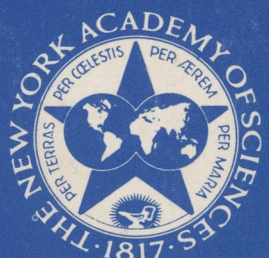
ANNALS OF
THE NEW YORK ACADEMY
OF SCIENCES

VOLUME 286

BIOCHEMICAL ACTIONS OF PROGESTERONE AND PROGESTINS



Editor
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UBLISHED BY THE NEW YORK ACADEMY OF SCIENCES
ANYAA9 286 1–449 (1977)

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

Volume 286

BIOCHEMICAL ACTIONS OF PROGESTERONE AND PROGESTINS

Edited by Erlio Gurpide

The New York Academy of Sciences
New York, New York
1977

Library of Congress Cataloging in Publication Data

Main entry under title:

Biochemical actions of progesterone and progestins.

(Annals of the New York Academy of Sciences; v. 286)

Includes bibliographical references.

1. Progesterone-Congresses. 2. Progestational hormones—Congresses. I. Gurpide, Erlio, 1927– II. Series: New York Academy of Sciences. Annals; v. 286. [DNLM: 1. Progesterone—Physiology—Congresses. 2. Progestational hormones—Physiology—Congresses. W1 AN626YL v. 286 / WP530 B615 1976]
Q11.N5 vol. 286 [QP572.P7] 508'.1s
ISBN 0-89072-032-0 [599'.01'6] 77-3994

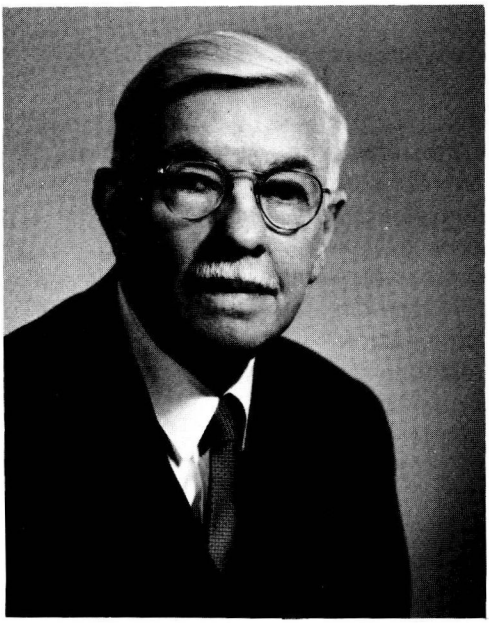
SP

Printed in the United States of America

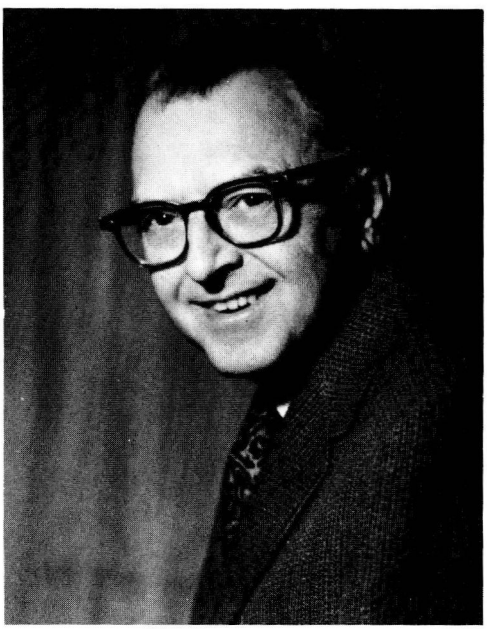
ISBN 0-89072-032-0

Financial assistance was received from:

- ALZA RESEARCH
- THE CAMILLE AND HENRY DREYFUS FOUNDATION, INC.
- HOFFMANN-LA ROCHE INC.
- THE LILLY RESEARCH LABORATORIES
- MEAD JOHNSON
- NATIONAL FOUNDATION MARCH OF DIMES
- ORTHO PHARMACEUTICAL CORPORATION
- SCHERING CORPORATION
- SEARLE LABORATORIES
- THE UPJOHN COMPANY



Dr. George W. Corner, Sr. (*top*) and Dr. Willard M. Allen (*bottom*), pioneers in progesterone research (see Dr. R. O. Greep's article in this volume), were guests of honor and speakers at the banquet held in occasion of the Conference on Biochemical Actions of Progesterone and Progestins, May 6, 1976.



THE GENESIS OF RESEARCH ON THE PROGESTINS

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To this group of biochemically oriented reproductive endocrinologists, progesterone and its collective family of chemical cousins the progestins are as familiar as apple pie. Noting, too, that approximately 100 million women have at one time or another used a progestin for the prevention of conception, it might be rightfully assumed that the term "progestin" had become a household word. It is not so. Although the future welfare of humankind rests more heavily at this point in history on the progestins than it does on atomic weapons, few of the world's citizenry, even of the intelligent laity, have any knowledge as to what a progestin is or can do. Still less do they realize that without progesterone they would not have been brought into this world. Should there be among you any who disagree, I suggest that the next time your seatmate on an airplane trip inquires as to your line of work you answer *progestins* and watch the expression that comes over the face of your questioner as he or she takes your measure. The gulf that separates science and society will suddenly widen and bring the conversation to a close. What surprises me more and fills me with lamentations is that many students emerging from college and heading for a career in medicine or biology have either never heard of progesterone or have only the haziest notion as to what it really is, much less what function it serves in the biology of the mammalian world.

What the progestins *are* and what they *do* are now well understood. What occupies the attention of this body of scientists is *how* they do it. The pursuit of this line of investigation is in the hands of a new generation of progestinologists skilled in molecular biology and genetics. An old era has passed into history, and a new one has been opened. There is an old axiom to the effect that those who do not know history are doomed to repeat it. That, however, does not necessarily hold true in scientific research. Were it so, all of our new recruits to this field would initiate their research by studying the history of progestins rather than molecular biology. In science, as we know all too well, it is possible to ignore history beyond the past five years and continue to work effectively at the most advanced level of research. History is something that is left to those who have passed the menopause of their professional career and are presumed to have acquired what some are inclined to call perspective.

That brings me to the business at hand. I was asked to speak to the genesis of research on the progestins, which means that I am expected to provide some historical perspective to the substance of this conference. The dimensions of perspective vary over a very wide range, and I suspect that the answer one would get to the genesis of research on the progestins would depend on the age, experience, and interest of the respondent. The fledgling investigator would likely guess that the genesis of research on progestins occurred about five to 10 years ago with concern as to their interaction with receptors. A more advanced teacher-investigator type who had found it necessary to dig back into the ancient literature might well come across some relevant work in the early 1950s by Pincus and Chang[1] and Rock *et al.*[2] A now greying and distinguished authority having been

invited to write a textbook would discover among the dust-laden and discoloring volumes dating back to the Prohibition Era works of great historical importance by such pioneering investigators as Corner and Allen,[3] Fevold and Hisaw,[4] Fels,[5] Slotta *et al.*,[6] Butenandt,[7] and Makepeace *et al.*[8] These works pertain to the extraction, purification, isolation, structure, synthesis, and function of a luteal secretion, the existence of which had already been substantiated. Let me interject here that my participation in research on the progestins around 1930 was limited to snipping thousands of corpora lutea out of sow's ovaries as raw material for the extraction studies of Hisaw *et al.*[9] What I received in return as a graduate student was the exhilarating privilege of being part of a team, plus a lot of what falls into the hopefully beneficial category of experience. Was this, then, the era in which it all began? Not at all. This was but the culminating thrust that capped a long series of important advances made during the previous 30 years by such stalwarts as Prenant, Born, Fraenkel, Marshall, Heape, Loeb, Ancel, and Bouin.

Employment of the experimental method as a means of elucidating the origin and functional significance of the luteal body stems only from the beginning of this century. Beyond that point lie 2000 years of the dialectical approach—of bewilderment, curiosity, erroneous observations, and unfounded speculation. The wheels of progress sometimes stood still for very long periods. A piece of dogma propounded in the third century B.C. by Heterophilus of Alexandria[10] held that the generative organs in women were female testes. This totally erroneous concept was still accepted as late as the sixteenth century by Andreas Vesalius,[11] the great Flemish anatomist and author of one of the early classics in anatomy, *De Humani Corporis Fabrica*. Vesalius illustrated the female testes as being connected to the uterus by a duct not unlike the male vas. Fallopius,[12] in 1561, was the first to determine that the tubes that still bear his name are not attached to the testes. Fabricius studied the hen and in 1621 reported that he could see very clearly that the structure of the generative organ of the female bore no resemblance to that of the testis and proposed the term "ovarium."[13] As is well known, it was Reinier de Graaf,[14] the Flemish physician, who in 1673 provided an authentic description of the ovarian vesicles that also still bear his name. He was a contemporary and compatriot of van Leeuwenhoek, who in 1677 observed the sperm in semen.[15] de Graaf named these large fluid-filled vesicles "ova" due to their strong resemblance to the ova seen in bird ovaries. de Graaf also took note of certain other structures on the ovarian surface, which he referred to as globules. By a stroke of what must be considered observational genius at that juncture in history, de Graaf also determined that the number of these globules corresponded with the number of fetuses in the various mammals that he dissected. These globules had also been seen somewhat earlier in human ovaries by Fallopius,[12] who described them as yellow bodies. More than 100 years later, Malpighi, in 1687, coined the term "*corpus luteum*."[16] He deduced quite incorrectly that they gave rise to the follicles and that the yellow substance, like egg yolk, served to nourish the ovum. Mind you that at this time no knowledge existed as to the true ovum or of ovulation. Nearly another 150 years were to pass before the mammalian ovum was first observed in 1827 by the Russian anatomist Karl Ernst von Baer,[17] whose observations also revealed the actual relationship of the ruptured follicle to the formation of the corpus luteum. von Baer was severely criticized by a succeeding generation of anatomists who through faulty techniques and timing failed to find the ovum in the fallopian tubes. Later, in 1857, Robin advanced the notion that the corpus luteum served only to heal the ruptured follicle and prevent the formation of scar tissue.

Following von Baer's epoch-making discoveries, a new problem arose. It

concerned the presence of corpora lutea in virgins. Because the corpora lutea were known to be associated with pregnancy, it was natural to assume that their presence was related to coition. Since it was unknown that corpora lutea exist only during the second half of the cycle, their presence was reported by some observers and as vehemently denied by others.[20] As an interesting sidelight to this controversy, George Corner[21] noted an instance in which the innocence of a dead woman was once sworn away in an English court because her ovaries were found to contain a corpus luteum.

The relationship of corpora lutea to the menstrual cycle was not established until early in this century, by Meyer.[22] These bodies were first considered false or abnormal and were termed "corpus luteum spurium," as opposed to the true corpus, or luteum gravidarum or verum.

It was not until near the end of the last century that any serious attention was given to the possible physiologic significance of the corpus luteum. Keep in mind, however, that at this stage, the ovary had no known function other than the production of ova. This was an amazing circumstance, considering that an endocrine function of the male gonad had been established 50 years earlier.[23] All that was needed was a study of the effects of removing the female gonad. Similar surgical ablations had been practiced on males since ancient times. This discrepancy can no doubt be accounted for on the basis of accessibility. In fact, surgical removal of the ovaries became quite a common practice around 1895, but the results were highly controversial. In 1896–1900, Emil Knauer[24,26] reported that the involution of the genital tract seen after ovariectomy could be repaired by ovarian transplants. His results were challenged by an outpouring of reports that claimed that ovariectomy in sheep, dogs, cats, monkeys, and even women did not interrupt the cyclic appearance of heat or menstruation. Marshall[19] cites a series of clinical papers published between 1895 and 1904 that reported the occurrence of pregnancy after removal of both ovaries and in one case of the tubes as well. By hindsight, one would assume that the women were already pregnant at the time of surgery, but the recurrence of menstruation or heat is another matter. In 1905–1907, Marshall and Jolly confirmed Knauer's work and established beyond doubt that bilateral removal of the ovaries in rats and dogs led to involution of the tract and cessation of all reproductive functions.[27–29] They also proved the effectiveness of ovarian transplants and, with typical British genteelness, attributed the contrary findings of others to "inadequate surgery." Thus came the first clear evidence that the ovary exercised control over the rest of the genital system, but the mechanism was still unknown. In 1905, Walter Heape[30] postulated that a "generative ferment," probably from food or the environment, acted on the ovary, stimulating it to secrete a hypothetical material, which he termed "gonadin." His "generative ferment," of course, became the pituitary gonadotropins of today, and his postulated "gonadin" was the ethereal forerunner of our present-day estrogens.

Prenant,[31] impressed by the unfailing presence of corpora lutea during pregnancy, proposed in 1898 that these structures might be ductless glands and concurred with Beard's[32] earlier suggestion that they might also be responsible for the inhibition of ovulation during pregnancy. Much credit must go to Gustav Born, a German embryologist, who in 1900 conceived the idea that the corpus luteum might aid in the attachment of embryos to the uterus by providing a nourishing substance. Being terminally ill, Born bequeathed his idea and how to test it to his student Ludwig Fraenkel. The idea was to mate rabbits and then within the first six days remove the ovaries or destroy the corpora lutea by cautery. The experiments were performed, and the rabbits did not become

pregnant. The results were published in 1903[33] and became the first firm landmark on the road to the discovery of the progestins. The choice of the rabbit for these experiments was fortuitous, because pregnancy in that species is dependent on the continuing presence of the ovaries. In several others it is not. Fraenkel came under such severe criticism by others who demonstrated that removal of the ovaries, especially from pregnant guinea pigs and monkeys, had no effect on the course of pregnancy that he was forced to repeat his work in 1910. The results were the same.[34] The key to the difficulty was species difference and the timing of the surgery during gestation. The impact of Fraenkel's work was, nevertheless, so great that it placed the corpora lutea in the position of appearing to produce the one and only ovarian hormone. Marshall[19] remained skeptical. He knew that seasonal breeders very often came into heat with no corpora lutea in their ovaries. He very cautiously proposed the possibility that the ovary might produce two kinds of secretions, one of which would induce the phenomenon of heat. The true picture was beginning to emerge, but the outline remained hazy.

What these suggestive studies at the turn of the century did was open an unsavory era of ovarian organotherapy. Saline or glycerin extracts were prepared from fresh or dried ovaries of any source and were administered to women for every conceivable reproductive dysfunction. The results were generally satisfactory, especially to the doctors. Such extracts could not have contained more than a trace of hormonal activity at best. This inglorious chapter in the history of reproductive endocrinology persisted into the 1930s, when authentic ovarian hormones became available for therapeutic use.

This bleak era was relieved by a few notable contributions. In 1907–1908, Leo Loeb demonstrated that the corpus luteum was essential for the development of deciduoma,[35,36] and in 1910, Ancel and Bouin[37] described the remarkable modification of the uterine mucosa that occurs in rabbits made pseudopregnant by mating with a vasectomized buck.

What opened the field of reproductive endocrinology to an explosion of meaningful research was the discovery by Allen and Doisy in 1923 of the estrous-inducing hormone in follicular liquor obtained from hog ovaries.[38] Asdell and Marshall[39] demonstrated that this new estrogenic hormone could not induce what Corner[40] termed "progestational proliferation." The succeeding demonstrations[3,41,42] in the late 1920s that crude extracts of corpora lutea would produce many of the normal physiologic functions attributed to that body were electrifying. They forged the final link in the chain of evidence that pointed to the existence of a luteal hormone and opened the way to its isolation. Early entrants to this second great quest of the ovarian hormones were Hisaw and Fevold, Corner and Allen, Slotta and Fels, and Parkes and Bellerby. The latter pair dropped out early but not before preparing an ether extract that inhibited estrous and ovulation in mice.[41] The extract prepared by Hisaw's group was shown to induce deciduoma formation,[42] inhibition of ovulation,[42a] and a premenstrual endometrium in monkeys.[43] Hisaw at that time was also much concerned with the effect of another luteal activity, relaxin.[44] Allen and Corner put their luteal extract to the ultimate test as a substitute for the natural hormone by showing that in rabbits castrated 18 hr after mating, it would permit implantation and sustain fetal development to term.[45,46] They also developed a suitable quantitative bioassay based on graded modification of the uterine mucosa induced in castrated adult rabbits by different dosages of the luteal extract.[3,47] This bioassay proved to be an enormous aid in their purification studies.

The hot methyl alcohol used by Corner and Allen as the initial extraction medium[3,47] was a modification of that employed much earlier with moderate but

unrecognized success by Hermann.[48] Hisaw's group started out using an acid-alcohol extraction[3] medium. Although this medium gave a good yield of the relaxative hormone, the yield of progestational activity was less than optimal, whereupon they changed to a slight modification[50,51] of the extraction procedure employed by Allen and Corner.[3,47] By 1930, several laboratories were able to report progress in purifying the hormone termed "progestin" by Corner's group, "corporin" by Hisaw's group, and "luteosterone" by Slotta *et al.* The major purification efforts took place between 1930 and 1934.

Discounting the poorly characterized crystals reported by Fels[5] in 1931, credit

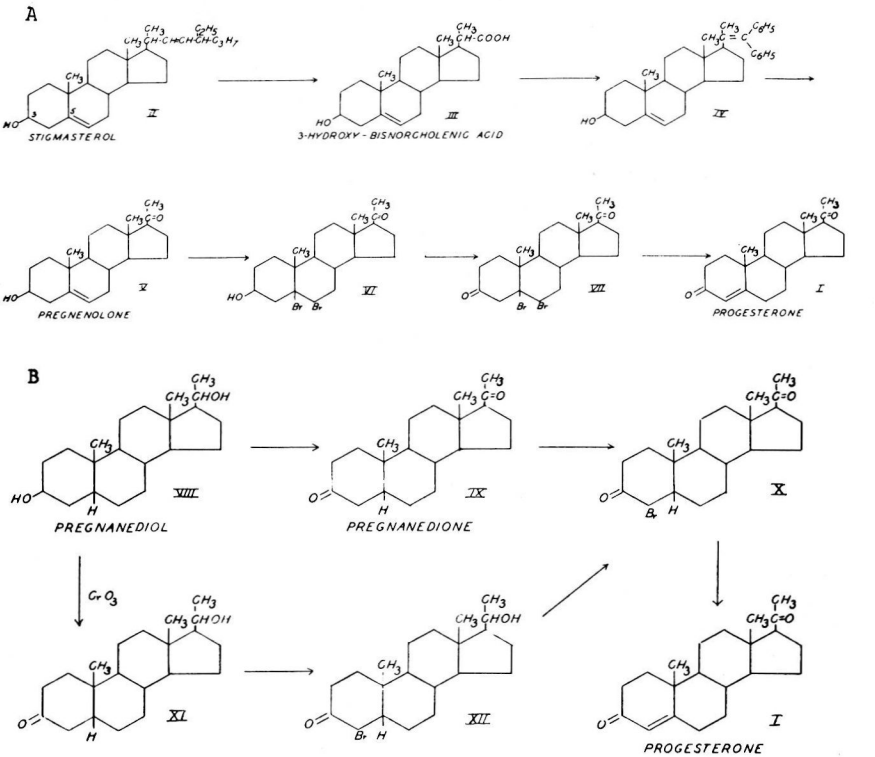

FIGURE 1. The historic synthesis of progesterone from stigmasterol and pregnanediol by the combined chemical ingenuity of Butenandt and Westphal[57] and Fernholz.[58] Stigmasterol was chosen because Fernholtz had already demonstrated a means of cleaving the side chain. Pregnanediol was an especially promising choice, because Butenandt had previously determined its chemical structure and knew that it was excreted in abundance during periods of active luteal function. A: The unsaturated diphenyl compound (IV) was produced by treatment of compound III with phenylmagnesium bromide. Oxidation yielded the 3-hydroxy-20-ketopregnene (V). The use of bromination to protect the double bond during further oxidation to progesterone was a technique conceived by Fernholz. B: The simple conversion of pregnanediol to progesterone made similar use of bromination of the diketone (IX) or the 3-keto-20-hydroxypregnene (XI) to yield the 4-bromodiketone (X). The bromine was removed with anhydrous pyridine to yield progesterone. (From Allen.[59] By permission of The Williams and Wilkins Co.)

for having achieved the first progestationally active crystalline product goes to Fevold and Hisaw,[4] as reported in 1932. The crystals proved not to be chemically pure, and further attempts at isolation of the luteal hormone by these workers were essentially discontinued. In the same year, Allen[49] also obtained a crystalline product. His crystals were of much greater potency than those of Fevold, but, again, they did not stand up to additional tests of chemical purity.

Further purification work performed by Allen left him with two crystalline products of about equal potency but with different melting points. He then turned to a collaborative effort with Wintersteiner at Columbia University. By fractional crystallization, they obtained four crystalline compounds, termed A, B, C, and D. A and D were inactive, while B and C were about equally active. B and C melted at 128° and 120–121°C, respectively. B, the luteal hormone, had the empirical formula $C_{21}H_{30}O_2$. The time was 1934, and it appeared the race had been won, but their results were not yet published. As in many dramatic situations, a glittering dark horse from the continent, Butenandt, came up from behind and sprinted to the finish line. He announced to a meeting of the Deutsche Gesellschaft für Innere Medizin on April 11th that he had obtained crystals with potent progestational activity and with a melting point of 128°C. The details of his work appeared in July[7] and gave the formula as either $C_{21}H_{30}O_2$ or $C_{20}H_{28}O_2$. A few days later, he and Westphal reported the definitive formula as $C_{21}H_{30}O_2$.[52] Allen and Wintersteiner's preliminary announcement appeared on August 24th,[53] and that of Fels *et al.* was dated only one day later.[54] Compounds C and D of Fels *et al.* proved to be identical with compounds B and C of Wintersteiner and Allen,[55] and both agreed with the findings of Butenandt.[7] The conclusion was that progesterone is a polymorphous substance.

The structure of progesterone as proposed in August by Slotta *et al.*[6] on a purely speculative basis was proven correct, and its synthesis was achieved almost simultaneously and in record time by Butenandt *et al.*[56,57] and by Fernholz[58] (FIGURE 1).

By common agreement of the Advisory Committee on the Nomenclature of Endocrine Principles of the Council on Pharmacy and Chemistry of the American Medical Association, this new jewel in the endocrine crown was officially named "progesterone."[58] Both progesterone and its related compounds that have a similar action were given the generic name "progestins."[58] There you have the genesis of research on the progestins as seen by one observer. Considering that the progestins had been in existence for hundreds of millions of years before being brought into the light of human knowledge, it is fitting and proper that we celebrate their delayed birth to science and ask now how it is that they accomplish the benefits that they have so long conferred on the mammalian world.

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PROGESTERONE BINDING TO SERUM PROTEINS*

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Progesterone is bound in the blood serum of humans and most other mammalian species to three proteins: albumin; corticosteroid-binding globulin (CBG), or transcortin; and α_1-acid glycoprotein (AAG), or orosomucoid. Whereas human serum albumin (HSA) consists of a folded polypeptide chain free of carbohydrate, AAG and CBG are glycoproteins with relatively high sugar content (TABLE 1). The binding of the steroid hormones to the serum proteins is mediated essentially by hydrophobic forces and hydrogen bonds; noncovalent complexes are formed that are dissociable. The rate of dissociation increases with rising temperature.

An inverse relationship exists between the serum concentrations of the three proteins and their affinity for progesterone. The association constant of CBG for progesterone at 37°C is approximately 500 times higher than that of HSA, but there are about 800 HSA molecules for each molecule of CBG in normal blood serum. This is the reason why the participation of HSA in the binding of steroid hormones is substantial[1,2]; about half of the progesterone in pregnancy serum is associated with albumin (TABLE 2). A smaller percentage is albumin bound in the case of cortisol, a steroid of more hydrophilic nature than is progesterone.

The biologic significance of protein binding of the steroid hormones[3] is seen in several ways. The steroids are being transported by the serum proteins. This function does not include, however, the need for solubilization, because the maximal levels[4] of even the least soluble progesterone are within the limits of aqueous solubility.[5] An important aspect of the protein complex formation is the protection of the steroid hormones from chemical or enzymatic attack, resulting in decreased metabolic clearance. This steroid-conserving mechanism has been emphasized recently[6] for the progesterone-binding globulin (PBG) of the pregnant guinea pig, a serum glycoprotein to be discussed below. The steroids are biologically inactive as long as they are associated with protein; they can be "activated" by dissociation to the unbound hormone. In this manner, a relatively large amount of steroid can be carried in an indifferent storage form and can be made available immediately at the target tissue. For progesterone, nearly all of the hormone circulates in the body as a biologically inactive species (TABLE 2).

The most desirable objective of the study of steroid hormone interaction with proteins would be the investigation of steroid receptor proteins.[7,8] Unfortunately, none of these receptors has been available in purified form in sufficient amounts to make such study practical. Therefore, we have concentrated our efforts on the exploration of the chemical basis for the interaction of steroid hormones with serum proteins, preferably those of high affinity and specificity. Earlier results from our and other laboratories on the binding of progesterone to the three human proteins

*Supported by Grant AM-06369 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

†Research Career Awardee of the United States Public Health Service.

TABLE 1

PROGESTERONE-BINDING PROTEINS IN HUMAN SERUM

Protein	Molecular Weight (daltons)	Carbohydrate (%)	Concentration (μM)	Association Constant (μM^{-1}) 4°C	37°C
HSA	69,000	—	550	0.36	0.18
AAG	41,000	42	18	1.5	0.6
CBG	52,000	26	0.7	700	90
PBG*	88,000	71	13	2200	350

*From pregnant guinea pig serum.

listed in TABLE 1 have been summarized[9]; additional results on HSA,[10] AAG,[11,12] and CBG,[13-16] have been published.

Studies on CBG, the serum protein that binds progesterone in human serum with highest affinity, are handicapped by its instability to heat and acidic milieu and by its low concentration in the blood. It was fortunate, therefore, that we discovered in the serum of the pregnant guinea pig a protein, different from CBG, that binds progesterone with high affinity and specificity and that occurs in relatively high quantity.[17] The existence of such a protein was postulated in independent studies.[18] In the present report, some recent results on this PBG will be discussed.

PURIFICATION OF PBG

PBG occurs in pooled serum of pregnant guinea pigs at the relatively high concentration of 1.2×10^{-5} mol of progesterone binding sites per liter,[19] which corresponds to more than 1 mg/ml. It has considerable heat stability. This feature is evident from FIGURE 1, which shows that more than 50% of the PBG activity survives a 2-day exposure to 60°C.[20] The heat resistance is greater than that of CBG, from which it can be separated.[21,22] Noteworthy also is the remarkable indifference toward acidic and alkaline milieus, as seen in FIGURE 2; incubation for 48 hr at pH 2 or 11 results in very little inactivation.[23]

Purification of PBG has been reported from several laboratories[19,24,25]; ion-exchange chromatography, gel filtration, ammonium sulfate precipitation, and electrophoresis were the principal methods applied in the initial studies. A useful simplification of the purification procedure was achieved[26] by taking advantage of the acid stability of PBG and its extremely low isoelectric point of 2.8[23]: chromatography at pH 4.5 of the serum over a column of sulfopropyl (SP)

TABLE 2

BINDING DISTRIBUTION OF PROGESTERONE AND CORTISOL IN HUMAN PREGNANCY SERUM[1,2]

Protein	Progesterone (%)	Cortisol (%)
CBG	43–48	60–80
HSA	50–54	13–32
AAG	~1	—
Unbound	1–2	7–8

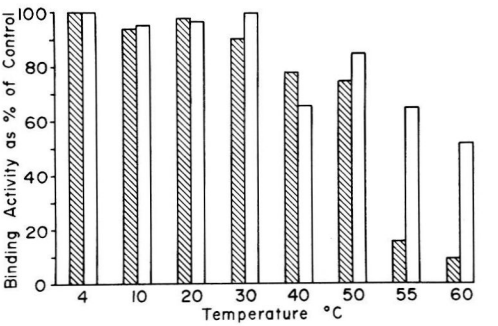

FIGURE 1. Effect of temperature on progesterone-binding activity and stability of PGB in serum diluted 1:10 with citrate-phosphate-borate buffer of pH 7.0. Specific binding was determined by charcoal adsorption at the temperatures indicated after 30 min of incubation with saturating levels of [^{3}H]progesterone at the same temperatures (hatched bars). Companion tubes were incubated for 48 hr at the temperatures indicated, and specific binding was then determined at 4°C (open bars). Binding activity is expressed as a percentage of the control value incubated for 30 min at 4°C and measured at 4°C. (From MacLaughlin & Westphal.[20] By permission of *Biochimica et Biophysica Acta*.)

Sephadex®, a strong cation exchanger, results in adsorption of most serum proteins, except PBG, which is eluted in the void volume of the column. A single run gives a PBG preparation free of albumin and of CBG, which presumably is inactivated under the acidic conditions.

The prepurified PBG preparations were subjected to affinity chromatography on columns of immobilized steroids.[27] Sepharose® 4B was activated with cyanogen bromide, coupled with diaminodipropylamine as the spacer, and the product was condensed with the 17-hemisuccinate of 19-nortestosterone, with formation of an amide bond. This steroid derivative was selected because the binding affinity of 19-nortestosterone to PBG is close to, but lower than, that of

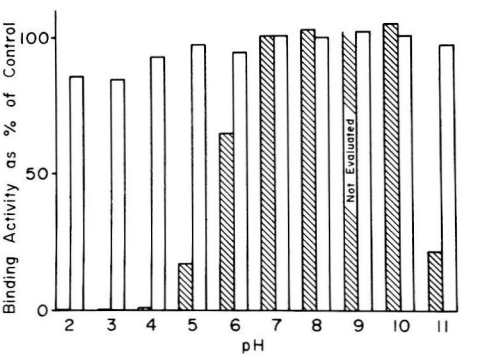

FIGURE 2. Effect of pH on progesterone-binding activity and stability of a purified PBG preparation in citrate-phosphate-borate buffer at 4°C. Binding parameters were determined by equilibrium dialysis at the pH stated (hatched bars) or at pH 7.0 after incubation for 48 hr at the pH indicated (open bars). Binding activity is expressed as a percentage of the control value determined at pH 7.0, 4°C. (From Harding *et al.*[23] By permission of *Life Sciences*.)