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Role of Spiral Steroids in Pregnancy and Pre-Eclampsia

Fred Chasalow

Abstract

My laboratory discovered a new type of steroids. The structure of these steroids is unique in three ways: (i) they have 23, 24 or 25 carbon atoms – no other known vertebrate steroid has more than 21 carbon atoms; (ii) they are phosphodiesters – no other steroid phosphodiesters are known; and (iii) some of them have a spiral steroid at carbon 17 – no other endogenous spiral steroids are known. In total, our laboratory had elucidated the structure and path of biosynthesis for more than 20 related compounds. We have developed an LC–MS method and a MS–MS method to measure the compounds in small samples (< 1 ml). Synthetic compounds with similar spiral steroids (e.g., spironolactone) function as potassium sparing hormones but there were no known endogenous hormones with this function. We propose that the natural spiral steroids have that function. Endogenous compounds with these functions would have important roles in the physiology of pregnancy, pre-eclampsia, and eclampsia. This chapter will review the proposed physiology and pathology of the spiral steroids during pregnancy. There are many details to confirm but this is a useful paradigm.

Keywords: hypertension, proteinuria, hypokalemia, edema, spiral steroids, Ionotropin

1. Introduction

Here is a brief history of the milestones on the discovery path that led to the discovery of phosphodiester spiral steroids and the recognition of their function as potassium sparing hormones (KSH):

- In the 1950s, Szent-Gyorgyi proposed that digoxin was not a drug but was an analog of a natural hormone [1].

- In the 1970s, Walsh and others developed RIAs for digoxin [2].

- In the 1980s, Graves observed that, during the third trimester, patients with pre-eclampsia had unknown materials in their serum that cross-reacted in his assay for digoxin [3].

- Chasalow observed that patients with Smith-Lemli-Opitz Syndrome (SLO) were K+ wasting, had high levels of two polar steroids, of which one was a Digoxin Like Material (DLM). By two weeks of age, both compounds were undetectable. In normal infants there were four polar steroids, three of which

were DLM and all four were not detectable by 2 weeks of age. We proposed that SLO was an enzyme defect in a previously unknown pathway that produced a compound that was potassium sparing [4].

- Bradlow observed that some human breast fluids had high K+ levels [5]. Chasalow and Bradlow speculated that the high K+ levels were caused by a DLM the SLO patients did not make. This started a collaboration to identify the DLM [6].

- In the 1990s, Hamlyn claimed to have isolated 13 µg of 'ouabain' from 80 liters of human plasma and proposed that it was the DLM hormone anticipated by Szent-Gyorgyi [7]. Hamlyn has not been able to confirm that the material he isolated was present in serum samples from a patient with pre-eclampsia by any method other than by RIA. If it were ouabain that he isolated, the concentration in serum that he reported would have little or no consequences [8]. The chemical properties of his material differed from the unknown DLM we found in newborn serum and in human breast cyst fluids [6, 8].

- In the 2000s, based on the identical assay for digoxin, Chasalow isolated six novel steroids from animal and human serum [9].

- In the 2010s, Chasalow identified the structures of the first 6 steroids and proposed a pathway for biosynthesis with the added atoms derived from malonyl-CoA [10]. Three of the compounds are phosphodiester conjugates of spiral steroids. The other 3 are potential precursors. Later, we corrected the biosynthetic pathway [11].

- In the 2020s, we observed that as early as 22 weeks of gestational age, precursors of spiral steroids were elevated in serum from women with pre-eclampsia but were not elevated in serum from normotensive women of similar gestational age. We confirmed by MS–MS spectrometry that these were steroid phosphodiesters, like those present in newborn serum and human breast cyst fluids [12].

- Precursors were elevated in 11 of 19 women with pre-eclampsia and in only 1 of 20 normotensive pregnant women. No other proposed marker correlates with more than 35% of affected women [13]. We propose that this divides patients with pre-eclampsia syndrome into two diseases. This would be a major advance in developing treatment protocols [14].

In summary, this chapter proposes a new paradigm to account for the symptoms of pre-eclampsia. The paradigm also accounts for the long-term increased risk of both cardiovascular disease and end-stage renal disease in affected women and their offspring [15, 16].

2. Biochemistry of steroid phosphodiesters

This section describes the biochemistry of steroid phosphodiesters. I have used Ionotropin as a key word in every paper about steroid phosphodiesters. I suggest other investigators do likewise.

Ionotropin was the name we assigned to the steroid phosphodiester that was present in human serum and not present in serum from infants with SLO syndrome.

We now know that there are two compounds that fit the definition of Ionotropin – C339 and C341. C339 and C341 are both present in human blood and were not present in serum from an infant with SLO. This usage would be equivalent to using glucocorticoid as a hormone type name and cortisol and corticosterone as specific compounds.

2.1 Symbol convention

Based on the steroid fragment observed on mass spectroscopy, we assigned a symbol with four characters in the 'Zabc' format [11]. The Z identifies which phosphodiester is present in the molecule: (a) C = phosphocholine, (b) P = phosphoethanolamine and (c) X = unknown. The 'abc' identifies the mass of the steroid fragment observed in a positive ion mass spectrum. The method is not antibody dependent. Anyone with a mass spectrometer can identify the appropriate symbol for any steroid phosphodiester. Note that, potentially, there could be isomers that share the same mass ion and phosphodiester fragment.

C339, C341, E339, and E341 were all present in bovine adrenal extracts but neither E339 nor E341 were detectable in serum from any species that we tested. This observation points to adrenal cortex as the site of synthesis.

2.2 Numbering convention

When only phosphodiester steroids with 23 carbon atoms were known, it did not make much difference which carbon was designated # 22 or # 23. Both are part of the E-ring. However, when we recognized that the added carbon atoms were derived from acyl-CoEnzyme A, we have revised the numbering scheme to reflect their common origin (**Figure 1**).

2.3 Mass spectrometric methods

Two basic methods were used. The first method used LC–MS with Atmospheric Pressure Chemical Ionization (APCI) in the positive ion mode [9]. Voltages were
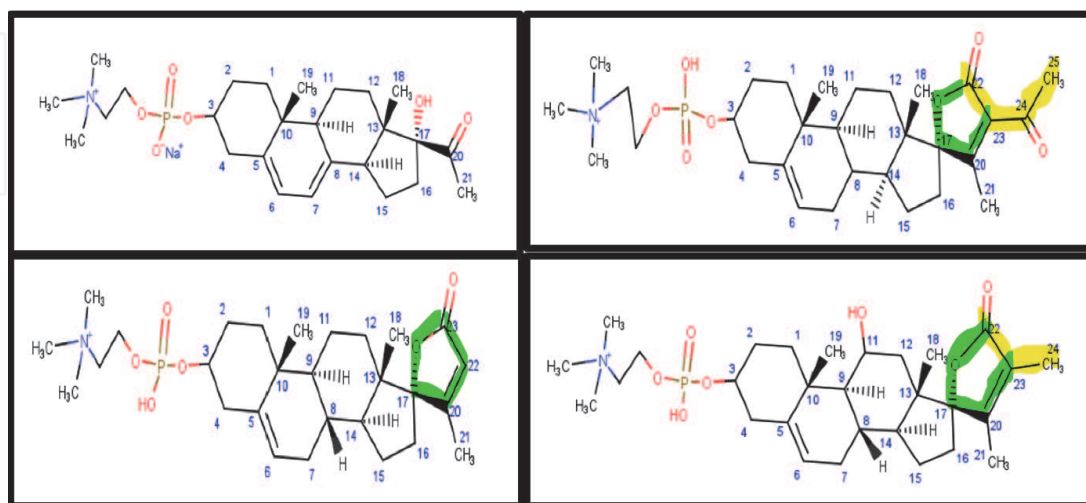

Figure 1.
Structures of representative steroid phosphodiesters. Starting in the upper left and going counter-clockwise, the compounds are C313, C339, C351, and C381. Carbon 17 is the spiral steroid. Ring E is painted green and the extra side-chain carbons are painted yellow. C339 is shown with the original, classical numbering scheme [17]. The revised scheme is shown for both C353 and C381. The new scheme recognizes carbon 22 as the carboxyl carbon of the CoEnzyme A acyl group. This numbering scheme clarifies the proposed common origin of the extra carbon atoms.

selected to minimize fragmentation. The second method used direct injection into a quadrupole ion trap mass spectrometer [12]. Spectra were collected both with and without an additional fragmentation voltage. MS–MS analysis was also used to confirm parent-fragment ion relationships.

Steroids have molecular masses in the range 270 to 400 Da. The smallest steroid fragment from a phosphodiester has a molecular mass of 297 Da and the largest fragment thus far identified has a mass of 413 Da.

2.4 Trial and error (T&E) determination of chemical composition

Table 1 illustrates the use of the T&E method to propose a composition for C381. As shown on Line 7, only one composition of carbon, hydrogen, and oxygen atoms can form a molecule with a mass of 398 Da - $C_{25}O_4H_{34}$. Readers are invited to test other molecular compositions to generate a molecule with a mass of 398 Da. Similar T&E tables have been used for each of the steroid fragments we identified. The observation that only one composition fits the mass may be a coincidence but it certainly was useful. Occam's razor suggests that the phosphodiesters are all related, as precursors and/or metabolites. If this is not true, then there must be other, as yet undetected, phosphodiester steroids.

Line	Carbons	Oxygen	C + O	H-maxHH H-Max	H-Req	m/z	Delta
1	23	5	356	48	42	398	3
2	23	6	372	48	26	398	11
3	24	4	352	50	46	398	2
4	24	5	368	50	30	398	10
5	24	6	384	50	14	398	18
6	25	3	348	52	50	398	1
7	**25**	**4**	**364**	**52**	**34**	**398**	**9**
8	25	5	380	52	18	398	17
9	26	2	344	54	54	398	0
10	26	3	360	54	38	398	8
11	26	4	376	54	22	398	16
12	27	2	356	56	42	398	7
13	27	3	372	56	26	398	15

Line: Each line describes a trial of a possible composition.
Carbons: The number of carbon atoms in this specific trial.
Oxygen: The number of oxygen atoms in this specific trial.
C + O: The contribution of the carbon and oxygen atoms to the mass.
Hmax: Maximum number of hydrogen atoms – 2+ 2 for each carbon atom.
Hreq: Difference between m/z and "C + O".
m/z: mass of the steroid fragment plus 17 Da- the fragment has lost an OH.
Delta: the number of delta necessary to complete a molecule. Delta is ½ the difference between Hmax and Hreq.
Delta is the number of rings and double bonds in the molecule. The basic steroid structure has four rings. Ring E
contributes 3 delta – ring, alkene, and the carboxylic acid. Thus, delta must be 7 or larger.
Conclusion: Line 7 (in bold) shows the molecular composition is $C_{25}O_4H_{34}$ and delta must be 9.
Isomers for the proposed structure of C381 are not eliminated by the T&E analysis. The same analysis has been done
for each steroid fragment.

Table 1.
Trial and error (T&E) analysis of composition of C381.

2.5 Spiral steroid biosynthesis

All of the newly discovered compounds are either phosphocholine (PC) steroid diesters or phosphoethanolamine (PE) diesters. The presence of the choline phosphodiester was confirmed by ^{31}P-NMR (**Figure 2**) and by the presence of a characteristic fragment at m/z = 184 Da in mass spectra. In humans, both choline and ethanolamine may be essential nutriments. The phosphodiester could be added to a steroid by condensation with CDP-serine and subsequent decarboxylation (see **Figure 2**). Based on the phosphodiesters we have identified, the acceptor steroid seems to be 17α-hydroxy-pregna-5,7-dienolone. Shackleton has isolated this compound from patients with SLO [19] and Slominski has confirmed that enzymes exist to convert 7-dehydrocholesterol to the same precursor [20].

The working theory is that the extra carbons are added by condensation of C313 or E313 with an acyl CoA (**Figure 3**). The three most common CoA acyl groups are: (i) acetyl, (ii) propyl, and (iii) acetoacetyl. The three lead to steroids with 23, 24, and 25 carbon atoms, respectively (**Table 2**). The three carboxylic acid intermediates were identified by their mass spectra. We can identify compounds which have hydroxy groups by MS–MS fragmentation (by loss of 18 Da). However, it does not identify which specific carbon atom had been hydroxylated.

C341 is the major spiral lactone in adult serum with lessor quantities of C337 and C339. These compounds differ by stepwise reduction of the two alkenes in their common C313 precursor. For cholesterol biosynthesis, the Δ7–8 bond must be reduced first because cholesterol has a Δ5–6 alkene but not a Δ7–8 bond. The same enzyme could be responsible for the reduction of Δ7–8 alkene to reduce C337 to C339. A second reduction step is necessary to reduce the Δ5–6 bond. Although testosterone is reduced to form the 5α derivative, that enzyme substrate specificity requires a Δ4, 3-ketone. As the phosphodiester blocks the ketone at carbon-3, that enzyme could not reduce C339. There is a reductase that generates 5β-metabolites. It forms cholic acid for bile. Thus, an enzyme with this specificity would produce the 5β-C341 isomer. Note that digoxin is also a 5β steroid.

The takeaway lesson from **Figure 4** is that the 5β isomer would fit like a key into a specific binding site in which the 5α isomer would not fit. The stereo-specificity of C341 is probably significant because the major weak androgen in humans (but not in most other species) is DHEA-S, which is a 5α-steroid. If C341 were a 5α- steroid, then both DHEA and 5α-dihydrotestosterone could both interfere with its function by binding at the receptor for C341, whatever it might be. Recall that spironolactone also binds to both the androgen receptor and the KSH receptor. In fact, this cross-
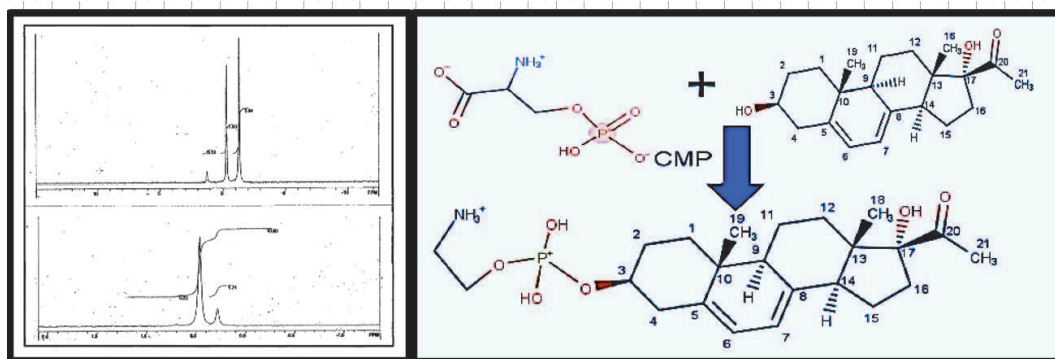

Figure 2.
Biosynthesis of steroid phosphodiesters. Left panel. ^{31}P–NMR of synthetic DHEA-phosphodiester [18] and of C341 obtained by isolation from bovine adrenal extracts [9]. The three peaks are caused by the three cations (H+, Na+, and K+). Right Panel. Condensation of serine-CDP with 17α-OH-pregna-5,7-dienolone [19, 20] to form E313. We do not know the order of the two reactions – Decarboxylation and esterification. Mass spectroscopy confirmed E313 was present in adrenal extracts [9].
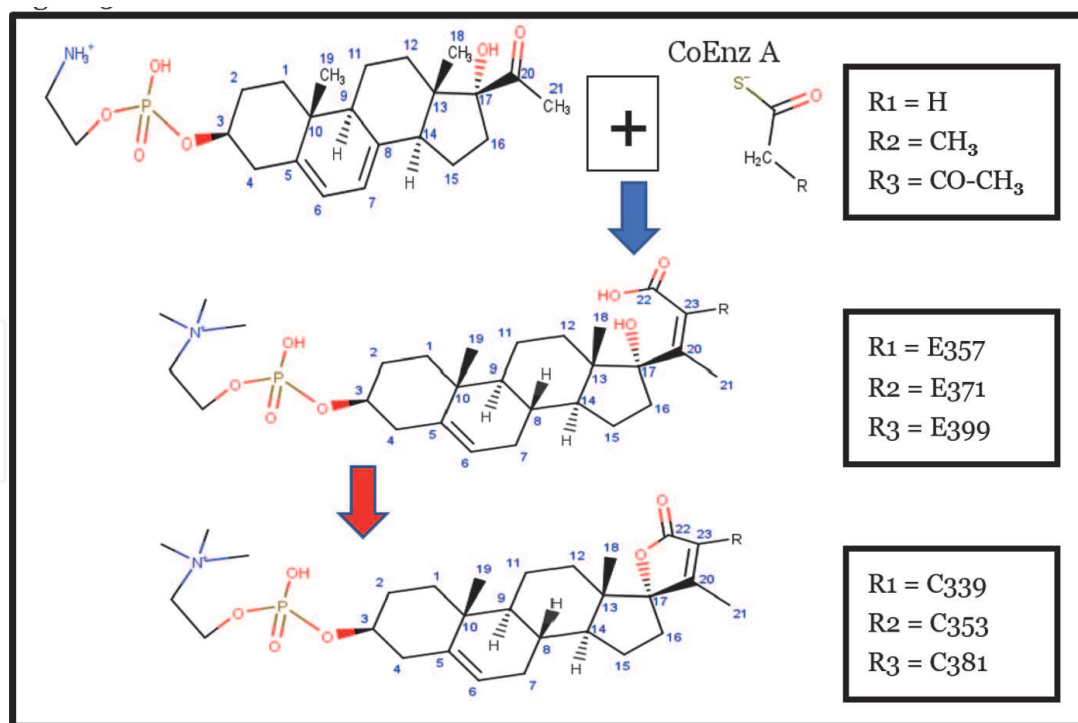
Figure 3.
Synthesis of phosphodiester spiral steroid lactones. Starting with E313, this figure shows the formation of the spiral steroid lactones. There are several steps at the Blue arrow: N-methylation, Δ7–8 reduction, and condensation with acyl-coenzyme A. The lactone ring formation occurs at the Red arrow. The order of the steps has not been positively determined. The three boxes show how the three Acyl groups lead to the three different side chains at carbon 23. The conversion of PE compounds to PC compounds (N-methylation) may occur at any step.

binding makes spironolactone a less desirable pharmaceutical. Chickens and turkeys do not use DHEA as a weak androgen. This may explain why their serum has C339, but not C341, as the major spiral lactone [21].

2.6 Tissue specificity

Question: why do we need all three classes of spiral lactones?
Answer: Tissue specificity. Pre-pubertal children only have 23-carbon lactones. Gonad extracts and serum from pregnant women have 24-carbon lactones. Milk and high K+ breast cysts have 25-carbon lactones. There are multiple forms of the NaK-ATPase. We need to isolate each of the forms and evaluate their binding constants to the different spiral steroids at the different forms of NaK-ATPase.
Question: why do need both PE and PC phosphodiesters?
Answer: Best answer at present is the PE compounds are for storage until needed. N-methylation is ACTH dependent. Thus, as part of the stress response the epinephrine increases glycolysis and the spiral lactone increases heart efficiency [10]. We suggest (without direct proof) that the same process occurs during childbirth.

2.7 Summary of biochemistry

The last discovery of a novel steroid was of aldosterone and that occurred in the 1950s. The general consensus has been that all of the steroids were already known. Hamlyn's claim to the discovery of endogenous ouabain has not been widely accepted [7]. They reported isolating 13 μg from 80 liters of plasma (0.2 ng/ml). Blaustein, one of his colleagues, has even published a paper asking, "Why is endogenous ouabain not more widely accepted?" [22]. Nicholls replied saying, "Ouabain, a circulating hormone secreted by the adrenals, is pivotal in cardiovascular disease,

Symbol	Alkenes	Composition	Other features
Steroids with 21 carbon atoms			
C313*@	Δ5; Δ7	C21O3H30	
C329*#@	Δ5; Δ7	C21O4H30	Hydroxy
Steroids with 23 carbon atoms (formed by condensation with Acetyl-CoA)			
C337*@	Δ5; Δ7; Δ20	C23O3H30	!
C339*@	Δ5; Δ20	C23O3H32	! Ionotropin
C341*@	Δ20; 5β	C23O3H34	! Ionotropin
C361*@+	Δ5	C23O4H36	22 - carboxyl
C363*@+	5β reduced	C23O4H38	22 - carboxyl
Steroids with 24 carbon atoms (formed by condensation with Propyl-CoA)			
C353	Δ5, Δ20, 23-CH3	C24O3H34	!
C369#@	Δ5, Δ20, 23-CH3	C24O4H34	! hydroxy
C371#@	5β, Δ20, 23-CH3	C24O4H36	! hydroxy
C389@+	Δ5, 23-CH3	C24O5H36	22 - carboxyl
Steroids with 25 carbon atoms (formed by condensation with Acetoacetyl-CoA)			
C381¶	Δ5, Δ20, 23-CO-CH3	C25O4H34	!
C413#&	Δ5, Δ20, 23-CO-CH3	C25O6H34	! di-hydroxy

*Compounds purified to near homogeneity.
@ Mass spectrum also identified phosphoethanolamine (Exxx).
! Spiral steroid lactone.
Site of hydroxy unconfirmed. Likely possible sites are at carbons 11 & 16. Compounds with an extra hydroxy fragment by loss of water (18 Da). This eliminates hydroxy groups at the axial carbons −18, 19, and 21.
+Carboxyl compounds must be protonated in the positive ion spectrum.
¶ This fragment was detected in milk extracts from cows, sheep and goats.
& This fragment was only detected in fetal calf serum extracts.

Table 2.
Steroid phosphodiesters identified by Mass Spectroscopy.

Figure 4.
3D images of C341, a spiral steroid. Color code: carbon – grey; oxygen - red; phosphorus – orange; nitrogen – blue. Hydrogen atoms are not shown in these images. From bottom to the top, the ring designations are A, B, C, D, & E. Panel A and B show two different views of the 5β stereoisomer of C341. Ring A and Ring E, the spiral ring, are both perpendicular to the plane generated by Rings B, C and D. Panel C shows the 5α stereoisomer. Note that in the 5α stereoisomer, Ring A, B, C, and D are co-planar and only the plane of Ring E is perpendicular to the plane of the four rings. In both stereoisomers, the PC fragment has free rotation around the steroid plane.

fact or fantasy?" [23]. Nicholls described two criteria required for an endogenous hormone: [a] biosynthetic pathway and [b] a method of assay not dependent on antibody specificity. Endogenous ouabain satisfies neither criteria. In fact, Baecher developed an ultrasensitive LC–MS method to measure serum levels of "endogenous ouabain" down to less than 2 pg./ml and could find none [24]. This section describes both a biosynthetic path to the spiral steroids and methods to measure spiral lactones by mass spectroscopy. It is unclear what Hamlyn measured but it is time to consider the role of the spiral lactones as the real endogenous KSH.

3. Physiology of spiral steroid phosphodiesters

This section describes our knowledge of the function of spiral steroids. As spiral steroid phosphodiesters are also present in oysters, the function is not limited to mammals but is probably common throughout the animal kingdom [25]. Plants seem to use cardiotonic glycosides for the same function. Amphibians use marinobufagenin and related compounds as poisons to discourage predators [26]. We have not measured spiral steroids in amphibian serum to identify which spiral steroid is used in their internal physiology. Note that marinobufagenin can only be obtained from amphibian skin after extensive hydrolysis. As such, it would not be expected to be present in serum.

3.1 Why do we need to regulate intracellular K+

For creatures living in the sea, ocean electrolytes are 460 mM Na + and 10 mM K+. This ratio closely resembles the electrolyte ratio in plasma 145 mM Na + and 4 mM K+. In contrast, intracellular electrolytes are 10 mM Na + and 140 mM K+. Although we know about the role of mineralocorticoids to recover needed Na+, until 2016, there were no known mechanisms to maintain intracellular K+ levels or to recover K+ in the kidney.

Most plants and animals have high levels of both Na + and K+ in their tissues and/or fluids. Thus, there is little need for a concentration mechanism for life forms that have free access to environmental foodstuffs. However, *in utero*, fetuses only have access to maternal serum electrolytes via the placenta. The fetus must concentrate K+ about 20-fold and must maintain the intracellular levels, despite passive diffusion of K+ from a high K+ intracellular fluid to a low K+ extracellular fluid.

3.2 Background to endogenous K+ sparing hormones or diuretics

None known.

3.3 Background to synthetic K+ sparing diuretics (KSD)

There are two types of chemicals classified as KSD and they function by different mechanisms. The Steroid-type, represented by spironolactone, activates K+ transport by the NaK-ATPase. The AT type, represented by amiloride or triamterene, interfere with passage of Na + ions through the epithelial sodium channel (ENaC). This reduces the need to 'pump' Na + out of cells [27].

Steroid-type KSDs include: digoxin, ouabain, spironolactone, eplerenone, marinobufagenin. Common features include:

- E-ring lactone with 5, 6, or 7 atoms

- Binding to most digoxin specific antibodies

- Inhibition of NaK-ATPase in the usual assay

- Pressor activity in vivo

Spiral steroid phosphodiesters have all four features.

AT Type compounds function by interfering with Na + passage through ENaC. This activity reduces the diffusion of Na + from high Na + extracellular fluids to low Na + intracellular fluids. This leads to lower intracellular osmotic pressure and 'spares' intracellular K+. The net affect is to generate a positive inotropic response [27].

3.4 Potassium accumulation in human breast cyst fluids

Earlier, because breast cysts were suspected of being precursors for breast cancer, the biochemistry of the cysts was investigated [5]. Based on electrolyte composition, there were two types. Type 1 had high K+ levels (60–100 mM) and Type 2 had potassium electrolyte levels resembling normal serum (∼5 mM). We investigated DLM levels in cyst fluid samples obtained in the normal course of patient care [6]. DLM was only present in the Type 1, high K+, samples and the levels were 10 times the levels detected in serum from normal women or men. We proposed that the basis for the high K+ levels was the presence of a K+ regulating hormone. Type 1 fluids were used to develop methods for extraction and chromatography. The new methods were different from that used to isolate 'ouabain' or 'digoxin' from plasma [7]. Doping experiments confirmed that the new method would not extract authentic cardiotonic glycosides. However, we could not collect sufficient Type 1 cyst fluid to purify the steroid phosphodiesters. In retrospect, it seems likely that C381 was the spiral steroid actually present in the Type 1 fluids.

4. Biochemistry and physiology of spiral steroids during pregnancy

Spiral steroids function as endogenous KSH and regulate both intracellular K+ levels and K+ recovery [27]. Regulation of K+ is particularly important during pregnancy because the fetus receives all of its nutrition via the placenta and does not have access to K+ rich foods (**Figure 5**).

4.1 Fertilization

After ova are fertilized, the cells divide and multiply. The growing cells need K+ for their intracellular fluids. We detected C369 and E369 in bovine ovarian extracts. C369 is a spiral steroid with 24 carbon atoms and a hydroxy group at an unidentified location (**Figure 6**). I propose that C369 is the spiral lactone that functions as KSH for fertilized ova. Both C329 and C353 are present in serum from pregnant women. C369 was present in serum from 10 out of 10 (5 males and 5 females) obligate heterozygotes for SLO [29]. At present, there is no explanation for the presence of C369 in serum from the heterozygotes but not in other men or women.

4.2 Maternal spiral steroids during second trimester

At 22–24 weeks of gestational age, there are five steroid phosphodiesters in maternal serum: C313, C329, C341, C353 and C381 (**Figure 7**). C313 and C329 have

Figure 5.
Schematic regulation of potassium during pregnancy. The figure shows the proposed relationship between potassium and spiral steroids during pregnancy. Most of the processes are known, but the significance of the steroid phosphodiesters had not been recognized [28].
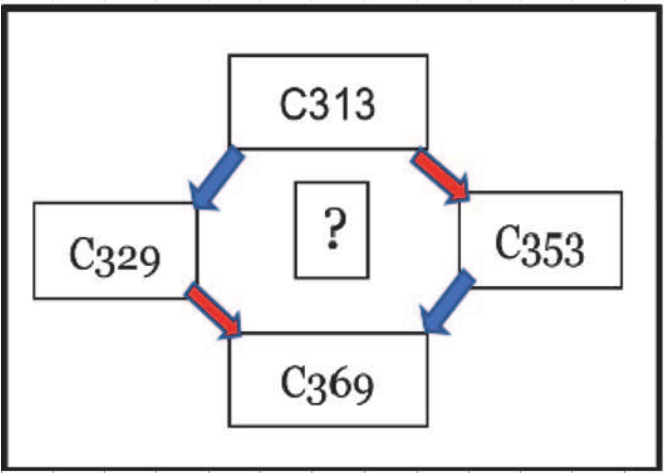

Figure 6.
Biosynthesis of C369. Red Arrows: condensation with Propyl-CoEnz A; Blue Arrows: Hydroxylation at unconfirmed carbon atom. C353 and C369 are both spiral steroids.
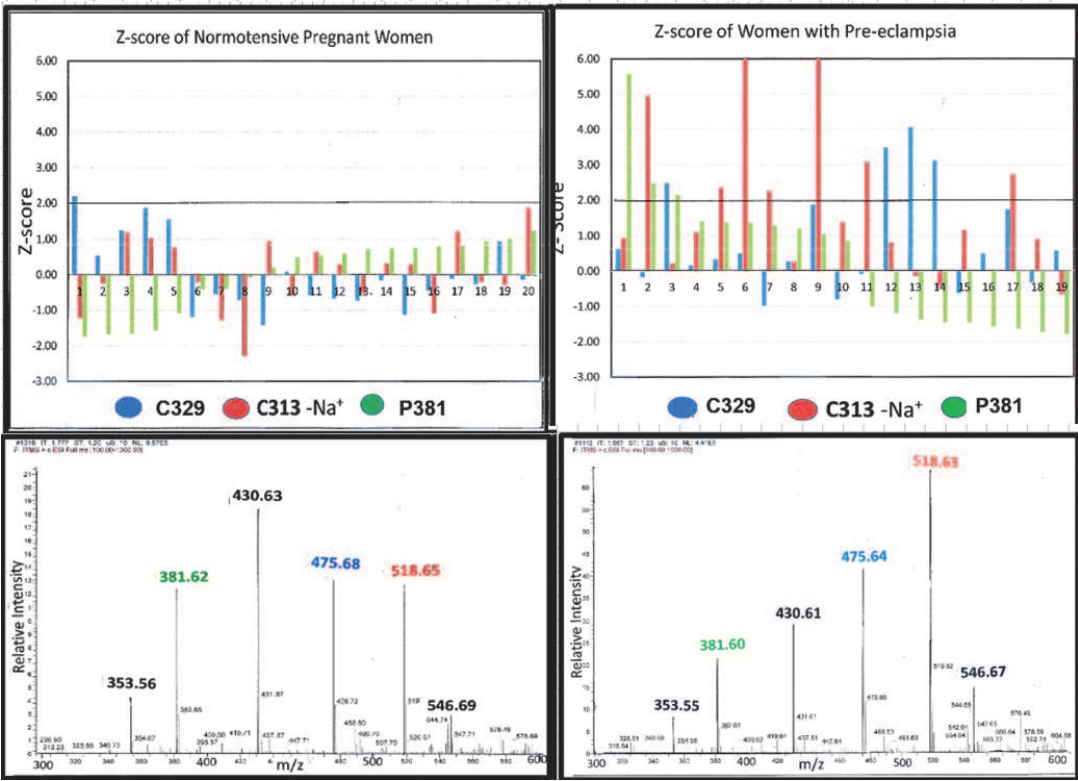
Figure 7.
*Steroid phosphodiesters during the second trimester. Serum samples (22–24 weeks of gestational age, n = 20 normotensive women; n = 20 women with pre-eclampsia) were obtained from Global Alliance for the Prevention of Prematurity and Stillbirth (GAPPS). Left column: normotensive women; Right column: women with pre-eclampsia. Top: Z-scores based on intensity of miltefosine as internal control. Bottom: Representative mass spectra. Method of analysis: For each spectrum, the intensity of each ion was compared to the intensity of the ion generated by miltefosine (hexadecyl-phosphocholine). The mean and standard deviations of ions from the normotensive women were calculated and used to generate Z-scores for each of the 40 samples. The Z-scores for each sample were graphed as a cluster [12]. Ions are identified in **Table 3**.*

21 carbon atoms. The other three are spiral steroids with 23, 24, and 25 carbon atoms, respectively.

4.3 Aldosterone signaling changes in the 3rd trimester

During the third trimester, there is a so-called 'aldosterone-signaling defect.'

In fact, there are actually two distinct aldosterone-signaling *changes* during pregnancy and these do not resolve until 2 weeks post-natal [30].

One change reduces the activity of the ENaC. This is equivalent to an AT type activity of KSD. This leads to Na + wasting in the fetal kidney and is a key, necessary step in producing the electrolytes for the amniotic fluid.

The second change is equivalent to the S Type of KSD, leading to increased activity of the NaK-ATPase pump. The increase leads to increased intracellular K+ in the fetal and maternal heart. The K-Ca- exchange mechanism increases calcium levels in the heart and results in a calcium-dependent, increased pressor response in both the fetal and maternal compartments.

The increased fetal pressor response is necessary because, as the fetus grows, the arterial resistance increases due to the increased length of the arterial bed. The increase in the maternal pressor response is needed because of the increased size of the vascular bed in the placenta. For both processes, the biology was known but the relationship to endogenous KSH was unknown because the existence of a KSH was unrecognized.

In summary, the aldosterone signaling changes are not a defect but are normal changes that are necessary during the second and third trimester.

4.4 Preparation for milk production

Milk is unique in that it is the only major extracellular fluid with K+ levels higher than Na + levels – 12-17 mM of K+ vs. 5–6 mM Na+ [31]. A KSH function should be necessary to concentrate K+ from plasma (4–6 mM) to the higher K+ levels in milk. In fact, milks from goats, cattle and sheep all had high levels of C381, suggesting that it is the KSH required to accumulate high levels of K+. This observation suggests that the NaK-ATPase isoform in breast tissue may be specific for C381, rather than for any other spiral lactone.

4.5 Post-natal

Post-natal, infants are fed milk, which is high in K+, and the need for a KSH ends but serum levels of spiral steroids remain detectable for about two weeks. Infants remain Na + wasting and usually lose about 10% of their birth weight. By two weeks of age, the need for KSH is over; the spiral steroids have been metabolized; aldosterone function is restored; Na + wasting ends; growth resumes [30]. Mother and infant "live happily ever after."

4.6 Summary of the role of spiral steroids during pregnancy

Spiral steroids, acting as KSHs, play a key role in K+ regulation during pregnancy. Ionotropin with 23 carbon atoms is the primary KSH for maternal function. The 24 carbon atom compounds, C353 and C369, function in the gonads and in the fetal-placental compartment. As the mother prepares for milk production, C381, the spiral steroid with 25 carbon atoms, directs the accumulation of K+. Ionotropin (C339 and/or C341) and C381 are DLM. C353 and C369 have the same spiral lactone epitope and are probably DLM, but we have not confirmed that suggestion by isolation and testing of extracts. All of these compounds are phosphocholine steroid diesters. The corresponding phosphoethanolamine steroid diesters are present in extracts from tissues that ordinarily synthesize steroid hormones but are only present in trace amounts (if at all) in serum [9].

m/z (Da)	Symbol	Color	# of C	Origin of ion	Comment
353*	C353	Black	24	Fragment	Spiral steroid
381*	C381	Green	25	Fragment	Spiral steroid
475!	C329	Blue	21	Loss of TMA	Precursor
518	C313	Red	21	Na + ion	Precursor
546	C341	Black	23	Na + ion	Spiral steroid

The identify of each steroid ion was confirmed by MS–MS analysis. All parent ions were Na + ions. C313 is the precursor for C341; C329 is the precursor for C369. One of the precursors was elevated in 11 of 19 samples from women with pre-eclampsia.
* The ion detected is the steroid fragment after loss of the phosphocholine.
! The ion detected is derived from Na + ion after loss of trimethylamine (TMA).

Table 3.
*Identification of phosphodiester steroids in serum (**Figure 7**).*

5. Physiology of spiral steroids in pre-eclampsia

Pre-eclampsia is a syndrome, not a disease [32]. As a syndrome, the diagnosis is made by hypertension and proteinuria. The symptoms can begin as early as 20 weeks of gestation [33–35]. For many patients with pre-eclampsia, there is little consequence during pregnancy. Monitoring and bed rest are often recommended. However, about 6–10% of affected women develop life-threatening hypertension and/or seizures. The only treatment is immediate C-section [15]. After C-section, the seizures and hypertension usually resolve.

In addition to the classical symptoms, during the third trimester, many affected patients also develop hypokalemia [36, 37]. In fact, there is a statistically significant ($P < 0.05$) inverse relationship between maternal serum K+ levels and maternal blood pressure [35]. Publications from 3rd world countries describe hypokalemia in patients with pre-eclampsia but publications from 1st world countries do not recognize hypokalemia as a symptom or risk factor.

There are several things to note in **Figure 7**.

- At 22–24 weeks of gestation, serum DLM is undetectable by most assays [38].

- Although C353 (an ion at m/z = 353 Da) and C341 (an ion at m/z = 546 Da) were detected by mass spectroscopy, both ions were of low intensity and might not be detected in a DLM assay. The variability of the intensities for these two ions did not correlate with disease status.

- Neither C381 (detected at m/z = 381 Da) nor C329 (detected at m/z = 475 Da) were detected in outdated human plasma [9].

- For both C313 (detected at m/z = 518 Da and C329 (detected at m/z = 475 Da) there was a significant increase ($P < 0.05$) in mean serum levels6in women with pre-eclampsia and many samples had Z-scores greater than 2.0.

We used three different statistical methods to evaluate the ion intensity of the C313, precursor of Ionotropin. First, we compared the mean and standard deviation of the of the samples from the normotensive women with the corresponding data from the women with pre-eclampsia. Second, we used Rank sum analysis. This method does not assume a normal distribution and is considered more robust than methods that imply a normal distribution. Third, we used the mean and standard deviation of the normotensive women to show Z-scores for all 40 samples. This set of data is presented in the clusters in **Figure 7**. There were 12 samples from the women with pre-eclampsia with Z-scores over 2 for either C313 or C329; there was only one sample from a normotensive woman with Z-score over 2. This distribution is statistically significant at the $P < 0.01$ level. With all three methods, the differences are statistically significant at the $P < 0.05$ level. Although there was an increase in the concentration of precursors, DLM was undetectable at this stage of gestation [39].

This data portends converting pre-eclampsia from a syndrome to at least two diseases. One disease, Type A, characterized by elevated levels of at least one of the spiral steroid precursors (either C313 or C329), a second disease, Type B, characterized by normal levels of the precursors. The takeaway lesson from this study is hypertension and proteinuria seem to be symptoms of more than one disease [40].

5.1 Proposed biopathology of pre-eclampsia

- Inadequate implantation leads to inadequate fetal K+.

- To compensate, the placenta secretes excess spiral steroid precursors – either C313 or C329.

- Mother responds by converting the spiral steroids to C341 or C369.

- C369 acts as a KSH in the fetal-placental unit, which further depletes maternal K+.

- Fetal hypokalemia prevents normal growth. This may be the process that leads to low birthweight infants.

- Hyperspirolemia (high serum levels of C341 or other spiral steroid phosphodiester) functions as an KSH and leads to maternal hypertension and proteinuria. Hyperspirolemia would be detected as a DLM.

- Continuous hyperspirolemia would lead to life-threatening seizures.

- Sustained hyperspirolemia damages heart and kidney and increases life-long disease risk (**Figure 8**).

The initial underlying biopathology seems to be inadequate placental implantation [41, 42]. Investigators have measured many, many hormones as possible risk factor or mediators, but none predict more than 35% of the patients who develop the symptoms, none predict hypokalemia, none predict risk of life-threatening hypertension [43–45], and none provide a biochemical basis for the increased life-long risk of renal or cardiac disease.

5.2 Significance of changes in aldosterone signaling

If fetal K+ levels were inadequate, the placenta should synthesize spiral steroid precursors, C313 and C329 [12]. This leads to their increase in the maternal circulation. In turn, these compounds are converted to spiral steroids which function as KSHs. Elevated KSH has been documented in patients with pre-eclampsia as increased DLM levels. This seems to be a normal third trimester process occurring during the second trimester. The preeclampsia symptoms would be caused by interference in function of the ENaC in the kidney and by increased pressor activity in the heart due to the secondary increase in Ca++.
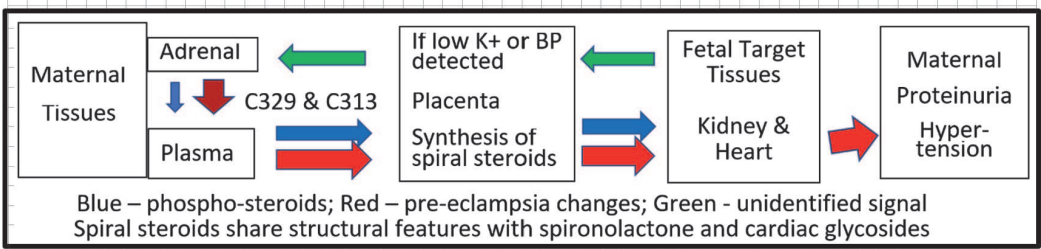

Figure 8.
Changes in spiral steroids during pre-eclampsia. This figure integrates the regulatory process with the normal role of spiral steroids acting as KSH. The biology has been known. The underlying biochemistry was unknown prior to the discovery of the spiral steroids. The shuttling back and forth of steroid phosphodiesters between the mother and the fetal-placenta unit is similar to the synthesis and function of estriol.

5.3 Pilot study results

C313, the precursor for C341, was detected in serum from pregnant women by the ion at m/z = 518 Da (generated by {a} 313 Da from the steroid fragment, {b} + 183 Da from the PC fragment, {c} + 23 Da from the Na+, and {d} -1 Da from the loss of the H+ = 518 Da.). 7 of 20 women diagnosed with preeclampsia had elevated levels (Z > 2) of C313 in serum collected at 22–24 weeks of gestation. Just like overdose of KSDs, elevated levels of C341 in maternal serum would be expected to lead to maternal hypertension, proteinuria and hypokalemia.

C329, the precursor for C369, was detected in serum from pregnant women by the ion at m/z = 475 Da. This ion is generated by loss of trimethyl amine (59 Da) from the Na + ion at m/z = 534 Da. 4 of 20 women diagnosed with preeclampsia had elevated levels (Z > 2) of C329. There was a statistically significant increase in concentration of C329 in the affected patients when compared to the normotensive control group. This would be expected to lead to increased levels of a KSD in the fetal circulation without corresponding increases in the maternal circulation. However, the incidence of samples with Z > 2 for C329 did not reach statistical significance; a larger sample size will be needed.

C329 is the precursor of C369. C369 was not detected in pre-pubertal children but was present in 10 of 10 obligate heterozygotes for SLO. There is no report of increased incidence of maternal hypertension or proteinuria in this group. Three of the 40 samples had high levels of C369, presumably associated with heterozygote carrier status for SLO.

A third group, 9 of 20 women with preeclampsia, had normal levels of both C313 and C329 at 22–24 weeks of gestation. There may be three different patterns: [a] high levels of C313 leading to maternal hypokalemia and life-threatening hypertension, [b] high levels of C329 leading to self-treatment of the fetal hypokalemia without generating maternal life-threatening hypertension, and [c] a 3rd group of patients with an unrelated origin of their symptoms. Overall, only about 5–10% of women with pre-eclampsia develop seizures and/or life-threatening hypertension later in pregnancy. The existence of 3 diseases sharing symptoms of proteinuria and hypertension might be the explanation for the lack of progress in developing therapy for these syndromes.

5.4 Post-partum

The green bars and peaks in **Figure** 7 show the C381 levels in serum from pregnant women. At 22–24 weeks of gestation, only one of the 40 samples had elevated levels of C381, characterized by a score of Z > 2. There was no significant difference between the serum levels of C381 of normotensive pregnant women when compared to the serum levels of C381 from women with pre-eclampsia. C381 could stimulate milk production without affecting maternal heart or kidney function.

If during gestation, the mother had pre-eclampsia, long-term damage may have occurred due to persistent hyperspirolemia. Animal models treated with plant-derived cardiotonic steroids develop long-term heart and kidney consequences [46, 47].

6. Therapy for pre-eclampsia

6.1 Failed therapies

6.1.1 Phosphodiesterase inhibitors

One hypotheses is pre-eclampsia can be treated with phosphodiesterase inhibitors, including sildenafil citrate [48, 49]. However, Podymow writes, "As currently

understood, the hypertension of preeclampsia is secondary to placental under per-fusion, thus lowering systemic BP is not believed to reverse the primary pathogenic process." [50].

6.1.2 Digibind

Digibind is an FAB isolated from an antibody to digoxin and is used to treat patients with hypertension caused by digoxin toxicity [51]. As there are elevated levels of DLM in serum from women with pre-eclampsia, Digibind has been tested to determine if it would reduce hypertension in women with preeclampsia [51]. Infusion with Digibind does lead to a prompt decrease in blood pressure in affected women. However, the effect is short lived. Within 12 hours, blood pressure has returned to pre-therapeutic levels. The interpretation was that ane unknown agent was bound to the FAB and excreted. However, additional amounts were synthe-sized, leading to continued hypertension. The effort, if any, to confirm the identity of the unknown agent has not been published.

6.1.3 Monoclonal antibodies to Marinobufagenin

Marinobufagenin is a poison originally isolated from toad skin extracts [52]. Abi-Ghanem, with polyclonal antibodies, developed a chemifluorescent immunoassay [53]. Agunanne used the assay to confirm elevated marinbufagenin levels in women with preeclampsia [54]. Fedorova developed monoclonal antibodies and observed there was an unknown factor in serum of Dahl rats that was detected by their monoclonal antibody [55]. In the first publication, it was characterized as marinobufagenin-like, then as endogenous marinobufagenin [56]; most recently, just as marinobufagenin [52]. However, there are no publications describing char-acterization of marinobufagenin, or any plausible precursor or metabolite, from any mammalian source, other than by immunoassay.

Despite not knowing the true identity of the 'factor' detected by these anti-bodies, investigators have proposed a role for marinobufagenin in pre-eclampsia in women [57]. I do not doubt that there is at least one unknown substance that cross-reacts with marinobufagenin-specific antibodies in serum from patients with pre-eclampsia. I doubt that it is marinobufagenin.

6.2 Proposed therapy

The Pilot Study showed increased levels of one of the spiral steroid precursors, C313 or C329, in the maternal circulation. The corresponding spiral steroids are C341 and C369. High levels of C369 were present in obligate heterozygotes with SLO but these women do not have pre-eclampsia [29]. Thus, the cause of hyper-tension and proteinuria would seem to be C341. This leads to two significant therapeutic suggestions: [1] monitor disease progression with C313 and [2] treat with C369 or its precursor, C329. The goal would be to stimulate KSH activity in the fetus without stimulating the function of a KSH in the maternal circulation.

6.3 Proposed diagnostic method

The pilot study was designed to maximize the chance of a clear positive response. In fact, statistically that was achieved. However, it is likely that the elevated level of C313 did not appear suddenly at 22 weeks of gestation. A large study is needed to determine when the elevated precursor levels begin and, later in

gestation, which spiral steroids are elevated in the patients who develop eclampsia or HELPP syndrome [15].

7. Conclusion

One general theme in endocrinology is, "One disease to a customer." If all symptoms experienced by a patient are not explained by the proposed biochemistry, the patient has a syndrome, not a disease. This chapter title tells the story, "It's all about potassium." None of the reviews that I found recognize the significance of hypokalemia as part of the disease.

In detail, several facts stand out: [a] there is little evidence that pre-eclampsia is a single disease, [b] the common characterization of pre-eclampsia as a syndrome does not include hypokalemia, [c] without considering the role of spiral steroids, there is no recognized mechanism that shows how inadequate placental implantation leads to all of the classical symptoms of pre-eclampsia, hypokalemia or to the long-term increased risk of coronary or renal disease.

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Conflict of interest

The authors declare no conflict of interest.

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