adrenal is capable of effecting this transformation, though the acid represented less than 1% of the total perfused steroid [90]. A 20 → 18 lactone was isolated from the adrenal vein blood of the baboon (*Papio hamadryas*). It was secreted at a concentration of 3 μg per 100 ml of adrenal venous blood [67].

B. *18-carboxylic acids.* A key step in the biosynthesis of aldosterone results in the oxidation of the angular C-18 methyl group to an aldehyde [91]. The 18-aldehyde is subject to further oxidation within the adrenal gland. Neher and Wettstein [92] isolated 20,20,21-trihydroxy-3-oxo-4-pregnen-18-oic acid 18 → 20 lactone from bovine adrenal glands. The acid was formed along with small amounts of progesterone-18-oic acid when 18-oxo-progesterone was incubated with adrenal slices from hog, sheep, rat, rabbit or bullfrog. Introduction of an 11 hydroxy group resulted in the formation of the 11,18-hemiacetal and protected the 18 aldehyde against 18-oic acid formation [93]. Whether aldosterone-18-oic acid serves any biological function is uncertain [93, 94]. Recent evidence suggests that fetal tissues and placenta of the guinea pig oxidize aldosterone to carboxylic acid metabolites. The location of the acidic group on the molecule has not been established [95].

Biosynthesis of 20-hydroxy-21-oic acids

A. In vivo *studies in humans.*

1. *Acids derived from cortisol.* A significant fraction of the cortisol metabolites in urine remains in the residual urine after hydrolysis with glucuronidase and extraction with ether, or is recovered in the alkali wash of the organic extract. From their solubility properties it seemed likely that the latter fraction contained acidic metabolites. Their nature remained conjectural until the development of Amberlite XAD-2 as an adsorbant for extracting steroids from aqueous media [96]. The steroid acids were quantitatively adsorbed by the resin, eluted with methanol, esterified with diazomethane, and subjected to a series of chromatographic separations and chemical characterizations. In this way seven acids were identified (97–99). The 17-hydroxy-21-oic acids, collectively designated "cortoic acids", together constitute 5–25% of the urinary metabolites of cortisol [97]. Cortolonic acid is one of the four most abundant metabolites of cortisol. Table 4 summarizes data on the excretion of the cortoic acids in selected subjects. In most instances 20α cortolonic acid excretion exceeds that of the 20β epimer. This is reversed in anorexia nervosa. There is more than 3 times as much 20α cortolic acid in the urine as 20β cortolic acid. In general more cortolonic acids are formed than cortolic acids. In contrast, the 17-deoxycortolonic acids (see below, p. 905) are excreted at about 1–2% of the dose independent of clinical status.

2. *Mechanism of formation of the cortoic acids.* Although some facets remain to be clarified a series of experiments with radioactive cortisol and suspected intermediates has established the broad outlines of the transformation of cortisol to cortoic acids [97, 99–102]. Any mechanism which purports to account for the transformation must fulfill the following requirements: (1) One half of the ^{3}H at C-21 is

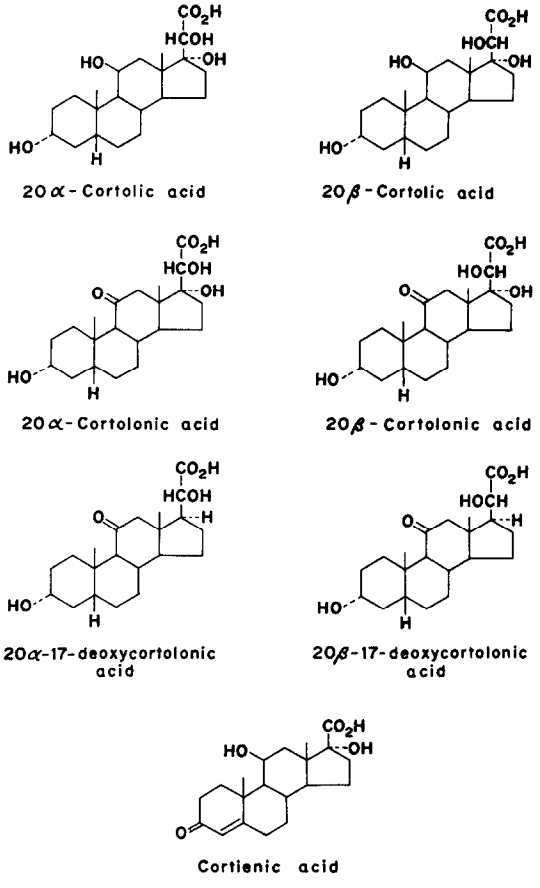


Fig. 1. Structures of acidic metabolites from human urine.

Table 4. Formation of cortoic acids

	Subject	17-deoxy cortolonic acid	cortolonic acid β	cortolonic acid α	cortolic acid β	cortolic acid α
Ca prostate	A	1.6*	2.4	3.3	0.3	1.5
	B	1.4	2.4	2.3	0.7	2.5
	C	0.7	1.0	0.8	0.8	2.5
Ca breast	A	0.9	1.7	1.8	0.3	0.7
	B	1.0	1.6	2.5	0.1	0.7
	C	0.6	1.7	2.3	0.1	0.9
Coronary disease	A	1.8	2.2	1.7	0.9	0.2
	B	1.6	3.0	4.0	0.5	1.7
	C	1.8	6.3	9.7	1.0	2.7
	D	1.2	2.4	3.6	0.3	1.3
	E	1.5	1.4	1.9	0.4	1.6
Normals	A	—	2.8	4.1	0.2	1.2
	B	—	2.0	3.5	0.2	1.7
Anorexia nervosa	A	—	1.9	1.2	0.5	1.0
	B	1.0	0.7	0.4	0.5	1.8
	C	1.0	1.0	0.5	0.5	2.3

* Values are per cent of labeled dose recovered as the acidic metabolite.

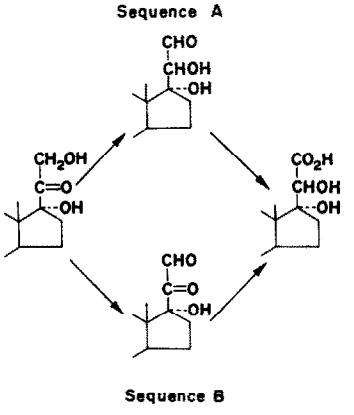


Fig. 2. Alternative metabolic sequences in the transformation of cortisol to cortoic acids.

lost and the remainder is specifically transferred to the C-20 position [97]; (2) 20-dihydrosteroids (Reichstein's E or U) are not transformed to cortoic acids [101]; (3) Tetrahydrosteroids are intermediates in the formation of cortoic acids [102]; (4) 21-dehydrocortisol, previously suggested as a possible intermediate, is not on the main route to the cortoic acids [98]; (5) Pathways exist for the hexahydrometabolites which are completely unrelated to the route leading to the tetrahydro metabolites [100] and (6) isosteroids are converted to cortoic acids, while simultaneously yielding both cortolone epimers [100].

Table 5. Retention of ^3H in acidic metabolites of [21-^3H]-cortisol

	Patient 1*	Patient 2*
Total acid	59	58
Cortolonic acid	57	59
Periodate cleavage of side chain	0	0
Cortolic acid	52	—
Periodate cleavage of side chain	0	

* Values are expressed as (^3H/mol product recovered/^3H mol steroid administered) × 100.

Requirement (1) is based on our studies with [4-^{14}C; 21^3H]-cortisol in which we found that the cortoic acids derived from this precursor retained about 1/2 of the initial tritium and that this label is specifically transferred to the C-20 position by an intramolecular process which is preceded or followed by a second oxidation at C-21 (see Fig. 2 and table 5). Moreover the isotope ratio of the tetrahydro metabolites showed that no loss of ^3H occurred during the conventional metabolic transformations (table 6).

Table 6. Retention of ^3H in neutral metabolites of [4-^{14}C; 21-^3H]cortisol

	Steroid administered [4-^{14}C; 21α-^3H]cortisol	Steroid administered [4-^{14}C; 21β-^3H]cortisol
Urinary metabolites	% of initial tritium content	
cortol	88	104
β-cortol	163	105
cortolone	95	100
β-cortolone	114	99
THF	100	128
THE	108	128
allo THF	95	114
allo THE	105	115
Recovered cortisol		108

Table 7. Relative formation of cortoic acids from ring A reduced metabolites of cortisol

	[1,2^{3}H]-THE + [4-^{14}C]F R*		[1,2^{3}H]-THF + [4-^{14}C]F R*	
Subject	A	B	A	C
β cortolone	2.81†	2.02†	0.15	0.07
THE	1.92†	1.72†	0.08	0.15
cortolonic acid	1.74†	1.89†	0.09	0.14
β cortolonic acid	1.52†	1.86†	0.12	0.22
β cortol	0.62	—	3.68†	1.25†
THF	0.53	0.54	3.99†	3.15†
β cortolic acid	0.64	0.58	1.19	2.91†
cortolic acid	0.48	0.90	3.96†	2.41†
cortolone	0.50	0.44	0.02	0.04
cortol	0.34		0.62	0.88

* Compound ^{3}H/^{14}C./.dose ^{3}H/^{14}C.
† Metabolites preferentially formed from the [1,2^{3}H]-tetrahydroprecursors.

The requirement for a 20-carbonyl is derived from studies on the metabolism of Reichstein's E or U from which acid formation was found to be negligible [101]. These experiments showed that oxidation at C-21 of a 20-hydroxy steroid does not occur, thus satisfying requirement [2].

Requirement (3) is supported by the observation that the related cortoic acids after the administration of labelled THF or THE had isotope ratios comparable to that of the recovered THF and THE. This suggests that these tetrahydro compounds are major if not obligatory intermediates in the formation of cortoic acids and that ring A reduction precedes side chain oxidation. These values are indicated by † in table 7. In the scheme which we originally proposed [97], reproduced in Fig. 2, two alternative pathways for the transformation of cortisol to the cortoic acids proceeding by way of isosteroids (sequence A) or 21-dehydro steroids (sequence B) were offered. Subsequent studies [98] have shown that route B via the 21-dehydrocortisol is not on the main route since it was a very poor precursor of the cortoic acids. However, it was preferentially converted to cortienic acid and α cortolone. These results are illustrated in table 8 and support requirement (4).

3. *The existence of a "long loop" pathway* leading to the hexahydro metabolites is suggested by two lines of evidence: (1) Comparison of the isotope ratios of the hexahydro metabolites derived from [4-^{14}C; 21^{3}H]-cortisol with those of the tetrahydro metabolites suggests that a pathway exists from the side chain in the course of the transformation (table 6). Because of the presence of the 21-^{3}H in the side chain, oxidation is diminished relative to the 21-^{3}H species and relatively more of the tritiated form is available for ring A reduction to neutral metabolites. As a consequence of this selectivity the neutral metabolites are selectively enriched in the ^{3}H isotope. This is reflected in the elevated ^{3}H/^{14}C ratios in table 6. (2) 21-dehydrocortisol serves as a preferential precursor of α-cortolone and α-cortol when compared with cortisol given simultaneously [98], but was less effectively converted to other neutral metabolies. We have attempted to rationalize these observations by pro-

Table 8. "Normalized" isotope ratio* of radioactivity from 21-dehydrocortisol in various metabolites

	Subject				
Metabolite	GA	GS	GE	CE	SY
Cortisol	1.13	0.84	1.00	†	†
THF	1.05	1.05	1.03	0.93	1.03
THE	0.95	0.95	0.97	1.07	0.97
β-Cortol	1.24	1.66	1.05	1.29	1.51
β-Cortolone	1.02	1.37	1.28	0.86	1.00
Cortol	3.13	4.19	3.44	6.93	6.70
Cortolone	2.78	3.35	2.55	3.79	2.92
Cortienic acid	‡	‡	‡	∝	∞
Cortolonic acid	(lost)	(lost)	0.50	†	1.13
β-Cortolonic acid	(lost)	(lost)	0.56	0.93	0.97
Cortolic acid	(lost)	(lost)	(lost)	‡	2.81
β-Cortolic acid	(lost)	(lost)	(lost)	†	7.18

Average isotope ratio of THF and THE = 1.00.
* In these ratios 21-dehydrocortisol is the numerator.
† Insufficient counts.
‡ ^{3}H-label lost during formation; isotope ratio meaningless.

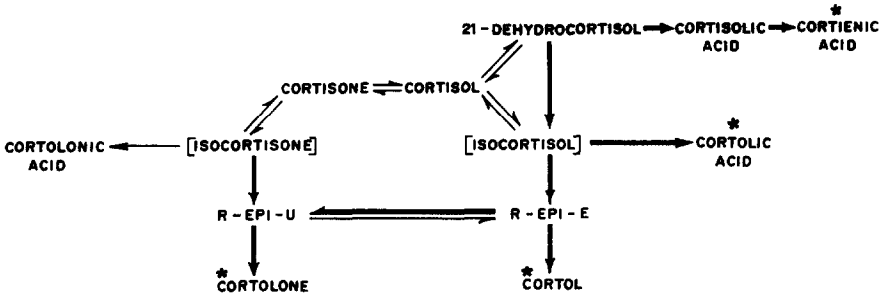
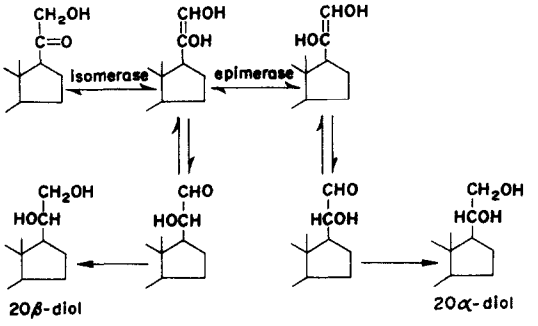


Fig. 3. Alternative pathways in the metabolism of cortisol. Bold-faced arrows delineate the "long-loop" sequence.

posing a metabolic sequence which bypasses the tetrahydro compounds. This hypothetical alternative route, which we have designated the "long loop" pathway (Fig. 3) invokes the intercession of either or both of the two new intermediates, 21-dehydrocortisol and isocortisol.

To test the sequence A in Fig. 2, the metabolism of 20β [1,2-^{3}H]-isocortisol and 20β [1,2-^{3}H]-isotetrahydrocortisone mixed with [4-^{14}C]-cortisol was studied in normal subjects. Normalized isotope ratios of the various metabolites are summarized in table 9. Isocortisol was preferentially converted to the cortols, cortolones and 20β cortolic acid while iso THE gave the cortols, cortolones and 20β cortolonic acid. Isomerization of the isosteroid side chain back to the ketol structure occurred to only a minor extent as shown by the low isotope ratios for the tetrahydro steroids. In both cases only the stereospecific cortoic acids were isolated in significant amounts. However both epimeric cortolones were isolated. The identity of the 20α epimer was confirmed by reverse isotope dilution assay. Since C-20 oxidation can be excluded in light of the miniscule conversion to THE or THF some form of epimerization without actual loss of tritium must play a role in the rearrangements that yield 20α cortolone. An epimerization mechanism which could account for the results obtained is offered in scheme 6. Further confirmation was obtained using [4^{14}C; 20^{3}H]-20β isocortisol as precursor; again both cortolone epimers were obtained. The 20α epimer still



Scheme 6. Mechanism of isomerization of isosteroids.

contained ^{3}H, confirming that epimerization had occurred without oxidation.

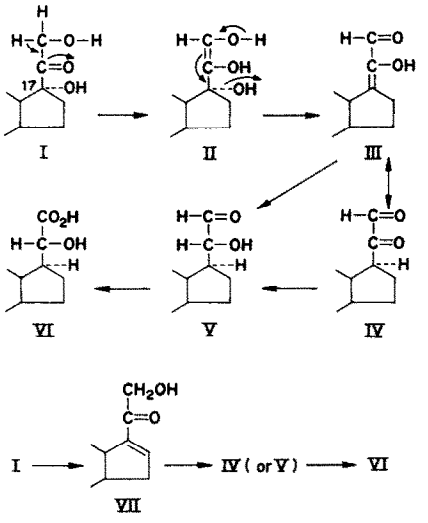
4. *Biological 17 deoxylation.* During the isolation of the cortolonic and cortolic acids a relatively nonpolar ester fraction was isolated and found to consist principally of two 17 deoxycortolonic acids: 3α,20α-dihydroxy,-11-oxo-pregnan-21-oic acid and its 20β epimer (Fig. 1). The structure of both acids was established by nuclear magnetic resonance, mass spectral analysis, reverse isotope dilution [99], and by comparison with authentic samples that had been rigorously characterized by Mattox and coworkers [103]. High pressure liquid chromatographic analyses of the labeled cortisol given to the subjects established the absence of sufficient corticosterone to account for the formation of these acids. Moreover, studies on the

Table 9. Relative isotope ratio of metabolites of isosteroids

	[1,2-^{3}H]-20-β-iso cortisol + [4-^{14}C]-F	[1,2-^{3}H]-20-β iso THE + [4-^{14}C]-cortisol
Dose	1	1
cortol α	0.71	—
cortol β	1.74	0.62
cortolone α	0.54	2.33
cortolone β	0.89	2.66
THF	0.006	0.029
THE	0.006	0.20
20α-cortolonic acid	0.042	0.078
20β cortolonic acid	0.084	0.90
20α cortolic acid	0.026	0.036
20β cortolic acid	0.44	0.34

Relative isotope ratio = (^{3}H/^{14}C Metabolite) (^{3}H/^{14}C Dose.)

metabolism of corticosterone failed to detect the formation of these compounds. The isolation of the 17-deoxy compounds represents the first demonstrated occurrence of 17 deoxylation in the metabolism of cortisol in humans. A plausible mechanism for the formation of these compounds is depicted in scheme 7.



Scheme 7. Proposed pathways for the formation of 17-deoxycarboxylic acids from cortisol.

5. In vivo *metabolism of [4-^{14}C; 21-^{3}H]-11-deoxycorticosterone (DOC) in man.* The oxidation of [4-^{14}C; 21-^{3}H] DOC at C-21 ranged from 7–9% of the administered steroid as measured from the tritium content of the body water. However, acid formation was minimal (0.5–2.0%). The isotope ratio of the recovered 21-hydroxypregnanolone was essentially identical to that of the dose, indicating the absence of an oxidation–reduction cycle at C-21 resulting in the loss of tritium during ring A reduction. However a decreased isotope ratio was observed in the smaller pregnanetriol fraction suggesting the possibility of a "long loop" in the side chain metabolism of DOC similar to that which we observed in cortisol metabolism. The low conversion of DOC to acids by humans was first observed by Senciall *et al.* [76].

B. In vitro *studies*

1. *Metabolism of [21-^{3}H]-corticosteroids.* The transformation of cortisol to carboxylic acids undoubtedly proceeds through a number of intermediates. Our ability to propose a sequence from cortisol to cortoic acids is largely based on our ability to follow the fate of the 21-tritiated side chain. The assumption which led to the synthesis of the tritiated corticosteroid side chain was that loss of hydrogen isotope should be a measure of the extent of C-21 oxidation of the side chain. It was recognized that carbon-21 represented a prochiral center (Fig. 4A) and that chemical reduction of 21-dehydrocortisol (Fig. 4B) with sodium borotritiide would create a mixture of

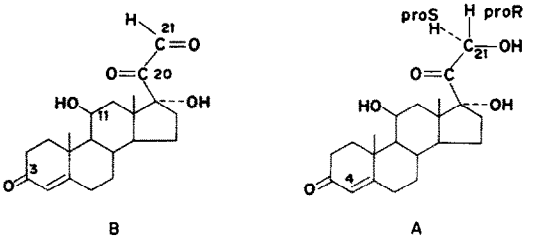


Fig. 4. Stereochemistry of the cortisol side chain.

two enantiomeric forms of the steroid: [21-S-21-^{3}H]-cortisol and [21-R-21-^{3}H] cortisol [104–106]. Although each epimer can be prepared independently [104, 105] we have performed most of our work with the chemically synthesized 21-tritiated corticosteroids in which the R and S forms are present in a ratio of approximately 4:6. Injection of [21-^{3}H]-cortisol or [21-^{3}H]-deoxycorticosterone into hamsters resulted in the appearance of tritium in all tissues as water [107]. Homogenates of tissues catalyzed the reaction with either [21-^{3}H]-cortisol or [21-^{3}H]-deoxycorticosterone, though the latter was by far the more active substrate (table 10). The most effective tissue was liver.

In hamster liver, an enzyme complex of mass 400,000 daltons is responsible for the transfer of tritium from steroid to water. The complex also catalyzes the oxidation of DOC to 3,20-dioxo-4-pregnen-21-oic acid, and to both 20-epimers of 20-hydroxy-3-oxo-4-pregnen-21-oic acid [108]. A similar enzyme is found in human liver [109]. As was observed with cortisol *in vivo* [98], the 21-dehydro form of the steroid is not an intermediate in any of these transformations. The enzyme does not contain, nor does it require, pyridine nucleotides which are obligatory for the 21-hydroxysteroid dehydrogenases (85–88) and aldehyde dehydrogenase activity [110]. Surprisingly, the enzyme-catalyzed loss of tritium from [21-^{3}H]-DOC was much greater than that accounted for by the net acid formed. Much of the tritium loss occurred with retention of the ketol side chain structure as illustrated below:

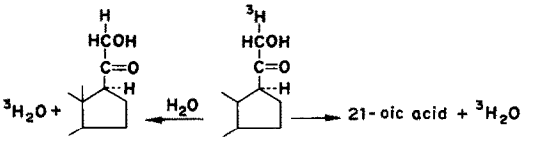


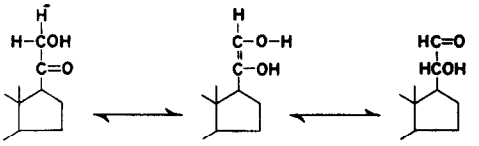
Table 10. Effects of some tissues *in vitro* on release of tritium from [21-^{3}H]-cortisol and [21-^{3}H]-DOC*

Tissue	[21-^{3}H]-Cortisol	[21-^{3}H]-DOC
Liver	5.65	21.43
Kidney	1.18	11.38
Adrenal	0.34	1.69
Brain	0.20	0.29

* Steroids (10 nmol, 0.5 μCi) in 25 μl of ethanol were incubated for 2 h at 37° with 2 ml of 12,000 *g* supernatant fractions of 25% (w/v) tissue homogenates prepared in 0.01 M potassium phosphate buffer. Reaction was stopped by freezing. Values represent per cent of total added radioactivity converted to water.

DOC recovered after 24 h of incubation with the enzyme had lost 97% of the tritium but was otherwise unaltered.

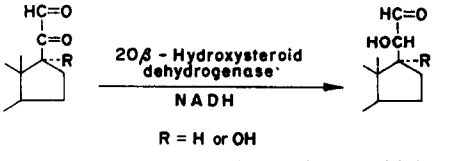
The loss of tritium from 21-[^{3}H]-ketol is probably due to an enzyme-catalyzed enolization of the side chain. The enol intermediate is, in its turn, in equilibrium with the iso-steroid, as shown below.



A number of observations support this mechanism (a) [4-^{14}C; 21-^{3}H] DOC is completely detritiated to [4-^{14}C]-DOC and ^3H_2O with the enzyme within 24 h; (b) unlabeled DOC incubated with ^3H_2O was converted to [21-^{3}H]-DOC; (c) [21-^{3}H] DOC, previously reduced to [21-^{3}H]-20β, 21-dihydroxy-4-pregnen-3-one was not detritiated [111].

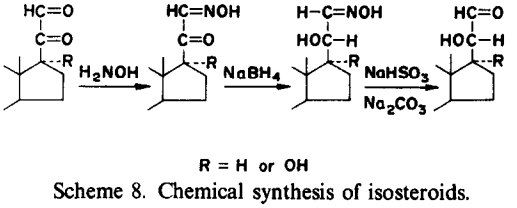
Synthesis of iso-corticosteroids

In order to test the role of isocorticosteroids in the above scheme and in the metabolic studies with human subjects, synthetic and enzymatic procedures of synthesis were developed. The enzymatic procedure is illustrated below.



Reduction of the 21-dehydrocorticosteroid by the hydroxysteroid dehydrogenase of *Streptomyces hydrogenans* is rapid and virtually quantitative. In fact 21-dehydrosteroids are the most reactive substrates of this enzyme [112].

The chemical method also starts with the 21 dehydrosteroid and follows the sequence outlined in scheme 8 [113]. In both enzymatic and chemical procedures, the 20β-epimer is the favored isomer.



Scheme 8. Chemical synthesis of isosteroids.

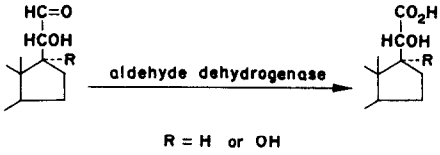
Enzymatic isomerization of isoDOC to DOC

The "detritiating" enzyme rapidly converts isoDOC to DOC. The rearrangement of [20-^{3}H]-isoDOC (prepared with sodium borotritiide as in scheme 8, was achieved with the transfer of tritium to water. The product, DOC, was recovered without label. IsoDOC could not be isolated when corticosteroid was incubated with enzyme. It is most probable that both isocorticosteroids and corticosteroids are in common equilibrium with the enediol although the ketol is greatly favored. The "ditritiating" enzyme may therefore be tentatively identified as an isomerase [114].

Synthesis of steroidal hydroxy acids from isocorticosteroids

It was pointed out above that the isomerase catalyzed the oxidation of DOC to the corresponding 21-oic acids. Over a period of several days of storage in the cold, the ability of the enzyme to catalyze acid formation was lost, while the isomerase activity remained unimpaired [115].

These observations suggest that the acid forming enzymes are unstable, and are therefore not an obligatory part of the detritiation process. If the unstable enzymes are aldehyde dehydrogenases, a straightforward route to the 20-hydroxy acids can be proposed:



Two aldehyde dehydrogenases, designated F-I and F-II were isolated from liver, using techniques described by Eckfeld *et al.* [116]. These enzymes oxidized isocorticosteroids with sharply differing degrees of specificity. The results are summarized in table 11.

The 21-dehydrosteroids were poor substrates for either enzyme. Ketoaldehydes, if they participate in the oxidative metabolism of corticosteroids, are probably converted preferentially to keto acids by ketoaldehyde dehydrogenase [86, 87] and subsequently decarboxylated oxidatively to etienic acids [97]. The F-I enzyme has no effect on isoDOC but rapidly oxidized isoF to its corresponding hydroxy acid. In contrast, isoDOC is oxidized to 20β-hydroxy-3-oxo-4-pregnen-21-oic acid by the F II enzyme, but isoF is not.

The specificity of the aldehyde dehydrogenases for the isosteroid side chain provides a test for the detritiase-catalyzed isomerization of corticosteroid to isocorticosteroid. Simultanous incubation of DOC with isomerase and dehydrogenase F-II increased acid formation consistent with a pathway depicted below:

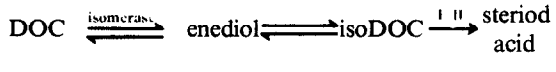


Table 11. Substrate specificity of aldehyde dehydrogenases

Steriod	FI	FII
	nmol NADH formed/ min/mg · protein	
iso DOC	1	18
iso F	45	1
21 dehydro DOC	7	2
21 dehydro F	11	0
DOC	0	0
F	0	0

SUMMARY AND CONCLUSIONS

The body of work reviewed above demonstrates that steroid acids are not to be regarded as artifacts but as normal enzymatically synthesized hormonal metabolites. Their formation from glucocorticoids parallels the formation of saccharinic acids from sugars. The enzymes which carry out these transformations appear to be ubiquitous and present in a wide variety of species and tissues, although in general the highest activity is present in the liver in all of the species tested. Since appreciable quantities of these acids are formed and are capable of binding to a variety of biological substances their possible role in promoting the biological action of corticoids remains to be explored. The work of Laurents *et al.* [37] has already established the topical activity of steroidal ketoacid esters without any central activity. As more of these compounds are identified and synthesized the systematic study of the biological properties of these compounds will be possible. With the development of improved analytical techniques the routine analyses of these acidic metabolites will permit a clearer definition of their role in health and disease.

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