RESEARCH ON STEROIDS

Proceedings of the Fourth Meeting of the International Study Group for Steroid Hormones

Edited by

M. FINKELSTEIN
C. CONTI
A. KLOPPER
C. CASSANO

Programme Committee

E. E. BAULIEU
C. CONTI
H. BREUER
J. K. NORYMBERSKI

VOLUME IV

1970

PERGAMON PRESS · OXFORD · NEW YORK
FRIEDR. VIEWEG + SOHN · BRAUNSCHWEIG

Library of Congress Catalog Card Number: 68–31485

ISBN 08 017573 2 (Pergamon)

ISBN 3 528 07673 9 (Vieweg)

Printed in Germany (W)
Druck: E. Hunold, Braunschweig

RESEARCH ON STEROIDS

Proceedings of the Fourth Meeting of the
International Study Group for Steroid Hormones

Volume IV

The Proceedings of the Fourth Meeting of the International Study Group for Steroid Hormones are sponsored by Schering AG, 1 Berlin 65, Müllerstraße 170

Preface

This is the fourth time that this 2-yearly gathering of research workers in steroid chemistry has been held. It has become for many of us an eagerly awaited occasion to broaden our friendship and stimulate our research imagination.

This year our attention is focused on the interaction between steroids and proteins, a study born fairly recently, when in 1958 Daughaday demonstrated the binding of cortisol by the alpha-globulin which he called CBG (cortisol binding globulin) and which was named transcortin by Slaunwhite and Sandberg. Our interest in these interactions has gradually grown with the identification of other binding proteins (albumin, orosomucoid etc.)

Whoever follows the study of the production, chemical characterization and metabolism of steroid hormones cannot fail to be attracted by the many aspects of steroid-protein interaction, an interaction which not only marks the first step in hormone action, but also the terminal event.

We are therefore dealing with a topic which like those discussed in previous meetings has significance in many fields.

Analysis of the physico-chemical alterations in the binding proteins, of their morphological changes in relationship to the different types of binding and the study of the binding sites at tissue level may all lead to a better understanding of the relationship between the structure and the function of the steroid hormones.

The specific proteins, in fact, appear to protect the circulating steroids from the clearance mechanism, carrying them towards the receptor organs, releasing them at tissue level and influencing their initial activity by means of the formation of hormone macromolecular complexes in the nucleus of the target cell.

With the increasing evidence available it has been possible to identify a transport pathology of steroid hormones, analogous with that already demonstrated for some polypeptide hormones. It is well known that there is an increase of transcortin in pregnancy and following estrogen treatment. Recently pathological conditions have been described in which one or more steroid hormones, otherwise considered normal from a blood concentration point of view, show a striking variation in peripheral behaviour which is related to the presence or absence of specific binding proteins. Typical of this finding are those forms of hirsutism in which a low level of protein binding 17BOH steroids is found or the case of the familial absence of transcortin passed as a dominating autosome.

It should not be forgotten that the steroid proteins binding are widely used for the determination of circulating hormones.

Perhaps the most striking implication of steroid-protein interaction is the possibility that these hormones acquire antigenic characteristics and that the way is open for their use in immunodiagnosis and immunotherapy. Studies in these fields which were begun about 10 years ago are still being developed and at the present time antibodies are available against various steroids, obtained by immunizing animals with hormone protein complexes. These are the well known antibodies which inhibit the circulating hormones and as such permit the study of the role played by the steroid hormones in various physiological processes. It should be possible to make a quantitative evaluation of these complexes by means of modern radio-immunological methods and in the not too distant future we may be able to correct the pathological picture caused by hypersecretion of steroids with specific antiserum treatment.

It is a pleasure once again to see that the International Study Group for Steroid Hormones has preserved the vitality which characterized its foundation and it is both comforting and stimulating to have so many scientists from all over the world drawn together by their mutual interest in scientific research.

I wish the meeting every success and take this opportunity to thank Schering AG. Berlin for supporting this Symposium.

My grateful thanks are extended to Prof. V. Caglioti, President of the C. N. R. (National Research Council) for allowing us to use the conference rooms, to the Dean of the Faculty of Medicine in Rome, Prof. A. Cimmino, who has honoured us by coming here today, and to all those who have contributed in the preparation and organization of the meeting.

December 1969

C. CASSANO

CONTENTS

General Aspects of Steroid-Protein Interaction

U. WESTPHAL

Biochemistry Department, University of Louisville School of Medicine, Louisville, Kentucky, USA

About 35 years ago B. Brunelli published the first paper on the binding of a steroid hormone to serum proteins (1). Its title, "Sulla 'Funzione Veicolante' delle Proteine Plasmatiche per l'Ormone Follicolare", i. e., on the carrier function of the plasma proteins for the estrogenic hormone, clearly shows the influence of the concept of the vehicle function of the serum proteins which had just been developed by H. Bennhold (2). The program of our present meeting illustrates to what extent the small door which Brunelli opened in Pisa in 1934 has been widened. New doors have been found and opened after the first one, and large new areas have been discovered in which interactions between steroid hormones and proteins play essential roles.

Figure 1 shows the main areas of steroid-protein interactions. Binding of steroids to *serum proteins* has been studied most extensively, and we know more about the properties of these complexes than about those of any others. The present discussion will be mainly concerned with the area of steroid-protein interactions, involving non-covalent bonding with different serum proteins. Perhaps of greatest biological interest is the interaction of steroid hormones with the *receptor proteins* of target tissues (3–6); this aspect clearly involves the mechanism of action of this important class of vertebrate hormones. However, our knowledge of the chemical nature of the receptor proteins is still limited.

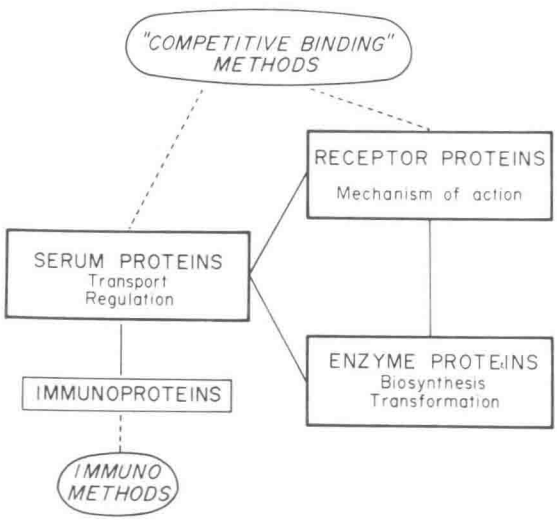

Fig. 1
Areas of research in Steroid-Protein Interactions

A special type of steroid binding to proteins is that to steroid-specific *enzymes* (7). Here the steroid plays the role of a substrate to an enzyme, and at the same time may be considered a ligand to a protein which has a high and specific affinity for the particular steroid. Obviously, the study of steroid-enzyme complexes is of interest for more than one reason, and results in recent years on the 3- and 17β-hydroxysteroid dehydrogenases, and especially the Δ^5-3-ketosteroid isomerase have demonstrated their usefulness for the investigation of fundamental problems of steroid-protein interaction. The Δ^5-3-ketosteroid isomerase of bacterial origin is the most highly purified steroid enzyme known today, it is also one of the most active catalytic proteins known. The homogeneous crystalline enzyme (Table I) [1] has a molecular weight of 40,800 and is composed exclusively of amino acids. It is free of cyst(e)ine and tryptophan residues. According to Talalay (7), the isomerase has 3 steroid-binding sites. These properties make the protein highly suitable for a study of the relationship between chemical structure of the binding site and its affinity for steroids. We may continue to expect valuable results on these fundamental problems from the analysis of steroid-enzyme interactions. A promising approach is seen in the affinity-labeling studies in Warren's laboratory (8).

TABLE I. Δ^5-3-Ketosteroid Isomerase (7)

Molecular Weight	40,800
$S_{20,w}$	3.3 S
Amino Acids	389; no cys; no trp.
Rate for Δ^5-androstene-	$1.7 \times 10^7 min^{-1}$
K_m 3,17-dione (25°, pH 7)	320 μM
K_i (19-nortestosterone)	5.2 μM
n	3

As may be expected from any fundamental research in a significant area, the study of steroid-protein interaction has contributed important fringe benefits to the field of steroid hormones (Fig. 1). One of them is the use of the binding proteins as reagents to measure very small quantities of steroid hormones. Displacement of radiolabeled steroid by the test steroid in a *competitive binding system* introduced more than 6 years ago by Beverly Murphy (9) is the basis for many procedures of highly sensitive steroid determinations. Increased specificity has been obtained by rigorous purification of the steroid prior to binding analysis. The principle of this competitive binding method has also been applied for an assay of the binding protein.

[1] Abbreviations used in this report: AAG, α_1-acid glycoprotein or orosomucoid; CBG, corticosteroid-binding globulin or transcortin; HSA, human serum albumin; k, association constant; n, number of binding sites; $\bar{\nu}$, average number of steroid molecules bound per molecule of protein; [S], concentration of unbound steroid.

A field of research closely related to the serum proteins may be considered as a second fringe benefit, namely the study of immunoproteins. *Immunoproteins* have been produced against protein-conjugated steroid hormones as antigens (10). These reactive antibodies have certain properties in common with specific steroid-binding serum proteins and with cellular receptor proteins. The unique design of these studies is to let nature produce binding sites which are tailor-made for a given steroid. The immunoglobulins can be applied for specific hormonal inactivation and for sensitive radio-immunoassays. The further development of this more recent application of steroid-protein interaction can be looked forward to with great interest and expectation.

Binding of steroid hormones to serum proteins was originally considered important for *transport.* A closer look at steroid solubilities in aqueous media showed, however, that such function is not necessary for steroid hormones under most conditions because of sufficiently high water solubility. Now there is renewed interest in a possible transport function of steroid-binding serum proteins in connection with the entrance of the hormone into specific target cells and permeation through nuclear membranes. This question and that of the chemical relationship between the specific carriers in the serum and the receptor proteins, as well as other problems are waiting to be answered.

Another consequence of steroid hormone interaction with serum proteins is perhaps more obvious. It has been found in all cases investigated so far that the formation of the protein complex suppresses the *biological activity* of the steroid hormone (Table II). This was shown many years ago for the corticosteroid hormones

TABLE II. Suppression of Hormonal Function by Protein Binding

Steroid	Protein	Authors	Ref.
Cortisol	CBG	Slaunwhite *et al.* (1962)	(11)
Cortisol	CBG	Sandberg and Slaunwhite (1963)	(12)
Corticosterone	CBG	Gala and Westphal (1965)	(13)
Corticosterone	CBG	Kawai and Yates (1966)	(14)
Cortexone (DOC)	Albumin	Blecher (1964)	(15)
Progesterone	AAG	Westphal and Forbes (1963)	(16)
Progesterone	Albumin	Hoffmann *et al.* (1969)	(17)
Progesterone	CBG	Hoffmann *et al.* (1969)	(17)
Progesterone	CBG	Billiar *et al.* (1969)	(18)

by Slaunwhite and Sandberg (11, 12), and subsequently by other investigators. The hormonal activity of progesterone is equally suppressed by complex formation with the three serum proteins, albumin, α_1-acid glycoprotein (AAG or orosomucoid), and corticosteroid-binding globulin (CBG or transcortin). It follows clearly as a consequence of the inactivation, that changes in the concentration of the binding proteins,

especially those with high binding affinity and low capacity, would provide a *regulatory mechanism* of hormonal function. Similarly, protein interaction may protect the circulating steroid hormones from chemical or enzymatic attack.

The steroid-protein complexes best known today are those between the steroid hormones and three *serum proteins,* which are available in pure form and are relatively well characterized: albumin, α_1-acid glycoprotein and corticosteroid-binding globulin. Table III shows that these three types of steroid-binding proteins have molecular weights of the same order of magnitude. It should be mentioned that according to unpublished results from Dr. Baulieu's laboratory, the sex steroid-binding β-globulin, which has a high affinity for testosterone and for estradiol, also has a molecular weight of 52,000 (19); about twice this size has been reported from other laboratories [2].

TABLE III. Human Serum

Components	Molecular Weight	Concentration	
		mg/l	10^{-7}M
HSA	69,000	38,000	5,500
AAG	41,000	750	180
CBG	52,000	36	7
Progesterone	314	0.01	0.3
Cortisol	362	0.1	2.8

The table shows the great differences in the concentration of the steroid-binding proteins in the blood serum. A comparison with the hormone concentrations, which are given only as orders of magnitude, shows that the CBG capacity for the corticoids and other steroid hormones is not much greater than the normal steroid level. A quantitative relationship of this order would appear to be required for efficient regulation of hormonal function. Table IV indicates that human serum albumin (HSA) has three binding sites for Δ^4-3-ketosteroids. The glycoproteins, AAG and CBG, have one binding site for the C_{21}-steroid hormones. The table also shows the great differences in association constants of the complexes, which seem to be in an inverse relationship to their serum concentrations. The loss of binding affinity at elevated temperature becomes greater for the complexes with higher association constants.

In all these cases, the relatively nonpolar progesterone is bound more firmly than cortisol with its five oxygen functions. This is an indication of the essentially hydrophobic nature of the non-covalent bonds between steroid and protein. Such binding

[2] cf. O. Crepy and J. Guériguian; report published in this volume

TABLE IV. Apparent Association Constants of Steroid-Protein Complexes; $M^{-1} \times 10^{-5}$

Protein	Temp. °C	n	Progesterone	Cortisol
HSA	4°	3	1.0	0.1
	37°		0.5	0.1
AAG	4°	1	10.0	0.20
	37°		4.0	0.15
CBG	4°	1	7,000	5,200
	37°		900	240

relationship is well known for interaction of many ligands with albumin; it was first noted for steroid associations with bovine serum albumin in Samuels' laboratory (20) and has been described by the polarity rule.

Validity of the *polarity rule* has been demonstrated for numerous steroid interactions with serum albumin and AAG, and has also been observed with human CBG. However, CBG's of other species may be different in their relative affinities (21), as evident in Table V. Whereas progesterone is bound more firmly than cortisol to human CBG (22), the association constants are about the same for rat CBG. The

TABLE V. Apparent Association Constants of CBG Complexes; $M^{-1} \times 10^{-7}$

CBG	Temp. °C	Progesterone	Cortisol
Human	37°	9	2.4
Rat	4°	30	30
Rabbit	4°	40	90

polarity rule relationship is reversed in the case of rabbit CBG which binds cortisol with higher affinity than progesterone. The CBG's of these three species, which have been isolated as pure homogeneous glycoproteins (23–25), thus reveal a *species specificity* which is also evident in their physical molecular properties, their amino acid composition and their carbohydrate content.

Representative *thermodynamic parameters* of the steroid-protein complexes are given in Table VI. The negative free energy change, ΔF°, indicates spontaneous association in all cases. The entropy changes, ΔS°, are positive for the HSA and AAG complexes. This may be interpreted as randomization of the water molecules which had been in an ordered state as hydration water surrounding the protein and the steroid molecules. In the CBG complex, a negative entropy change shows that this effect is overcome by a very tight fit between steroid and protein, accompanied by a decrease in heat content much greater than those seen with the HSA and AAG complexes. The thermodynamic parameters for complexes of other steroid hormones with these proteins closely resemble those in Table VI.

TABLE VI. Thermodynamic Parameters of Protein Complexes with Progesterone (P) and Cortisol (F)

Protein	Steroid	ΔF° 4°	ΔF° 37°	ΔH°	ΔS°
		kcal/mole		kcal/mole	cal mole^{-1} deg^{-1}
HSA	P	−6.3	−6.6	−4.0	+9
AAG	P	−7.7	−8.1	−4.1	+13
CBG	F	−11.0	−10.5	−15.7	−17

ΔF°, ΔH°, ΔS°: Apparent change of free energy, enthalpy and entropy, respectively

The stability of a steroid-protein complex and accordingly the binding distribution of a steroid in a system containing more than one binding protein should be adequately described by the *number of binding sites,* n, and the *association constant,* k, at a given temperature and pH. However, in practical experiments, and in an analysis of steroid interaction with the binding proteins in blood serum, additional factors have to be considered. One of them is the presence of *lipid contaminations* which inhibit steroid binding. A second factor is interference with the steroid-protein interaction by traces of *heavy metal ions* present as contaminants.

The effect of *lipid* is illustrated by the interaction of progesterone with HSA (Fig. 2); analogous examples have been observed with AAG complexes. It is known that pure crystalline serum albumin contains small amounts of fatty acids of various

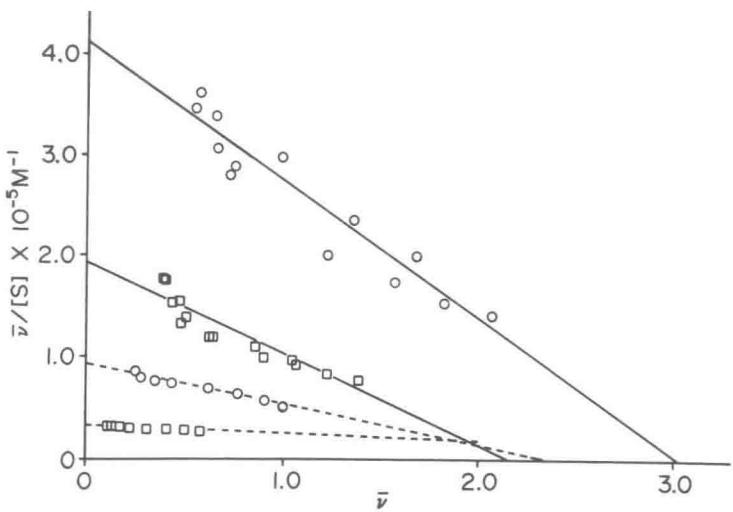

Fig. 2. Scatchard analysis of effect of addition of lauric acid to HSA (molar ratio 5:1) on its progesterone-binding activity. Phosphate buffer, 0.05 M, pH 7.4; 4 °C, ○ progesterone □ progesterone plus lauric acid ——— HSA delipidated - - - - - HSA, not delipidated