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Role of a Disordered Steroid Metabolome in the Elucidation of Sterol and Steroid Biosynthesis

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Abstract

In 1937 Butler and Marrian found large amounts of the steroid pregnanetriol in urine from a patient with the adrenogenital syndrome, a virilizing condition known to be caused by compromised adrenal secretion even in this pre-cortisol era. This introduced the concept of the study of altered excretion of metabolites as an in vivo tool for understanding sterol and steroid biosynthesis. This approach is still viable and has experienced renewed significance as the field of metabolomics. From the first cyclized sterol lanosterol to the most downstream product estradiol, there are probably greater than 30 steps. Based on a distinctive metabolome clinical disorders have now been attributed to about seven post-squalene cholesterol (C) biosynthetic steps and around 15 en-route to steroid hormones or needed for further metabolism of such hormones. Forty years ago it was widely perceived that the principal steroid biosynthetic defects were known but interest rekindled as novel metabolomes were documented. In his career this investigator has been involved in the study of many steroid disorders, the two most recent being P450 oxidoreductase deficiency and apparent cortisone reductase deficiency. These are of interest as they are due not to mutations in the primary catalytic enzymes of steroidogenesis but in ancillary enzymes needed for co-factor oxido-reduction A third focus of this researcher is Smith-Lemli-Opitz syndrome (SLOS), a cholesterol synthesis disorder caused by 7-dehydrocholesterol reductase mutations. The late George Schroepfer, in whose honor this article has been written, contributed greatly to defining the sterol metabolome of this condition. Defining the cause of clinically severe disorders can lead to improved treatment options. We are now involved in murine gene therapy studies for SLOS which, if successful could in the future offer an alternative therapy for this severe condition.

Keywords

Cholesterol synthesis; Steroid biosynthesis; Steroid metabolism

Introduction

The class of natural lipids we call steroids all have in common the perhydrocyclopentanophenanthrene structure, the core ring unit produced by the cyclization of oxido-squalene. This structure is used as a backbone of a remarkable suite of essential biochemicals with very diverse tasks, from cholesterol with its global responsibilities (membrane and neuron structure, steroid precursor, etc.), to bile acids with their cell

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signaling and detergent qualities, to skeletally essential vitamin D, to the gonadal steroids, and to the adrenal hormones that regulate our intermediary metabolism and salt and water balance. While all these vital components are derived from acetate, the first intermediate with the fused ring-structure is the C-30 sterol lanosterol and from that precursor there are likely more than 30 steps leading to the most distal steroid products such as estradiol and the terminal metabolites of cortisol.

Following the confirmation of the structure of cholesterol in 1932, defining the enzymes and other factors that are needed to progress from acetate to the most distal metabolites has taken more than 70 years and remains a work in progress. The study of the hormonal biosynthetic process started in the 1940s with the characterization of individual compounds within glandular tissues, mostly obtained from domestic animals. Radioactively labeled versions of these compounds (and their possible precursors) were synthesized and utilized in tissue perfusion studies or incubated with cellular fractions. Step by step a hypothetical biosynthetic scheme for cortisol evolved but its veracity proved difficult to confirm without in vivo experimentation.

The field that we now term metabolomics has been key to verifying the biosynthetic pathway leading to cholesterol, the hormonal steroids, and their metabolites. Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular and bodily processes leave behind—specifically the study of small-molecule metabolite profiles. In reality, relatively little information can be obtained solely by studying the blood or excretory metabolic profile of healthy individuals, it is those with biosynthetic disorders (inborn errors) that provide the necessary keys to understanding synthetic sequences as well as defining the biological role and importance of the biosynthetic end product.

In describing the elucidation of the steroid biosynthetic pathway through metabolomics it is useful to focus on cholesterol as the mid-point and discuss the pre-cholesterol (upstream) and post-cholesterol (downstream) processes separately. This author has been involved in research interpreting metabolic profiles of each process and three personal examples will be highlighted, two “Downstream” and one “Upstream”.

Downstream from Cholesterol

Cortisone was the first major adrenal steroid hormone to be identified and as soon as sufficient was synthesized for pharmacological testing it was found to ameliorate the symptoms of arthritis [1]. It was designated the “wonderdrug” of 1949 and for this discovery Kendall, Hench and Reichstein received the 1950 Nobel prize. A little later it was realized that a cortisone precursor, hydrocortisone (or cortisol) was in fact the true adrenally synthesized glucocorticoid. Following the identification of the major glucocorticoids there was a dramatic acceleration of studies of the biosynthesis of these hormones mostly carried out by groups in New York and the Worcester foundation in Massachusetts.

The initial breakthroughs in understanding adrenal steroid biosynthesis were uncovered independently of metabolomics. These studies were conducted in vitro, and generally consisted of isolating steroids from bovine adrenal tissues obtained from abattoirs, perfusions of such glands with individual labeled steroids, and finally metabolism studies utilizing sub-cellular fractions. By the mid 1950s there was already evidence that the likely synthetic route was pregnenolone oxidation to progesterone, sequential 17-hydroxylation, 21-hydroxylation and 11β-hydroxylation, finally producing cortisol. It was postulated at the time that cholesterol was the precursor of pregnenolone but there was no direct evidence to prove it. Proposing this biosynthetic pathway was a remarkable achievement based on the methodologies available at that time, and the story is well documented in the 1954 review by Hechter and Pincus [2].

The synthetic scheme, while attractive, still represented a series of deductions based primarily upon the ability of substrates to be converted. What was lacking was information derived from in vivo metabolism, and that was soon supplied by what we now refer to as metabolomics. It was the study of the steroidal products produced by patients with inborn errors of steroid biosynthesis that finally verified the postulated biosynthetic sequence. What follows is a brief history of these discoveries which are readily followed by viewing Fig. 1. For the benefit of readers less familiar with the numbering system of steroids, this is detailed in the structure of cholesterol illustrated.

Interestingly, the first metabolomic discovery pre-dated the actual discovery of the primary glucocorticoids (cortisol/cortisone) by a decade. This was the 1937 characterization of pregnanetriol in the urine of patients with the adrenogenital syndrome by Butler and Marrian [3]. The adrenogenital syndrome is a condition in which adrenal insufficiency is associated with masculinization of female fetuses. In their early paper they do not (and cannot) speculate on the reason for this overproduction as the terminal product of the adrenal steroidogenic pathway had yet to be identified. By today's standards the achievement of metabolite characterization was challenging. A great volume of urine was processed and in these pre-chromatography days identification comprised of nothing more than differential extraction, targeted chemical reactions and crystallization to constant melting point.

It took many more years before a clearer understanding of the significance of the pregnanetriol discovery was obtained. Eberlein and Bongiovanni in 1955 re-identified pregnanetriol as the major urinary metabolite in the adrenogenital syndrome (now referred to as congenital adrenal hyperplasia, CAH), but by then it was possible to connect this to cortisol deficiency [4]. Having deduced that pregnanetriol was a metabolite of 17-hydroxyprogesterone (17OHP) these investigators proposed a block between 17OHP and cortisol and suggested that 21-hydroxylase was deficient. It should be emphasized that in this era only urine analysis was practical, widespread use of sensitive immunoassay procedures that allowed serum hormone measurement were still two decades away.

Following this report on 21-hydroxylase deficiency the same investigators described a hypertensive form of CAH and the metabolic profile was defined by a high excretion of tetrahydro-11-deoxycortisol, pointing to a deficiency of 11β-hydroxylase [5]. Step by step these metabolite identifications were verifying the biosynthetic pathways proposed by the in vitro experimenters. In 1962 Bongoivanni described CAH due to 3β-hydroxysteroid dehydrogenase deficiency (3βHSD) hallmarked by high excretion of Δ^5 steroids such as pregnenetriol and DHEA [6]. In 1955, Prader and Gurtner [7] reported on neonates with a fatal condition marked not by the production and excretion of distinctive metabolites, but by the apparent absence of steroid hormone metabolites. At post-mortem examination the adrenal glands of these infants were found to be filled with cholesterol and its esters suggesting a deficiency in the conversion of cholesterol to the first C21 steroid, pregnenolone. This was attributed at the time to cholesterol side-chain cleavage (desmolase) deficiency, but now it is known to be due to a cholesterol transport protein deficiency. This condition was termed lipoid adrenal hyperplasia (LAH). The final example of disorders causing cortisol insufficiency was the description in 1966 of a second hypertensive form of CAH, 17-hydroxylase deficiency by Biglieri and co-workers [8]. The metabolome of patients with this disorder was based on the overproduction of 17-deoxycorticoids such as deoxycorticosterone (DOC) and corticosterone.

By the mid 1960s a basic understanding of the biosynthesis of adrenal steroids had pretty much been established, at least from the aspect of defining the primary transformations and the order in which they occur. These achievements have been summarized in Fig. 1.

Analyzing the Metabolome: The Author's Introduction

In metabolomic studies of urinary steroids the compounds quantified are not secreted hormones or precursors, but rather end products produced by renal or hepatic metabolism. For example, the 3-oxo-4-ene structure of a typical hormone or precursor is reduced to form a 3α-hydroxysteroid with either a 5α or 5β hydrogen. These metabolites are referred to as "tetrahydro-metabolites", for example *tetrahydro*cortisol (Fig. 1). A carbonyl group at position 20 may be converted to a hydroxyl and commonly hydroxyl groups at positions 17β and 11β are converted to carbonyls. Thus, it seems that whatever functional moiety an active hormone has is to a great extent oxidized or reduced to its "opposite" prior to excretion. Finally, essentially all metabolites with a 3α-hydroxyl are excreted as glucuronide conjugates and those with 3β-hydroxy-5-ene groups are excreted as sulfates.

While the earliest characterizations within the urinary steroid metabolome were made without the benefit of chromatography, by the 1950s paper chromatography came into own thanks to the painstaking developments by Ian Bush [9] and Zaffaroni [10]. The former in particular standardized methodologies to the extent that steroid metabolic profiles as we know them could be used clinically [11]. All of the first batch of steroidogenic disorder discoveries were documented by this technique.

My first introduction to the field was my undergraduate thesis entitled "cholesterol and cholesterol esters in the bovine corpus luteum during formation and regression", a study completed in 1964. My supervisor was the noted cholesterol researcher Robert Cook who published one of the first monographs on the subject [12]. My perseverance in the field was tested severely as I had to spend untold distressing hours at the slaughterhouse in Dundee, Scotland, collecting tissue samples. I started work on steroid metabolomics at the end of the golden age for steroid biochemists when the fundamental biosynthetic route to cortisol had largely been confirmed and primary defects described. I was employed in 1964 in the Dept of Clinical Chemistry, the University of Edinburgh to study the steroid metabolome of newborn infants and determine how it differed qualitatively and quantitatively from later life. My earliest experiments were conducted using paper chromatography for the identification and measurement of the steroids excreted by human newborns but soon realized that the rapidly developing TLC methodologies were much more effective and sensitive. By using this technique with three color reactions we could separate and quantify all the major excreted steroids of humans [13]. Although the methodology was effective it was soon overtaken by gas chromatography and the combined GC/MS technique. In 1966 I was privileged to use the first commercial GC/MS instrument purchased in the UK to identify some of the novel newborn steroids isolated by TLC [14]. This instrument belonged to Professor Charles Brooks of Glasgow University, a seminal steroid researcher of this era.

For post-doc training I went to the Karolinska institute in Stockholm to work with Professor Jan Sjövall, a previous recipient of the Schroepfer medal, to get a thorough education in this new field. Jan was a co-developer with Ragner Ryhage of the first practical GC/MS instrument which was marketed as the LKB 9000. By 1968, GC/MS was truly a metabolomic tool in the steroid field. By use of the combination of methyloxime protection of carbonyl groups and trimethylsilylation of hydroxyls, all neutral human steroids, whatever their origin and complexity could be analyzed in a single chromatogram. In 1970, I returned to the MRC in London and continued my studies on steroid biosynthetic disorders using GC/MS. Our group was at the forefront in introducing capillary column methodology in producing steroid profiles [15], in the use of GC/MS in athletic doping control [16] and in improving steroid recovery techniques by the first use of SPE (solid phase extraction) with cartridges [17].

In 1979 I moved to Berkeley, California to assist Dr Al Burlingame establish a national resource for biomedical mass spectrometry. While there we made the first utilization of the new Fast Atom Bombardment technique to the analysis of steroid of clinical relevance. With my colleague Ken Straub we showed that single spectrum profiles of urinary steroid conjugates could be used to diagnose five disorders of steroid biosynthesis [18, 19]. This was the first practical analysis of intact conjugates because in previous studies by paper, TLC and GC/MS steroid conjugates had first to be hydrolyzed prior to analysis. This technique was overtaken by development of HPLC/MS, first using the thermospray interface [20] soon followed by electrospray ionization. However, close to 50 years after the introduction of GC/MS for analyzing the steroid metabolome, this technique is still preferred for this role [21]. HPLC/tandem MS now has extreme sensitivity and is ideally suited to the measurement of circulating hormones and precursors but finds challenging the separation and quantitation of the characteristic steroids found in a urinary extracts.

Discovery of the 6th Form of Congenital Adrenal Hyperplasia (CAH)

The description of the five classical forms of CAH seemed to signal an end to the first chapter in the story of steroid biochemistry. As they covered the needed transformations in the ACTH-regulated pathway from cholesterol to cortisol and served to validate the pathway deduced from the original ex vivo investigations. In the late 1970s one more unique urinary steroid metabolome was documented by the late Dr Meta Nielsen and myself. Meta from the Glostrup hospital in Copenhagen was second-to-none in her ability to accurately diagnose steroid disorders by TLC of urinary steroids but she still needed a collaborator to confirm steroid identities by GC and GC/MS. A distinctive metabolome was seen in two sisters with masculinized genitalia, one who had been brought up as a boy. The unique feature was that there was elevated excretion of the metabolites of 17α-hydroxyprogesterone (pregnanetriol and pregnanetriolone, indicative of 21-hydroxylase deficiency) as well as elevated excretion of corticosterone metabolites (tetrahydrocorticosterone and others), a feature suggesting 17-hydroxylase deficiency. We termed the condition “combined 17-and 21-hydroxylase deficiency” but unwisely we did not pursue this to publication.

It was several years before another example of this metabolome was seen and then it was a patient of Dr Ralph Peterson and Julianne Imperato-McGinley at Cornell Medical School in New York. These investigators had an active program studying isolated kindreds in the Dominican Republic with high incidence of ambiguous genitalia and I had a role in diagnosing these individuals by GC/MS analysis. Almost all DSD (Disorders of Sexual Development) individuals had a disorder not directly related to cortisol synthesis called 5α-reductase deficiency, but one young virilized girl had a steroid profile of combined 17- and 21-hydroxylase deficiency. This patient was studied in detail and a resulting publication became the index case of the disorder [22]. Many years were to pass before the true cause of the disorder was determined but during that period there were isolated reports of other cases where patients were described with features of 17- or 21-hydroxylase deficiency. With my colleague Ewa Malunowicz of Warsaw we reviewed several of these papers and questioned the authors original interpretations as they all seemed to have a metabolome suggesting combined 17-and 21-hydroxylase deficiency [23]. Interestingly, several patients of this type described in the 1990s were not initially investigated because of DSD but because their dominant phenotype was consistent with Antley-Bixler syndrome, a skeletal disorder with features including skull, limb and finger abnormalities.

Enough was known of the genetics of steroidogenesis to render simultaneous errors in both 17- and 21-hydroxylase genes unlikely, so the focus was directed to ancillary factors that are required for the function of both enzymes. The most important co-factor for both 17-hydroxylase and 21-hydroxylase is cytochrome P450 oxidoreductase (OR), the enzyme

responsible for regenerating the NADPH required for hydroxylase enzyme function. In 2003 my colleague Prof. Wiebke Arlt at Birmingham started to sequence this enzyme in a few patients (from various clinicians) which we had identified by metabolic profile and a year later several mutations had been found and we published a paper describing the "sixth" form of CAH, a condition caused by deficiency of P450 oxidoreductase [24]. Simultaneous studies on Antley-Bixler syndrome and P450 oxido-reductase deficiency were carried out by Professor Walter Miller's group in San Francisco and Dr Hisao Hidachi in Japan; these were published the same year [25, 26].

There was one aspect of "combined 17-hydroxylase/21-hydroxylase deficiency" patients which confounded endocrinologists since the first description of the condition. The original forms of CAH (21- and 11β-hydroxylase deficiencies) were always associated with hyperandrogenism causing masculinization of female fetuses, a hyperandrogenism which persists for life in untreated patients. In this new form caused by oxidoreductase deficiency (ORD) (P450 oxidoreductase deficiency), females were virilized but males had ambiguous genitalia. While it was obvious that females were virilized we found that post-natal androgen levels were low, as one would expect due to the attenuation of 17α-hydroxylase and 17,20-lyase activity caused by the deficient OR. Hydroxylation of C17 and subsequent lyase activity are carried out by a single enzyme encoded by single mRNA. The OR enzyme is required to facilitate the transfer of electrons from NADPH to CYP17 allowing oxidation. In the presence of co-factor cytochrome b5 the lyase function oxidatively removes the side-chain, the rate-limiting step in the process (Fig. 2). Inactivity of OR attenuates the process and androgen formation is inhibited.

Clearly, the virilization of female fetuses was transient but how could this be explained? We formulated a hypothesis for the transient fetal hyperandrogenism by study of the pregnancy metabolic profile from a mother carrying an OR deficient fetus [27]. We had proof of the presence of ORD in this pregnancy because estriol (a primary product of pregnancy) excretion was almost negligible being replaced in urine by a pregnenolone metabolite. The conversion of pregnenolone to estriol requires active C17,20 lyase activity. In sequential maternal urine steroid assays carried out throughout an affected pregnancy a disproportionate increase in two 5α-reduced steroids androsterone and 3α,17α-dihydroxy-5α-pregnan-20-one compared to their 5β-reduced epimers was also noted [27]. We suggest that the ORD fetus uses a novel pathway where 3α,17α-dihydroxy-5α-pregnan-20-one is converted to androsterone which in turn is oxidized to the potent androgen 5α-dehydrotestosterone (DHT). The oxidation of a 3α-hydroxyl group has always been considered unlikely in human metabolism although this pathway has been shown to be important in the tammer wallaby [28]. The viability of our hypothesis rests on the finding that 3α,17α–dihydroxy-5α-pregnan-20-one is a much better substrate for residual 17,20-lyase activity than the conventional substrates 17-hydroxypregnenolone or 17-hydroxyprogesterone [29]. We have termed this "the alternative pathway" for fetal androgen synthesis (Fig. 3) and current studies are directed to proving this theory. This is an excellent example of a hypothesis developed by detailed metabolomic studies of urine; it would have been almost impossible to achieve by any form of conventional hormone analysis. The unique metabolic profile of ORD allows for GC/MS to be routinely used for diagnosing this condition both pre-and postnatally [27, 30].

While I have focused on the disorders of cortisol biosynthesis, investigations over the last 30 years have uncovered the causes of many other steroid endocrinopathies. In terms of intersex conditions there have been the description of 5α-reductase [31] and 17βHSD deficiencies [32] causes of incomplete male development and aldosterone (the mineralocorticoid hormone) biosynthetic defects such as aldosterone synthase deficiency

and glucocorticoid repressible hyperaldosteronism. The urinary steroid metabolomes of these disorders were first described by the late Dr Stanley Ulick [33, 34].

Studies of the Cortisol-Cortisone Interconversion

Oxidation

Cortisol, with its 11β hydroxy group is the primary glucocorticoid product of the adrenal glands. It can be interconverted with cortisone (which has a carbonyl at position 11) by means of 11β hydroxysteroid dehydrogenase (11βHSD). In the normal human metabolome there are virtually equivalent amounts of metabolites with 11-carbonyl and 11β-hydroxyl functions. The principal cortisone metabolites are tetrahydrocortisone (THE) and the cortolones (20α and 20β); while the principal metabolites of cortisol are tetrahydrocortisols (THF and its 5α-epimer, 5αTHF) and the cortols (20α and 20β).

In the late 1970s, GC/MS was used by us and Drs Stanley Ulick and Maria New to study the excretion of steroids by young patients with an unusual form of hypertension. These patients retained salt which suggested high production of a mineralocorticoid but atypically low aldosterone and low renin, the opposite of what was expected. It was found that the urinary cortisol metabolites essentially only had the 11β-hydroxyl group, the steroids with 11-carbonyl were virtually absent [35, 36]. Further studies showed that the deficient enzyme was a novel renal 11βHSD which was named 11βHSD 2. This enzyme operates exclusively in an oxidative direction and has the function of protecting the renal tubules against cortisol which competes with aldosterone for the mineralocorticoid receptor. In individuals with the deficiency cortisol acts as a potent mineralocorticoid giving rise to sodium retention and hypertension. We termed this interconversion the “cortisol-cortisone” shuttle and demonstrated its importance in blood pressure regulation. In the years following multiple patients with this disorder have been studied and many loss-of-function mutations genetic found. These studies were carried out with my colleagues Chris Edwards, Paul Stewart, Maria New, among others [37].

Reduction

It had long been known that there could be active regeneration of cortisol from cortisone. In fact, for the first years of adrenal hormone discovery it was assumed that cortisone was the glucocorticoid hormone. Kendall and colleagues received the Nobel prize for their clinical studies with this steroid, as it was successfully used for treatment of rheumatoid arthritis. In the beginning of the 1950s it was finally recognized that cortisol was the true hormone and that the therapeutic efficacy of cortisone was due to its reduction to cortisol. Studies in recent years have shown that regeneration of cortisol is conducted by a long-known liver 11β-hydroxysteroid dehydrogenase, now called 11βHSD 1 after discovery of the kidney enzyme termed 11βHSD 2. It is the concerted action of 11βHSD1 and 11βHSD 2 which gives rise to the quantitative equivalence between the tetrahydrometabolites of cortisol and cortisone in human urine.

In 1996, we did a detailed study of two sisters, patients of Dr George Phillipov in Australia, who had a unique metabolome where essentially only the metabolites of cortisone were present (11-carbonyl containing steroids), i.e., THE and the cortolones. Thus, the metabolome appeared to be exactly the opposite of the aforementioned AME syndrome [38]. Interestingly, these individuals clearly had huge cortisol production but showed no symptoms of hypercortisolism (Cushing’s syndrome). This was attributed to the rapid and irreversible metabolism to cortisone, almost certainly catalyzed by hepatic 11βHSD 1. The disorder has been termed Cortisone Reductase Deficiency (CRD) and several more patients with the condition have been described in recent years [39]. The apparent inability of these patients to regenerate cortisol from cortisone causes ACTH-mediated hyperandrogenism

which manifests in early pseudopuberty in males and midlife hirsutism and infertility in women.

My colleagues at Birmingham under the direction of Professor Paul Stewart spearheaded studies of the genetics of this disorder. Interestingly, no mutations were found in 11βHSD 1 in affected patients, so analogous to the ORD studies described above, attention was paid to the generation of NADPH, which in the endoplasmic reticulum lumen is carried out by hexose-6-phosphate-dehydrogenase (H6PD). Drs Gareth Lavery and Elizabeth Walker of our department have conducted these studies and have demonstrated loss-of-function mutations in H6PD in four patients with the disorder [39]. The disorder and its affect on the metabolome are summarized in Fig. 4. These studies led to the development of a mouse model for the disorder and study of their steroid metabolome. In contrast to humans the mouse glucocorticoid is the17-deoxysteroid corticosterone and the metabolic profile was composed of reduced metabolites of this steroid. H6PD knockouts have a high percentage of 11-dehydocorticoterone metabolites proving the importance of this enzyme co-factor for corticosterone regeneration, strong evidence for the importance of this enzyme in cortisone to cortisol conversion in man.

Upstream of Cholesterol

The first product of squalene cyclization is the C-30 sterol lanosterol and conversion of this to the C-27 cholesterol is a multistep process. Disorders of post-squalene cholesterol biosynthesis were unknown prior to 1993 but since then a few have been described in humans, all bar one extremely rare. These disorders can be considered more severe than those downstream of cholesterol as they are invariably associated with dysmorphology, mental retardation and are frequently fatal. The original descriptions of the conditions were usually based on increased concentrations of the precursors for enzyme conversion in serum or cellular preparations. Verification of the deficiency has been obtained by detecting mutations in genes for individual proteins and development of transgenic mouse models [40].

In descending order as the disorders appear in the Kandutsch-Russell cholesterol biosynthetic pathway [41] the first is Antley-Bixler syndrome a skeletal dysmorphology syndrome. It was initially proposed that it was caused by lanosterol demethylase (Cyp 51) inactivity although the enzyme itself has been shown to have no mutations in affected individuals [42]. The most likely cause of attenuated Cyp 51 activity is the previously discussed P450 oxidoreductase deficiency since the latter enzyme is required for NADPH regeneration. The second condition is HEM dysplasia initially considered to be thought due to Δ^{14} reductase deficiency although more likely now a laminopathy [40, 43] and the third desmosterolosis due to inactive Δ^{24} reductase [44]. The fourth condition, is CHILD syndrome probably caused by NHSDHL (4,4-demethylase) deficiency [45] and the fifth, CDPX2 due to deficiency in Δ^{7}–Δ^{8} isomerase [46] The sixth post-lanosterol disorder is lathosterolosis cause by deficient 5-desaturase [47] and the seventh and final is Smith-Lemli-Opitz syndrome (SLOS) caused by deficient 3β-hydroxysterol-Δ^{7}-reductase (7-dehydrocholesterol reductase DHCR7, EC 1.3.1.21) [48].

SLOS was the first of the cholesterol deficiency syndromes to be reported [48] and has by far the highest incidence. Biochemically, SLOS patients have reduced cholesterol synthesis and elevated levels of 7-dehydrocholesterol (7DHC) and its isomer 8-dehydrocholesterol (8DHC). Phenotypically SLOS presents with a variety of problems such as multiple malformations, delayed development and growth, cognitive deficiency, and autistic-like behavior. Affected individuals have a compromised quality of life and present a lifelong challenge to their families and the medical community. There is no cure for SLOS.

George Schroepfer in whose honor the AOCS medal is awarded, and to whom this paper is dedicated, played a significant role in defining the sterol metabolome of the condition. His studies gave the most comprehensive analysis of circulating steroids as a result of the enzyme deficiency [49]. For the last decade our laboratory in Oakland has focused on the study of this disorder concentrating on three questions: (1) How does the cholesterol deficiency and build-up of 7- and 8-dehydrocholesterol impact the production of hormonal steroids and their metabolites? (2) Does the unique steroid metabolome of the condition offer new diagnostic options, particularly in the form of prenatal diagnosis? and (3) could gene therapy be a treatment option for increasing the deficient cholesterol synthesis?

In studying the steroid metabolome of the disorder, we were indebted to Drs Richard Kelley and Lisa Kratz at the Kennedy Krieger institute for their collaboration, particularly in supplying needed samples from affected individuals they had diagnosed. The other participants were two synthetic chemists Dr William Wilson and Liwei Guo who painstakingly synthesized and authenticated important Δ^7 and Δ^8 reference compounds. These investigators were part of Dr Schroepfer's department at Rice University, which emphasizes how, even after his untimely death, his legacy in SLOS research was cemented through his group's continuing studies on this serious disorder.

In the urine of affected adults and children, we identified multiple steroids with additional 7- or 8-unsaturation indicating that many biosynthetic transformations could take place using the excess 7- and 8-dehydrocholesterol substrates. However, we found little or no formation of dehydro versions of cortisol metabolites hinting that 21-and 11β-hydroxylation may not utilize such precursors [50]. We found normal excretions of adrenal hormone metabolites in urine from affected individuals indicating surviving SLOS patients were unlikely to suffer from adrenal insufficiency.

An immediate consideration when we were studying the steroid metabolome in SLOS was the possibility that maternal steroids excreted could include novel components originating in the affected fetuses. Such compounds could provide the basis for developing a non-invasive prenatal test for the disorder. It was predicted that 7- or 8-dehydroversions of estriol (1,3,5(10)-estratrien-3,16α,17β-triol, Fig. 5) in particular would be present since this steroid is almost exclusively of fetal origin. We did indeed find such compounds in urine, also interesting in that they have structures appropriate for the long-known "equine" estrogens, derivatives of equilin and equilenin, prominent estrogens produced by mares [51, 52]. The Rice University researchers chemically synthesized 7- and 8-dehydroestriol and we were able to confirm that the major metabolite in the urine of mothers carrying SLOS fetuses was 8-dehydroestriol (53, Fig. 5). Measurement of this steroid together with a dehydro steroid of the pregnane series has formed the basis of a non-invasive test for this serious disorder. Its efficacy has been evaluated in a large study including a million participants [54]. Not only were we able to validate the test but we were able to provide updated information of the incidence of the disorder in the North American population [54, 55].

At the final analysis, studying the metabolome of specific disorders should lead to new therapeutic options, and we are pursuing this aspect. Current treatment for SLOS is dietary cholesterol supplementation and anecdotal reports show positive, albeit limited, effects of exogenous cholesterol on somatic growth and behavior, but development outcome does not appear to be altered. However, an effect of dietary cholesterol on behavior would be somewhat surprising as the brain is believed to be impervious to external cholesterol, and a recent clinical trial showed no behavioral differences between short-term cholesterol supplementation and placebo. Because both cholesterol deficiency and excess 7-DHC likely contribute to the pathogenesis of SLOS, the therapeutic goal of treatment has been to enhance cholesterol accretion while decreasing accumulation of potentially toxic cholesterol

precursors such as 7-DHC. Some studies report a positive effect of combining a high cholesterol diet with administration of a statin inhibitor of sterol biosynthesis.

My institutional colleague Gordon Watson and I considered that the "single enzyme deficiency" nature of SLOS made it an attractive experimental model for gene therapy. We have been conducting experiments on a mouse model of the disorder and have introduced a recombinant adeno-associated virus (AAV) vector containing the human gene to young affected animals and controls. These studies were moderately successful, as we have shown the presence of human DHCR7 DNA and mRNA in liver for several weeks after administration. In addition it was shown that the introduction of a functional gene does increase the amount of cholesterol while simultaneously decreasing the amount of accumulated 7DHC, i.e., the C/DHC ratio was increased in both liver and serum [56]. While promising, the potential impact of gene therapy must be kept in perspective. Cholesterol synthesis may by stimulated, and potentially toxic precursors limited but the major dysmorphological and developmental outcomes of the disorder cannot be reversed as they result from fetal cholesterol deficiency in the earliest days of pregnancy.

In Summary

I sense we are closing in on the end of a long and remarkable era in the study of the formation and disposition of cholesterol in mammals. On the basis of study of the circulating and excreted steroid metabolome, as well as tissue conversion experiments, we have now a good understanding of the biosynthesis and further metabolism of cholesterol, the steroid hormones and the genetic errors that cause disease. While the original studies were on the primary enzymes (typically hydroxylases and dehydrogenases) involved in structurally altering a molecule, recent exciting developments have stressed the importance of partner enzymes and other factors that are essential for effecting the transformation. Examples are our own studies on ORD and H6PD without which steroid hydroxylases and 11βHSD would not function, and the studies by others of StAR transport protein which is essential for enabling cholesterol side-chain cleavage in most steroidogenic tissue, deficiency of which causes LAH [58]. Steroid metabolomics has been a key player in these discoveries.

Table 1 lists most of the known disorders and their proven or putative causes. These are illustrated in the simplified biosynthetic and metabolic scheme shown in Fig. 6. This scheme pays no attention to the tissue specificity of the many transformations. The known (and in a couple of cases, postulated) errors of transformative proteins (mostly enzymes) are listed in Table 1. Even this scheme is not comprehensive as major pathways have not been included, primarily of those leading to the formation of oxysterols and bile acids. These topics have been discussed by previous recipients of the Schroefer medal such as Professors Björkhem and Sjövall. Unexpected outcomes from detailed study of these metabolic products suggest that not only are they involved in cholesterol disposition but can be important signaling molecules interacting with receptor proteins.

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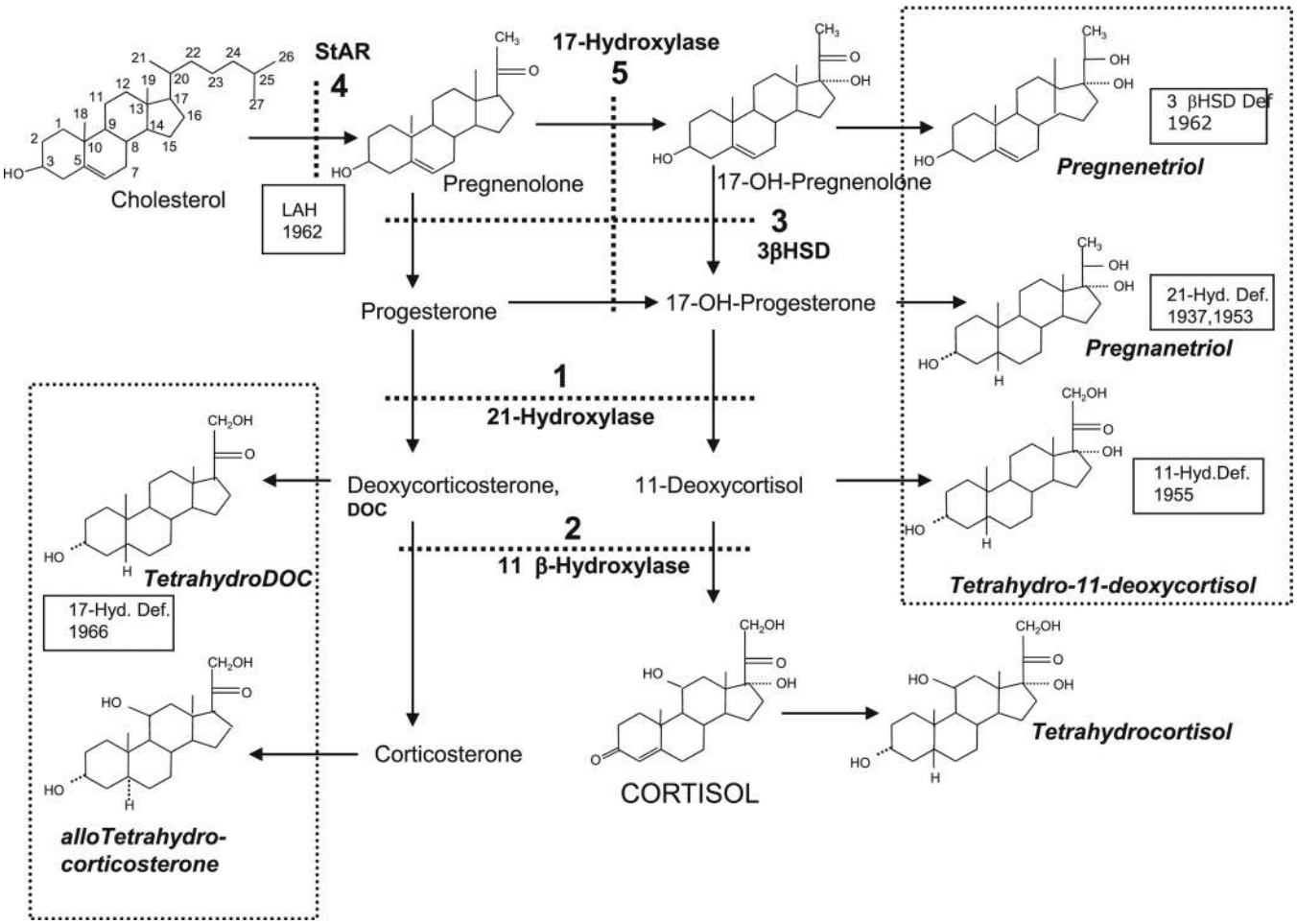

Fig. 1.

Cortisol biosynthesis and the first reported forms of Congenital Adrenal Hyperplasia (CAH). Identifying urinary metabolites and defining these conditions confirmed the postulated adrenal biosynthetic pathway determined by in vitro experimentation. The first form of CAH investigated was 21-hydroxylase deficiency [1], followed by 11β-hydroxylase deficiency [2] and 3β-hydroxysteroid dehydrogenase deficiency [3]. LAH patients [4] essentially had absence of urinary metabolites, with adrenal cholesterol build-up. 17-Hydroxylase deficiency [5] was the final form defined during the classical era. The major urinary metabolites are shown in italics, steroid hormones and their precursors in conventional script

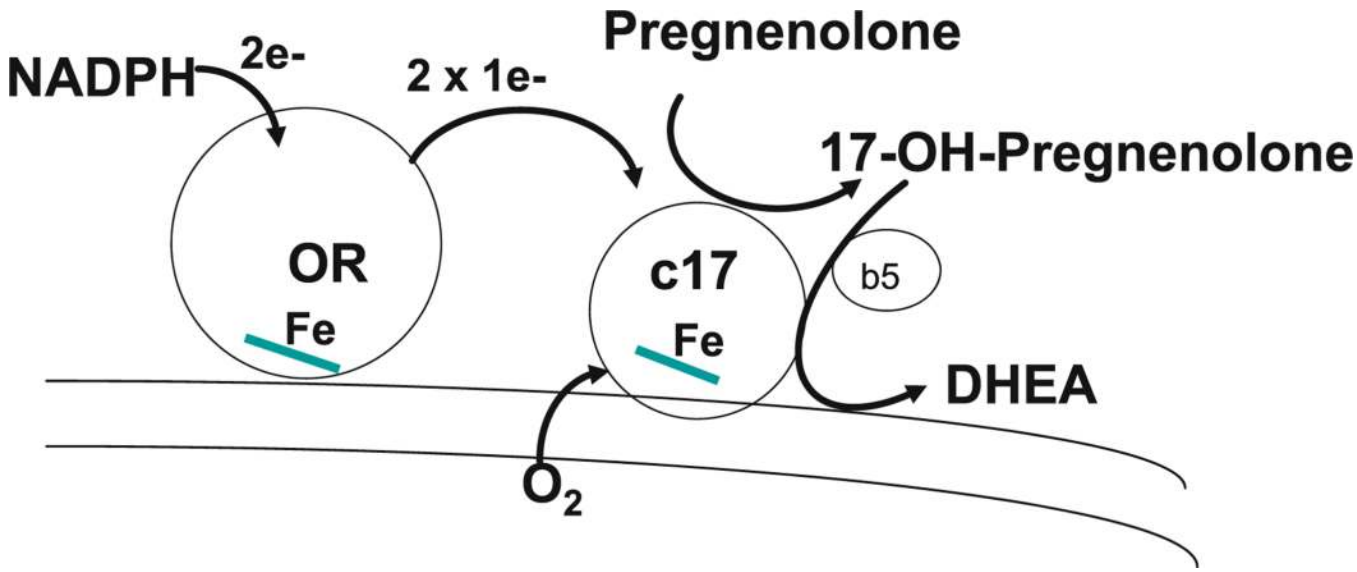

Fig. 2.

Oxido-reductase and 17,20-lyase activity. 17-Hydroxylase and C17,20-lyase activities reside in a single protein encoded by a single gene. This enzyme only works effectively in concert with P450 oxido-reductase which is necessary for providing the electrons required for oxidation. The lyase function of the hydroxylase enzyme also needs cytochrome b5

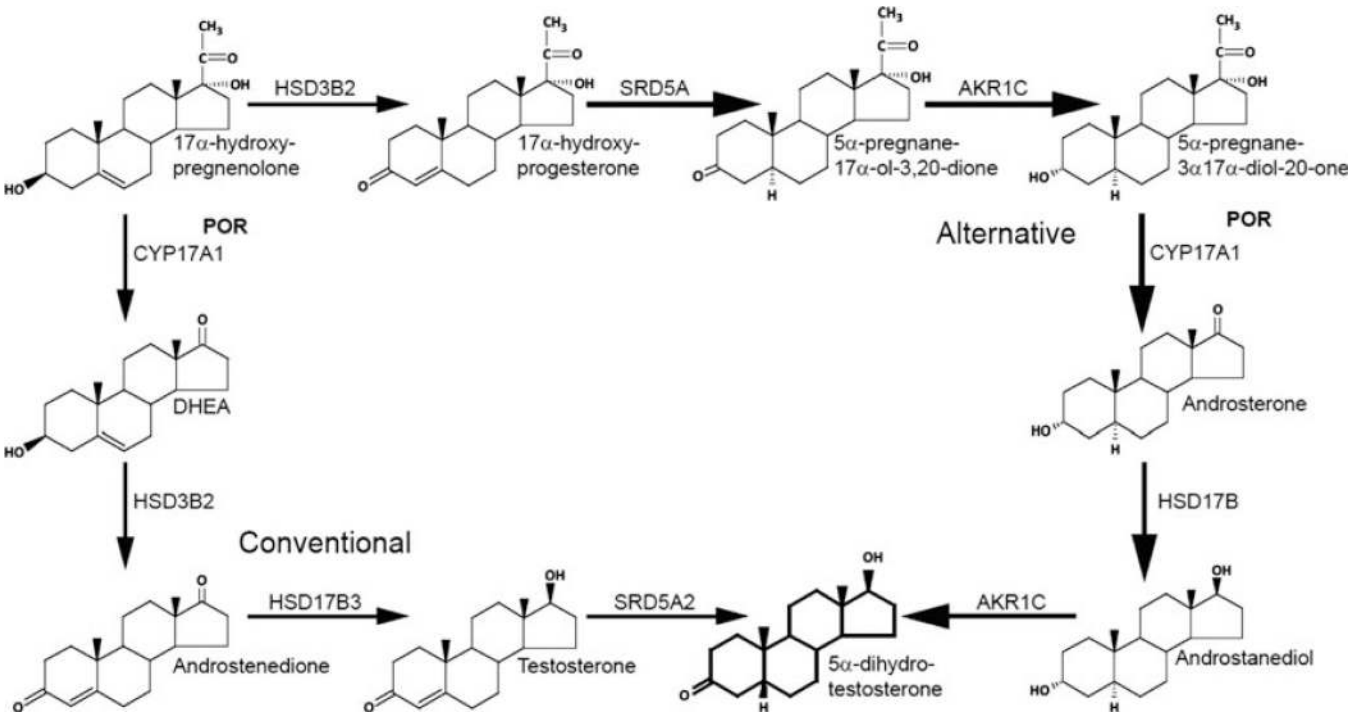

Fig. 3.

Proposed alternative pathway of feto-placental androgen synthesis from 17-hydroxypregnenolone. The *lower-left corner* represents the conventional pathway of androgen synthesis requiring sequential side-chain removal, oxidation by 3β, 17-keto reduction by 17βHSD and finally 5α-reduction terminating in DHT. The alternative pathway (*upper right corner*) starts with the formation of 17-hydroxyprogesterone and its reduction to 5α-pregnane-3α,17α-diol-20-one. In spite of attenuated 17,20-lyse activity due to POR deficiency this steroid can be converted to androsterone and likely on to DHT

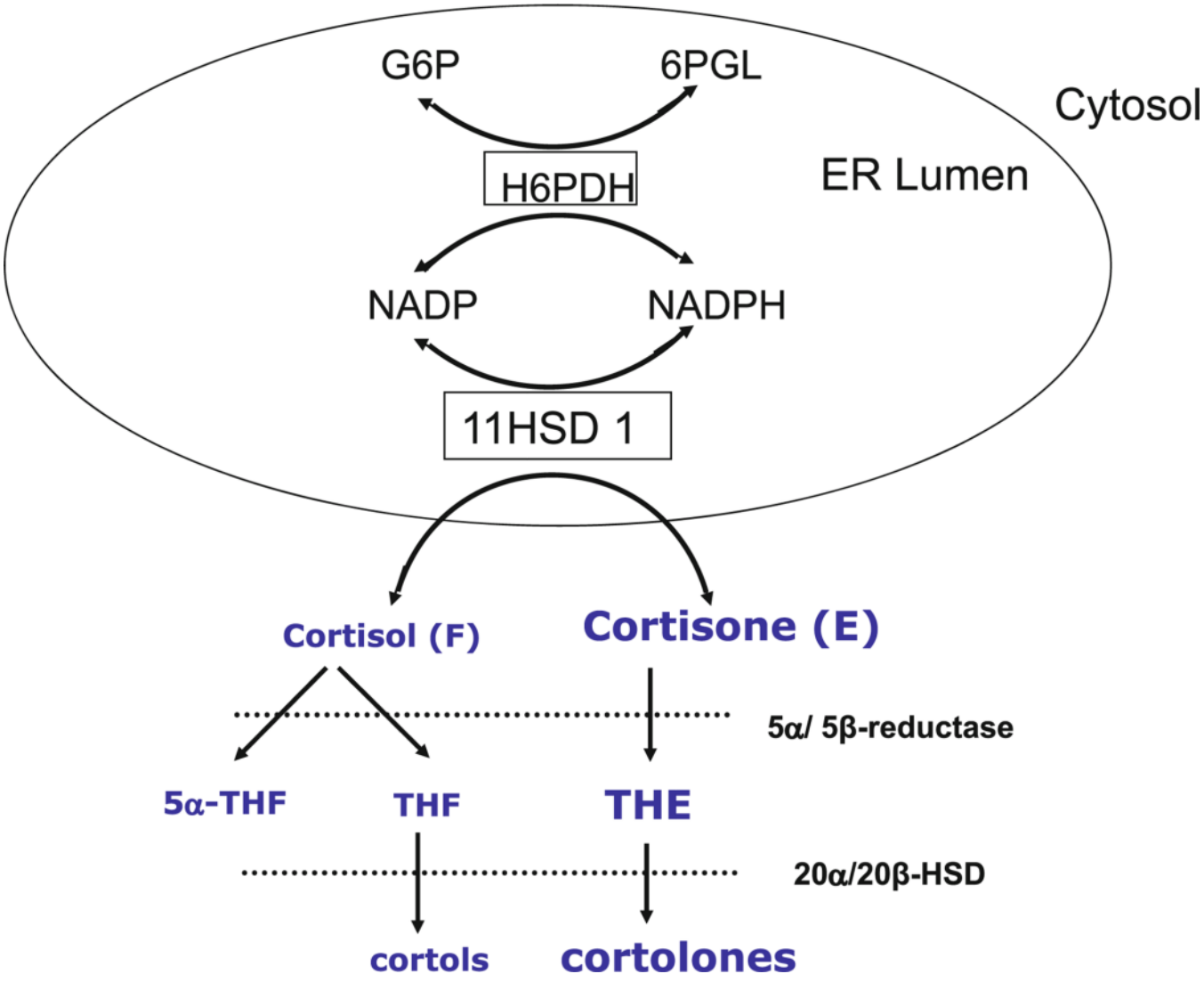

Fig. 4.

Cortisone reductase deficiency. This disorder was discovered in affected patients through the domination in urine of THE and the cortolones, corticosteroids with 11-carbonyl function. The analogous steroids with 11β-hydroxyl function, the epimeric tetrahydrocortisols (THFs) and cortols were severely diminished. This reduction was attributed to attenuated 11βHSD1 activity preventing hepatic regeneration of cortisol from cortisone. Reduced activity was shown to be caused by mutations in H6PDH responsible for NADP oxido-reduction

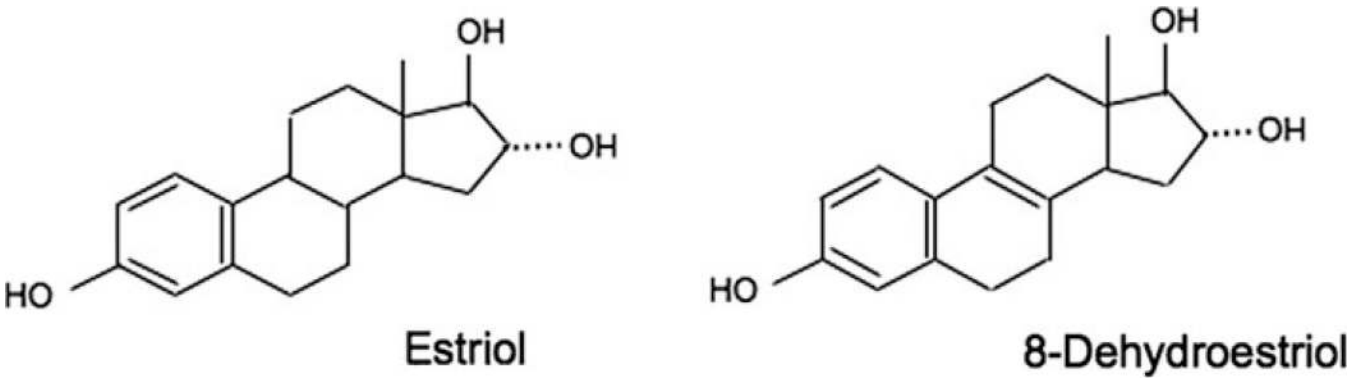

Fig. 5.

Structures of Estriol and Dehydroestriol. Estriol together with pregnanediol are the two dominant steroids excreted during pregnancy, products of feto-placental steroid synthesis. Women with a SLOS affected steroids excrete dehydroestriols, predominantly Δ^8. Measurement of 8-dehydroestriol can be used for prenatal diagnosis of the condition

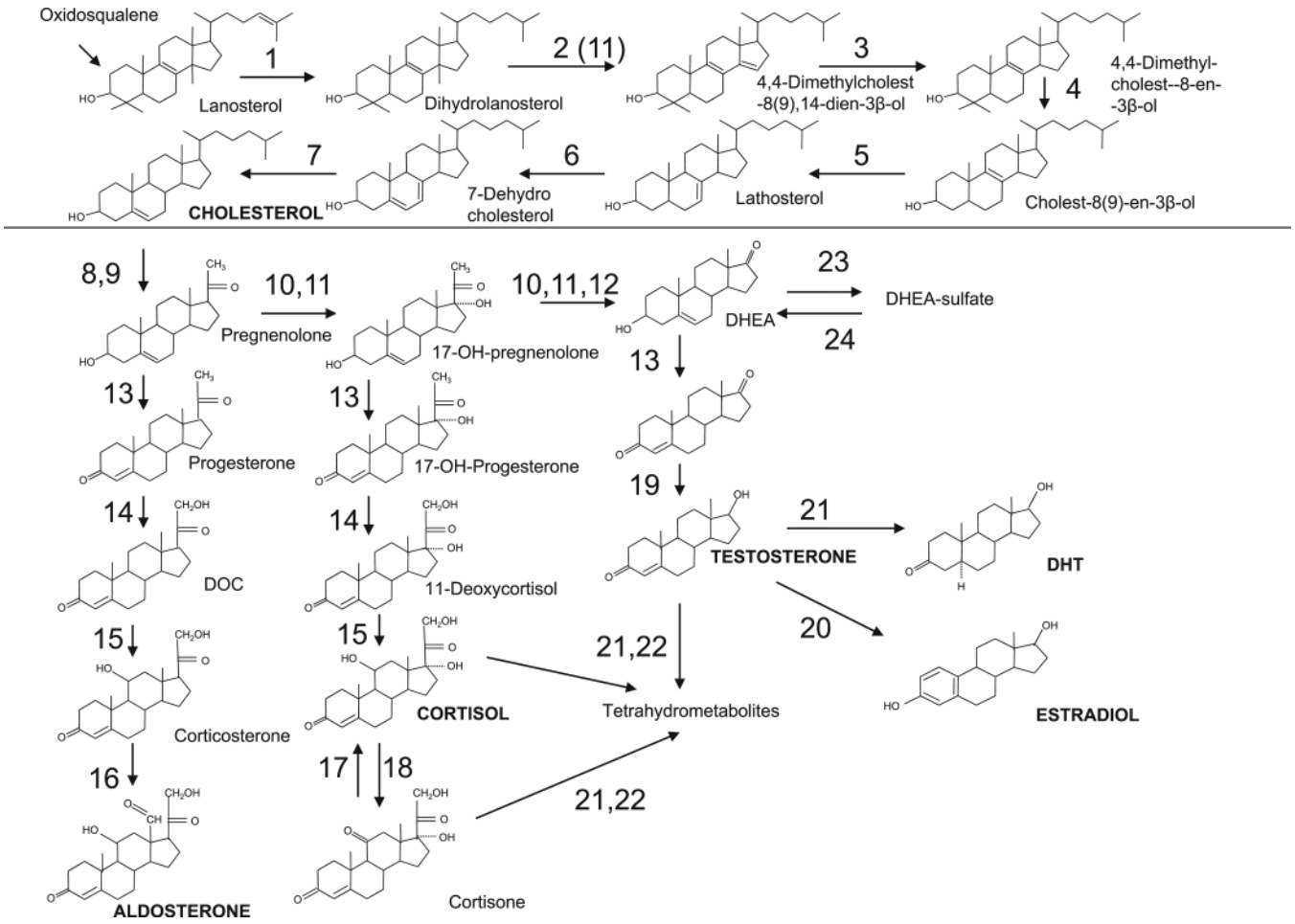

Fig. 6.

Sterol and steroid synthesis and metabolism. Simplified scheme showing reported deficiencies in steroid synthesis and metabolism. A list of disorders and the causes attributed are given in Table 1

Table 1

Disorders of sterol and steroid synthesis and metabolism

#	Disorder	Attributed to
1	Desmosterolosis	Sterol Δ^{24} reductase (DHCR24) deficiency
2	Antley-Bixler syndrome	CYP51 (sterol C14 demethylase) probably attenuated. Suggested cause P450 oxidoreductase deficiency (#11)
3	Greenberg skeletal dysplasia	Sterol Δ^{14} reductase deficiency
4	CHILD syndrome	4-demethylase (NSDHL) deficiency
5	CDPX2 syndrome	Δ^{7}–Δ^{8} isomerase (aka. epamomil binding protein, EBP) deficiency
6	Lathosterolosis	Sterol 5-desaturase (Sc5D) deficiency
7	Smith-Lemli-Opitz syndrome (SLOS)	7-dehydrosterol reductase. (DHCR7) deficiency
8	CAH, Lipoid adrenal Hyperplasia (LAH)	Steroid acute regulatory (StAR) protein deficiency
9	CAH, LAH	Cholesterol side-chain cleavage. CYP11A1
10	CAH, 17-hydroxylase deficiency	CYP17A1
11	CAH, oxido-reductase deficiency	P450 Oxidoreductase, POR
13	CAH 3β-hydroxysteroid dehydrogenase deficiency	HSD3B2
14	CAH, 21-hydroxylase deficiency	CYP21A1
15	CAH, 11β-hydroxylase deficiency	CYP11B1
16	Hypoaldosteronism Aldosterone synthase	CYP11B2
17	Cortisone reductase deficiency (CRD)	Hexose-6-phosphate-dehydrogenase (H6PD)
18	Apparent mineralocorticoid excess (AME) syndrome	11β-Hydroxysteroid dehydrogenase, HSD11B2
19	17β-hydroxy-steroid dehydrogenase deficiency	HSD17B3
20	Aromatase deficiency	CYP19A1
21	5α-Reductase deficiency	SRD5A2
22	5β-Reductase deficiency	Aldoketoreductase. AKRD1
23	Sulfokinase “deficiency”	SULT2A1 attenuated. Caused by PAPSS2 mutations
24	Steroid Sulfastase deficiency	STS