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REGULATORY EFFECTS OF 5β-REDUCED STEROIDS

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Summary—The first section of this publication summarizes early work according to which 5β-pregnanedione is an important metabolite of progesterone in the early stages of the chick embryo's adrenal steroidogenesis, then decreasing gradually as corticosteroidogenesis increases. In the second section a model is described in which adrenal 3β-ol hydroxylase-isomerase of the 17-day-old chicken is suppressed pharmacologically, this suppression being correlated with that of the synthesis of aminoevulinic acid (ALA), the first and rate-limiting step of the heme pathway. 5β-Pregnanedione (10^{-7}–10^{-6} M) restored ALA synthesis in this inhibited model to normal values. The effect of 5β-pregnanedione was specific since other steroids tested: progesterone; 5α-pregnanedione; corticosterone or estradiol, did not stimulate ALA. Since heme formation by steroidogenic glands contributes to the synthesis of cytochrome *P*450 rather than hemoglobin, 5β-pregnanedione was also assayed as a stimulator of this enzyme system and was found to increase cytochrome *P*450 in adrenals and testes but not in the liver. In view of these results a hypothesis is advanced according to which 5β-reduced progestagens and androgens stimulate cytochrome *P*450 formation, i.e. the synthesis of progesterone and higher hydroxylated steroids, by steroidogenic glands in the event of an excessive precursor reduction.

The recognition that steroids with a reduced A ring might possess hormonal activities is a relatively recent one: in the early 1960's, the statement that a 5α-reduced metabolite of a steroidal hormone might be endowed itself with hormonal properties [1] was only reluctantly accepted. But 5α-reduced steroids acquired respectability and even fame when Wilson and Bruchovsky [2] catapulted 5α-dihydrotestosterone towards its actual position. From then on, and for many years, some 5α-reduced steroids were thought to be "active" while their 5β isomers were thought to be inactive metabolites or "catabolites" of steroid-hormones.

A little earlier, Barton had introduced conformational analysis to steroid chemistry [3]. Conformationally 5α-reduced steroids are molecules with an A/B-*trans* structure, fairly planar molecules with an unhindered α face. In contrast, 5β-reduced steroids torsion their A ring towards the α face, A and B rings vaguely adopting a *cis* position with respect to a plan perpendicular to the molecule's equator (see Discussion). At the beginning steroid physiologists did not pay much attention to these concepts since planar structures seemed to explain sufficiently most hormonal properties. The few who did, associated a more or less unhindered α face, flat or only slightly torsioned as in Δ 4-3-keto steroids, with hormonal effects. Irrespective of structural criteria, steroid physiologists of the 1960s tended to question such effects for 5β structures.

Probably the first biochemist to study the 5β-reduced pathway with biological functions in mind was Parsons in 1970 [4]. Parsons found intense 5β-reductase activity in the chick embryo as early as the blastoderm (day 3 of development). In contrast to others [5, 6], his research was not motivated by "pure" endocrinology, or enzymology, but by interdisciplinary studies undertaken years earlier in the porphyrin field [7, 8]. According to these early studies, 5β-androstane and 5β-pregnane derivatives stimulate avian hemoglobin synthesis from this very early embryonic stage on. As an interesting

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Abbreviations: pregnanedione, pregnane-3,20-dione; ALA or δ-ALA, δ-aminolevulinic acid; δ-ALA-S, δ-aminolevulinic acid synthetase; TPG, toluene-propylene glycol system; TLC, thin-layer chromatography; PBG, porphobilinogen; PPO, 2,5-diphenyloxazole; dimethyl POPOP, 1,4-bis[2-(4methyl-5-phenyloxazolyl)]benzene.

case of converging strategies, those reduced metabolites enhance, albeit through different mechanisms, the synthesis of the blood pigment's globin chains [9, 10], as well as heme nucleus [7, 8, 11]. We shall come back to this biological action after analyzing the ontogeny of 5β-reductase in the chicken.

Our group became interested in the embryonic development of 5β-reductase when Enrique Perdenera joined us, also in the early 1970's. Pedernera is an embryologist who had previously studied such unusual effects of corticosteroids as their trophic action on the chick embryo's duodenal mucosa [12]. It was only natural that he wanted to gain insight into his model's adrenal steroidogenesis.

Pedernera chose day 15 of ontogeny because many events occur at this stage: thus, functional interlocking between the chick embryo's endocrine glands commences slightly earlier [12], while the capacity of the embryo's glucocorticoids to induce phenylethanolamine *N*-methyltransferase [13] only develops on day 15.

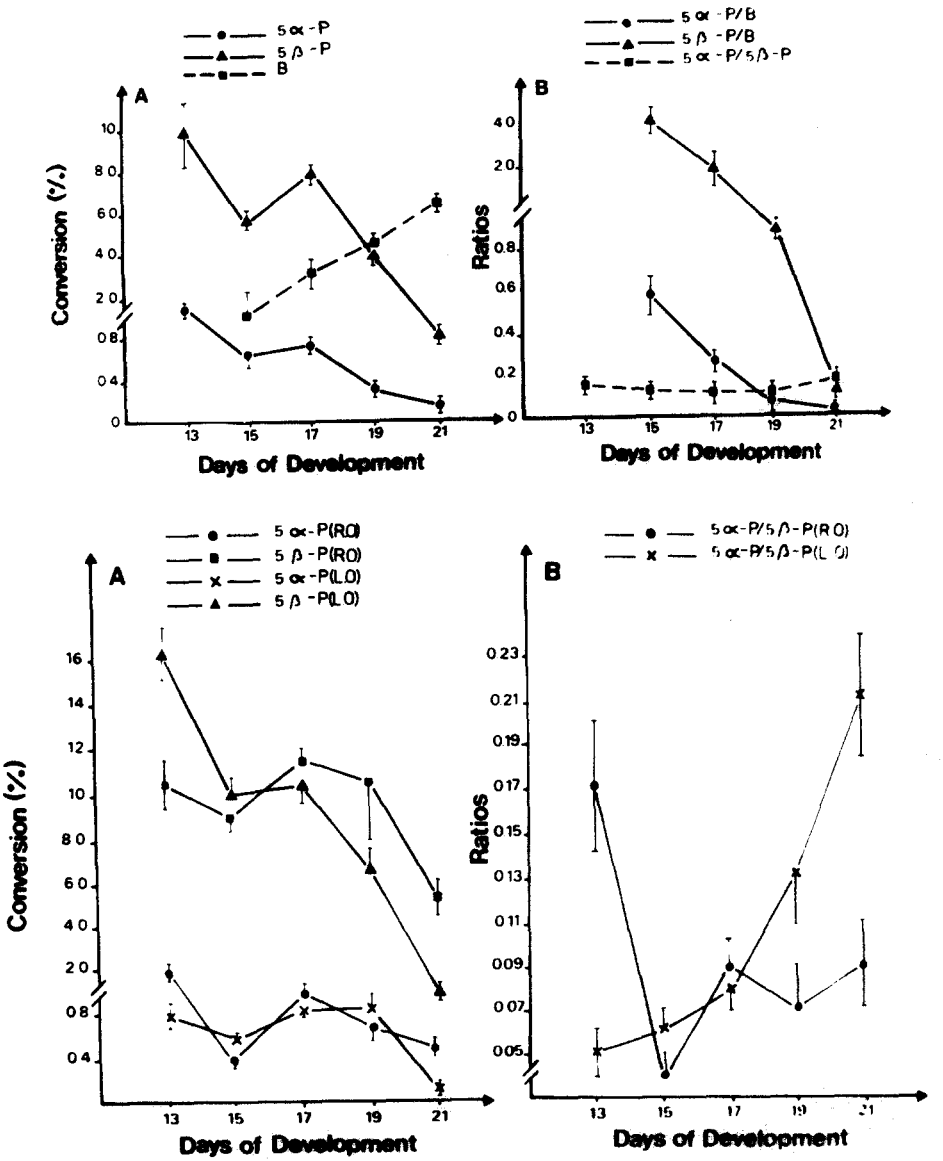

Fig. 1. Upper part: adrenal conversion and ratio curves. 5α-P, 5α-pregnanedione; 5β-P, 5β-pregnanedione; B, corticosterone. 5α-P (A) as well as 5α-P/5β-P and 5α-P/B curves (B) correspond to y values proportional to the lower part of the y axis. 5β-P and B (A) and 5β-P/B curves (B) correspond to y values proportional to the upper part of the y axis. Lower part: ovarian conversion and ratio curves. (A) Both 5β-P curves correspond to y values proportional to the upper part of the y axis, and both 5α-P curves to y values proportional to the lower part of the y axis. Weight of incubation pools: L.O. (left ovary) 34 ± 4 mg; R.O. (right ovary) 24 ± 3 mg. Adapted from Ref. [28].

In his corticoidogenic studies, Pedernera found the 15-day-old embryo's adrenal to convert progesterone to 5β-pregnanedione with far higher yields than conversions to other metabolites including that to corticosterone, the bird's main glucocorticoid. No 5α-pregnanedione could be found at that moment [14].

The upper part of Fig. 1 shows results obtained years later on the ontogenic development of both reductive pathways, compared to conversions to corticosterone [28]. Noticeable about these results are: the much higher biotransformation to 5β than to 5α-pregnanedione, the asymptotic flattening of 5α-pregnanedione/corticosterone—but not 5β-pregnanedione/corticosterone ratios and, most interestingly, the symmetric pattern of the developmental curve-pair 5β-pregnanedione vs corticosterone, the first curve descending gradually while the second one ascends as hatching approaches.

Of no less interest are both developmental curves and the ontogeny of 5α/5β ratios in each of the two embryonic ovaries (Fig. 1, lower part).

It has been known now for many years that the chicken embryo's right ovary develops differently from its left counterpart: while the left female gonad evolves anatomically and functionally to maturity, the right one regresses as hatching approaches, losing weight and whatever incipient hormonal function it might have acquired earlier [15]. In line with this functional diversity, only the left ovary exhibits an ascending 5α/5β curve. Nothing of the sort happens in the right one.

These findings are coherent with ideas discussed later, according to which A/B *cis* conformations can somehow be associated with very early life stages in the animal kingdom [16]. But even so, they raise questions, rather than informing on the biological usefulness of the 5β-reduced pathway. Is it a "catabolic" pathway, inactivating hormones in an organism as yet unprepared for their action, or do 5β-reduced steroids possess hormonal activity in these early stages? Assuming the second hypothesis to be valid, an obvious target to look for a hormonal action would be porphyrin synthesis stimulated, as mentioned above, by 5β-androstane and 5β-pregnane derivatives.

The first and rate-limiting step of porphyrin biosynthesis is the formation of aminolevulinic acid (δ-ALA) from glycine and succinyl CoA (see Fig. 2). The iron-containing heme molecule at the end of the pathway, well known as the prosthetic group, not only of hemoglobin but also of cytochromes, represses this step in a classical way described for metabolite repression, while xenobiotics such as pesticides, barbiturates and other drugs induce the synthase of δ-aminolevulinic acid (δ-ALA S) (for review see [17]). A/B-*cis*-reduced androgen and progestagen, but not corticosteroid derivatives possess this same inductive effect while their A/B-*trans* conformers are either devoid of, or even have opposite effects [18].

One of the preliminary series of experiments leading to those described in the next section related the ontogenies of 5β-reductase and δ-ALA synthase in the chick adrenal. The results of these show a fair relationship between both enzymic activities during embryonic development. At early *ex ovo* stages the relationship persists but 5β-reductase evolves at a much lower level than ALA S [19].

But those findings and the fact that mammal steroidogenic glands, such as rat adrenals [20] and testes [21], exhibit strong δ-ALA S activities pose a new question, that of the usefulness of porphyrin synthesis in those organs. Even if activities are higher than in the liver, a presumable contribution of endocrine tissues to hemoglobin synthesis is, in view of those organs small masses, highly unprobable.

Part of the work described in what follows focusses therefore on Tofilon's postulate according to which porphyrin synthesis in steroidogenic tissues leads to the formation of heme-containing proteins of a different sort: the cytochromes *P*450 necessary for steroid biosynthesis [21].

EFFECTS OF 5β PREGNANEDIONE ON ALA FORMATION AND MICROSOMAL CYTOCHROME *P*450 IN CHICK ADRENALS

In the present work: (a) We investigate the effect of inhibiting steroidogenesis* on ALA formation in the adrenal of young chickens and the ability of low concentrations of 5β-pregnanedione to restore ALA formation; (b) we compare this restoring effect of 5β-pregnanedione to presumably similar effects exerted by four carefully chosen steroids in order to study the specificity of the 5β-reduced metabolite and (c) we study the effect of 5β-pregnanedione on microsomal cytochrome *P*450 of our model's adrenals and testes.

*"Steroidogenesis" is here heterodoxically employed as a synonym of "post-isomerase-steroid formation".

EXPERIMENTAL

Animals

Male 17-day-old White Leghorn chickens of approx. 170 g body weight were used. The standard diet and water were supplied *ad libitum*. Animals were killed by decapitation. Chickens were opened and livers, adrenals and testes were removed, dissected free of adherent tissue and weighed. All steps were performed at 0–4°C. Homogenates were prepared as described below.

In experiments to study the effect of 5β-pregnanedione on cytochrome *P*450, chickens were preinjected with 0.2 ml dimethylsulfoxide or 5β-pregnanedione (10 mg) dissolved in 0.2 ml dimethylsulfoxide, 24 h prior to killing.

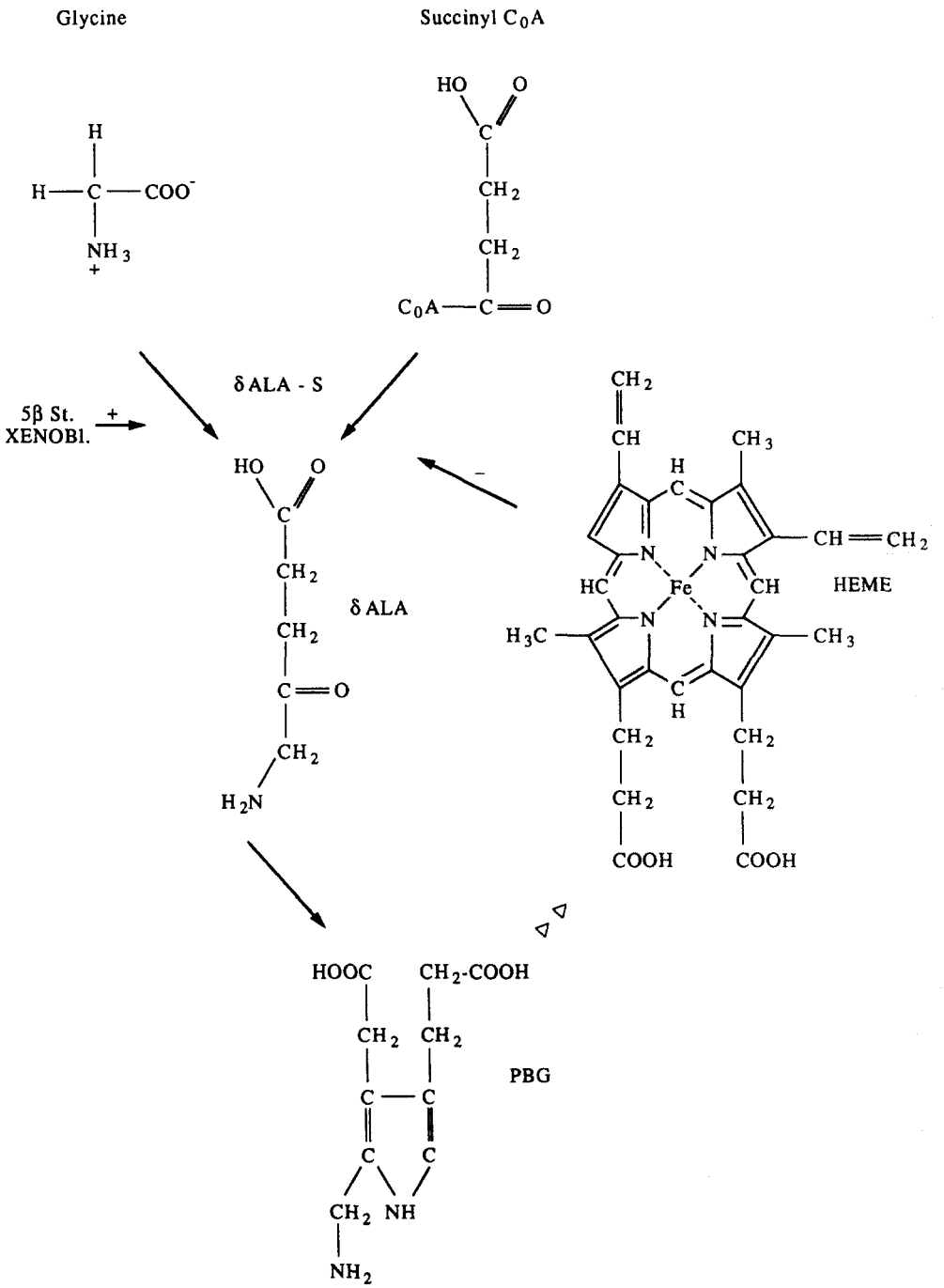

Fig. 2. Scheme illustrating the heme pathway. δ-ALA-S, aminolevulinic acid synthetase; δALA, δaminolevulinic acid; PBG, porphobilinogen; 5β-St, 5β-reduced steroid; XENOBI, xenobiotics.

Chemicals

Labeled compounds: 1,2[^{3}H]progesterone (50 Ci/mmol), 7[^{3}H]pregnenolone (19.3 Ci/mmol) and 1,4[^{14}C]succinic acid (53.5 μCi/mmol) were purchased from New England Nuclear (Cambridge, MA, U.S.A.), 5-δ amino [4-^{14}C]levulinic acid hydrochloride (42.2 mCi/mmol) was purchased from Amersham (Bucks, U.K.). δ-ALA, glutathione, ATP, CoA, pyridoxal 5-phosphate, Dowex 1 × 8 (200–400 mesh) and Dowex 50 × 4 (100–200 mesh) anion and cation exchange resins, as well as radioinert steroids were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Dimethylsulfoxide was purchased from E. Merck (Darmstadt, Germany). Sodium dithionite was obtained from Mallinckrodt (St Louis, MO, U.S.A.). All other chemicals were of analytical grade from various commercial sources.

Cyanoketone (2α-cyano,4,4,17α-trimethyl-androst-5-en-17βol-3-one), steroidal cyanoketone, was kindly donated by Winthrop Labs (Rensselaer, NY, U.S.A.) and spironolactone 3-(3-oxo-7α-acetylthio-17β-hydroxy-4-androsten-17α-yl) propionic acid δ-lactone was supplied by Dr Selva Cigorraga. Arcopal N-100 was a gift from La Química Hoechst S.A. (Argentina).

δ-ALA synthesis

The method of Tofilon and Piper [21] was adapted to the present experiments as follows:

Homogenates. Tissue homogenates 20% (w/v) were prepared in 0.2 M Tris–HCl buffer (pH 7.4) and centrifuged at 1000 *g* for 10 min.

Incubation mixture. The method used was the same as described previously [19]. The incubation mixture consisted of 1.5 μCi 1,4-[^{14}C]-succinic acid, Tris–HCl (pH 7.4) 100 mM, succinic acid 125 μM, glycine 100 mM, EDTA 1 mM, $MgCl_2$ 10 mM, ATP 0.5 mM, CoA 20 mM, pyridoxal 5-phosphate 0.1 mM, GSH 6 mM and 0.5 ml succinyl CoA according to Zaman *et al.* [22]. 1 ml Of supernatant from the homogenate was added to each incubation flask. Incubations were carried out in a Dubnoff incubator under an atmosphere of air at 37°C for 60 min.

To pertinent samples were added 10^{-5} M cyanoketone and/or spironolactone at the beginning of incubation.

Extraction and separation procedure. A two-step sequential ion-exchange column, as described previously [19], was employed to separate δ-ALA from succinate and other metabolites.

3β-ol dehydrogenase-isomerase assay

Incubation beakers contained 7-[^{3}H]pregnenolone, 2×10^6 dpm and pooled quartered chick adrenals. Some beakers contained 10^{-5} M cyanoketone, 10^{-5} M spironolactone or both inhibitors. Incubations were carried out as described by Goldman *et al.* [23] and [^{3}H]-progesterone formed during incubation was measured as follows: tissues and media were extracted in methylene chloride. To each extract were added 8000 cpm of [4-^{14}C]progesterone and the dried extracts were chromatographed in the TPG system [14, 19]. Overflows from these runs were developed in the Bush A paper chromatographic system. [7-^{3}H]progesterone was detected on paper strips by means of a Packard Model 7201 radio-chromatogram scanner. The radioactive progesterone zone was then quantitatively eluted, spotted on TLC and developed in heptane–ethyl acetate (3:1) at room temperature. The radioactive peak corresponding to progesterone was transferred to counting vials and counted in a Mark III model 6882 liquid scintillation counter fitted with a 4096 multichannel analyzer for quenching correction of each sample. The scintillation cocktail contained 4 g 2,5-diphenyloxazole (PPO) and 0.25 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] (dimethyl POPOP) per liter toluene.

Cytochrome P450 assays

Homogenates from livers, adrenals and testes were sedimented at low speed to remove debris, unbroken cells and nuclei with a Beckman J 2-21 refrigerated centrifuge. The mitochondrial fractions were obtained by centrifugation at 10,000 *g* for 30 min. The supernatant was further centrifuged at 105,000 *g* for 60 min in a Beckman L 8-55 refrigerated ultracentrifuge to yield the microsomal pellet. The sedimented cells were washed and suspended in 0.1 M potassium phosphate buffer solution (pH 7.4) containing 20% glycerol and 1.5 mM EDTA.

Cytochrome *P*450 was measured in microsomal fractions by the CO-binding spectrum according to Omura and Sato [24] using a Shimadzu UV-3000 recording spectrophotometer. The baseline was determined by scanning between 500 and 400 nm. Sodium dithionite was added to the microsomal suspension. The sample was then treated with carbon monoxide by bubbling the cuvette with CO for 2 min. The scan was repeated and the quantity of cytochrome *P*450 was determined by calculating the

differences (450–490 nm) in the presence and absence of CO. An extinction coefficient of 91 cm^{-1} mM^{-1} was used.

Protein determination

Homogenate and microsomal protein were determined by the method of Lowry *et al.* [25] using crystalline bovine serum albumin as standard.

Statistical analyses

Data were analyzed either by Student's *t* test or by a two factor ANOVA and Bartlett's homogeneity test. Contrasts *a posteriori* were performed for heterogeneous populations according to Tuckey–Kramer or Neumann Keuls as described by Stoline [26] and Sokal and Rohlf [27].

RESULTS

Comparison of the inhibiting effects of cyanoketone, spironolactone and the mixture of both inhibitors on progesterone and ALA synthesis

Figure 3 shows the effects of each inhibitor and their mixture on the production of [^{3}H]progesterone from [^{3}H]pregnenolone after 60 min of incubation.

It can be seen that: (a) cyanoketone is more efficient than spironolactone, and the mixture more efficient than individual inhibitors on [^{3}H]progesterone formation as well as ALA formation and (b) a fair linear correlation exists between the inhibiting effects on both syntheses. Neither cyanoketone, spironolactone or their mixture had any effects on ALA synthesis in the liver (Aragonés, Gonzalez and Lantos, unpublished results).

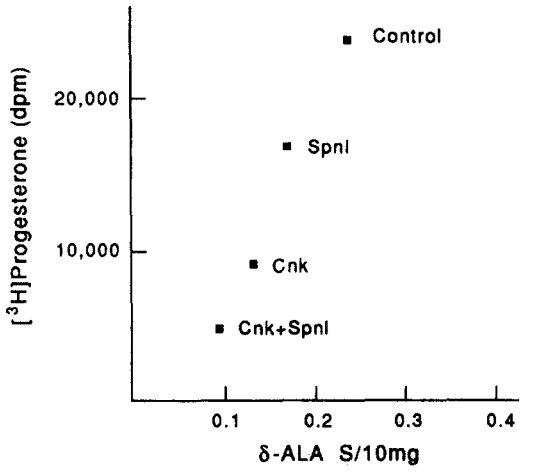

Fig. 3. Comparison of the inhibiting effects of cyanoketone, spironolactone and the mixture of both inhibitors on progesterone and ALA synthesis. Cnk, cyanoketone; Spnl, spironolactone. Y values: 2×10^6 dpm [^{3}H]pregnenolone were incubated with 60 mg tissue for 1 h; x values: nmol ALA/10 mg tissue/h. See Experimental for more details.

Table 1. Effect of 5β-pregnanedione on ALA synthesis after its inhibition by the mixture of steroid inhibitors

Group	ALA (nmol) formed per 10 mg tissue
Controls	0.020 ± 0.001
Inhibited	0.014 ± 0.001
Stimulated	0.038 ± 0.003
Restored	0.021 ± 0.06

Means ± SE of 4 samples. Inhibited: samples to which cyanoketone and spironolactone has been added at 10^{-5} M concentration; stimulated: control samples to which 10^{-7} M 5β-pregnanedione has been added; restored: inhibited samples to which 10^{-7} M 5β-pregnanedione has been added. Inhibitors and steroids were added at the beginning of the incubation. ANOVA (F: 6.50, $P < 0.01$) followed by *a posteriori* contrasts indicated that controls were higher than inhibited, and lower than stimulated samples ($P = 0.05$) while there were no differences between controls and restored samples.

Effect of 5β-pregnanedione on ALA synthesis after its inhibition by the mixture of steroid inhibitors

5 β-Pregnanedione at a 10^{-7} M concentration was able to restore ALA synthesis to control values in preparations in which this synthesis had been partially suppressed by the steroid-inhibiting mixture. The reduced steroid was also able to further stimulate ALA synthesis in uninhibited (control) samples (Table 1).

Effect of different steroids on ALA synthesis after its inhibition by the mixture of steroid inhibitors

In these experiments the ALA synthesis-restoring effect of 5β-pregnanedione was compared to presumably similar effects exerted by other steroids. In all cases two concentrations were assayed: a higher one, 10^{-6} M, corresponding to the contents of progesterone in adrenal tissue [28] and a lower one approaching tissue levels of each steroid. Hormone contents were chosen according to the following criterion: progesterone, average concentrations during development according to [28], 5β-pregnanedione, assuming 10% conversions of progesterone, according to [28]; 5α-pregnanedione, assuming conversions similar to those found for 5β-pregnanedione (actually these conversions are lower in the corresponding experiments [28]); corticosterone according to [29]; estradiol according to concentrations reported in ovaries [30].

The results show that only 5β-pregnanedione, at a 10^{-6} M concentration, increases ALA synthesis in the inhibited model. The other steroids slightly but significantly inhibit this synthesis further (Table 2).

Effect of 5β-pregnanedione on microsomal cytochrome P450 in adrenals and testes

Figure 4 shows the effect of a single dose of 10 mg 5β-pregnanedione on cytochrome *P*450 levels in adrenals and testes, as compared to hepatic levels. 5 β-Pregnanedione stimulates cytochrome *P*450 levels in both steroidogenic glands but not in the liver [31].

DISCUSSION

Early results analyzed in the first part of this publication give evidence that 5β-reductase is quantitatively important in early stages of the chick embryo's adrenal and left (functional) ovary, then decreasing as the levels of other hormones and metabolites increase. Findings from different laboratories are also mentioned according to which 5β-pregnane and 5α-androstane derivatives stimulate, as early as the blastodermic stage, the embryo's heme synthesis and its synthesis of globin chains corresponding to hemoglobins E and P [9, 10].

The blastoderm, on the other hand, already exhibits an A/B-*cis*-reductive pathway [9] and possesses cytosolic but not nuclear receptors for A/B-*cis*-reduced metabolites [10]. This embryonic biochemical and biological relevance of A/B-*cis* metabolism is by no means limited to the chicken. There is abundant evidence in the literature for the qualitative and quantitative importance of A/B-*cis* reduction in early life stages throughout the animal kingdom [16], even in arthropods, in which hormones eliciting molts from larval to pupal, and pupal to adult instars—ecdysones—are sterols with A/B-*cis* conformations. (For a review on function and structure of ecdysones see [32]). Figure 5 shows conformational similarities between 5β-pregnanedione and a widely distributed ecdysone, both exhibiting A/B-*cis* conformations as opposed to the A/B-*trans* conformation of 5α-pregnanedione. Because of this early appearance of the A/B-*cis* conformation and its ubiquity at larval stages spreading over a considerable portion of the phylogenetic universe, we usually refer to active A/B-*cis* metabolites as "protohormones" (see [16]).

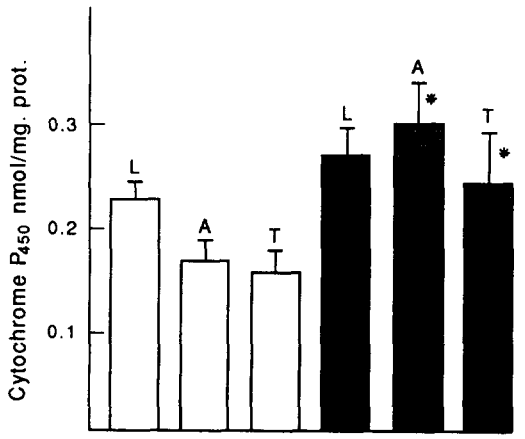

Fig. 4. Responses of liver (L), adrenals (A) and testes (T) to single injections (10 mg) of 5β-pregnanedione in 17-day-old chickens. Values are means ± SE of 4 experiments. *$P < 0.05$. □ controls, ■ plus 5β-pregnanedione. Redrawn from Ref. [31].

Table 2. Effect of different steroids on ALA synthesis after its inhibition by the mixture of steroid inhibitors

Steroid	ALA (nmol) formed per 10 mg tissue
Controls	0.025 ± 0.0001
5β-Pregnan-3,20 dione ○	0.044 ± 0.0008[a]
5β-Pregnan-3,20 dione ●	0.026 ± 0.0003[NS]
5α-Pregnan-3,20 dione (allo) ○	0.022 ± 0.0007[b]
5α-Pregnan-3,20 dione (allo) ●	0.022 ± 0.0006[c]
Progesterone ○	0.022 ± 0.0001[a]
Progesterone ●	0.022 ± 0.0004[c]
Corticosterone ○	0.019 ± 0.0009[c]
Corticosterone ●	0.019 ± 0.0007[c]
17β-Estradiol ○	0.018 ± 0.0004[a]
17β-Estradiol ■	0.018 ± 0.0007[a]

Means ± SE of triplicates. ○, 10^{-6} M, ●, 10^{-7} M, ■, 10^{-10} M. [a], $P < 0.001$; [b], $P < 0.02$ [c], $P < 0.01$; (Student's *t* test); [NS], not significant. Controls are samples containing the inhibitor mixture.

In line with these protohormonal characteristics, common 5β-reduced steroids of vertebrates, such as 5β-pregnanedione, regulate identical enzymes throughout the subphylum, a fact not only of evolutionary, but also of experimental importance since it makes models like the embryonic and young chicken more reliable for the study of human pathologies. In humans, research on the regulation of porphyrin synthesis by steroids has been largely motivated by certain hereditary forms of hepatic porphyrias whose common biochemical characteristics are: a defect in some of the later enzymes on the heme pathway; an accumulation of ALA and other low- molecular weight early porphyrin-precursors; as well as small 5α/5β ratios of urinary steroid metabolites. The best investigated form is acute intermittent porphyria (AIP), a disease linked to an autosomal dominant gene manifesting itself mainly, although not exclusively, in young postpuberal women. The patients exhibit neurological, psychiatric and CNS-dependent abdominal symptoms, and

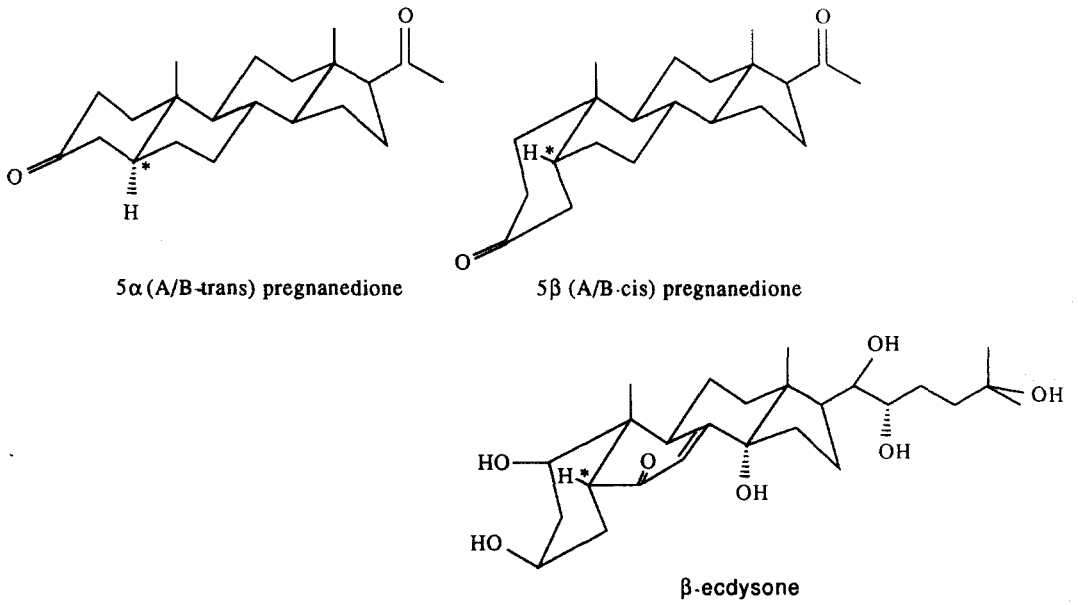

Fig. 5. Conformations of steroids described in the text. Although β-ecdysone has a 5β (A/B-*cis*) conformation, the greek letter indicates chronological order of product isolation.

a dominant pattern of inheritance associated with high levels not only of ALA but also of porphobilinogen (PBG). Associated enzyme abnormalities comprise an increase in ALA S and a decrease in uroporphyrinogen I synthase.

It is assumed that the resulting low heme production by the liver leads to a failure to repress the synthesis and activity of ALA S. But the disease expresses itself only in the presence of high concentrations of 5β-reduced steroids if they are both normal, as in two-thirds of adult human females [33], or caused by a deficient 5α reduction [34]. According to current ideas, low-molecular precursors of porphyrins consequently accumulate in the blood stream and may cross the blood–brain barrier, probably acting as precursors to neurotoxins in the CNS [35, 36]. The syndrome would be exacerbated by the stimulatory effects of certain 5β-reduced steroids on δ-ALA S (see [34] and Fig. 2).

We employed the 17-day-old chicken as a model for our recent studies. This model corresponds to young postpuberal vertebrate females [18] and has besides technical advantages sizeable amounts of adrenal tissue, as well as a presumably high stimulating activity (high ALA vs comparatively low reductase levels [19]).

We used all steroids at concentrations not surpassing 10^{-6} M and suppressed steroidogenesis at the steroid 3β-hydroxylase-isomerase level to a maximal degree, a procedure leading to a correlated decrease of ALA as well as progesterone yields (Fig. 3).

As indicated by lack of action on hepatic ALA, the inhibitor mixture did not inhibit *per se* ALA synthesis but depended, for this inhibition, on progesterone.

A second series of experiments demonstrated that the addition of 10^{-7} M 5β-pregnanedione to samples of the inhibited model restored in these samples ALA formation to normal values (Table 1).

A third series of experiments assayed the stimulating activity not only of 5β-pregnanedione but also of progesterone, 5α-pregnanedione, corticosterone and estradiol. These steroids were used in two concentrations, one corresponding to the endogenous progesterone contents of adrenals [28] and the other one, to the endogenous contents of each steroid in adrenals or ovaries. Only 5β-pregnanedione at 10^{-6} M was able to stimulate ALA formation in the inhibited model. In this experiment, at variance with the former, the A/B-*cis*-reduced steroid was inactive at the lower (10^{-7} M) concentration and we have no explanation for the lower sensitivity. One reason could be a slight difference in age between both chicken populations.

The important conclusion of this last experiment, however, is that at or around 10^{-6} M concentrations only 5β-pregnanedione stimulated ALA formation. Progesterone, as well as its 5α-reduced metabolite, corticosterone, and

estradiol inhibited ALA formation (Table 2). This observation had not been reported before.

Employing a suppression and replacement model classically used in physiology, these experiments indicate a specific role of 5β-pregnanedione, and probably also other 5β-reduced progestagens and androgens [11] for the stimulation of ALA in endocrine glands of the developing chicken.

An obvious question arising from the combined results of Fig. 1 and Table 2 is why the suppression of progesterone biosynthesis should inhibit ALA formation while added progesterone, at variance with added 5β-pregnanedione, would be inefficient to restore ALA formation. In other words, if the lack of 5β reduction of progesterone, in Fig. 3, is adequate to explain the lack of ALA synthesis, why then is 5β-reduction of added progesterone (Table 2) not sufficient to restore this synthesis while its added 5β metabolite accomplishes this function? The explanation may reside in differences of availability to the sites of 5β-reduction: endogenously biosynthesized progesterone (Fig. 1) might be more readily available to 5β-reductase, hence undergoing reduction earlier and therefore to a more significant degree than exogenously added progesterone. Moreover, the correlation found in Fig. 3 and the immediacy of the restoring effect in Tables 1 and 2 speak in favor of a non-genomic effect of 5β-pregnanedione. This hypothesis, although in need of direct experimental demonstration, is coherent with the lack of nuclear receptors, but presence of cytosolic receptors for 5β-reduced steroids in chicken embryos reported by several groups [9, 10].

The last series of experiments (Fig. 4) strongly suggests that 5β-pregnanedione stimulates ALA synthesis in order to increase cytochrome(s) *P*450 in the young animal's steroidogenic glands.

In line with the findings a hypothesis is cautiously advanced, according to which the progesterone 5β metabolite activates the synthesis of its hormonally active precursor, progesterone, and pathways leading to active glucocorticoids and androgens (Fig. 6). Since these experiments were carried out on microsomes, the second part of this working hypothesis has for the time being received better experimental confirmation. Preliminary experiments suggest a similar effect on mitochondrial cytochrome(s) *P*450. These ideas, while not denying the "inactivating" nature of the A/B-*cis* pathway from the gland's major functional point of view [37], would imply that an excessive deviation towards 5β metabolism could be homeostatically corrected by an A/B-*cis* metabolite-mediated activation of the main hormonal pathways.

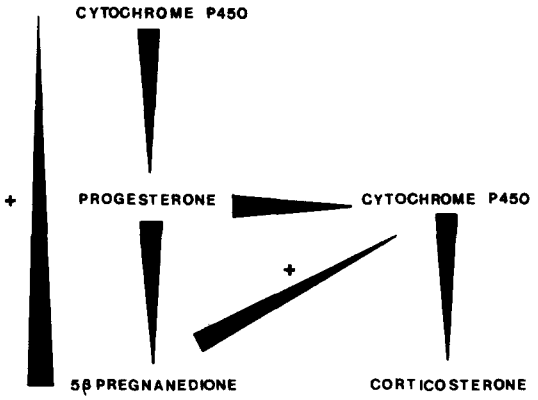

Fig. 6. Proposed mechanism through which 5β-pregnanedione regulates cytochromes *P*450. ▶+ indicates stimulation. (More details in the text.)

There can be no doubt that 5β-reduced steroids affect brain function. Of the steroidal effects published, however, only certain ones, reported by Kubli-Garfias *et al.* [38] require preferentially an A/B-*cis* conformation. The authors studied progesterone and 7 of its metabolites on EEG and multiunit activity of various limbic structures in the flaxedil-immobilized cat and found 5β-reduced progestagen steroids to inhibit neuronal discharge and synchronize EEG activity with shorter latencies than either their 5α epimers or progesterone. Although suggesting a blockade of calcium influx [39], the authors open a debate on the mechanism of steroid action in this neuromodulatory model. Since Hu *et al.* [40] have recently demonstrated cytochrome *P*450 scc and steroid formation in rat-brain cells, and since 5β-reductase has been known for years to be present in the brain of many species (see for example [41, 42, 37]) a mechanism similar to the one proposed in Fig. 6 should not be ruled out in these and other neurological and behavioural models and diseases.

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