A Discussion of the Mechanism of Action of Steroid Hormones*

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The steroids may be classified as hormones when they originate in one group of cells and regulate the physiologic behavior of other cells in the same multicellular organism. Through the classical methods of endocrinology, involving extirpation of various organs and hormone replacement, a number of primary sites of synthesis of such steroids have been identified as well as the chemical agents which they produce. That other steroid hormones will be found which are selectively produced in cells not nearly so susceptible to removal from the host seems highly probable, although it does not pertain to the present discussion.

As hormones, the steroids elicit biological responses in susceptible cells and tissues; the latter are often referred to as target organs when the response is sufficiently obvious and characteristic of a particular hormone. These responses, for the most part, represent rather complicated changes of physiology in a group of cells and usually require a considerable period of time for overt manifestation. This physiologic behavior, which the steroid agents modify, is the product of interaction of many enzyme systems and other functional units of the cell. Therefore, when we inquire how hormones act we are really asking how do these substances induce shifts in the pre-existing balances of integrated enzyme systems. Unfortunately, in many cases the basic systems are not yet well established and their manner of interaction only inferred. Obviously then, the question of mechanism must be asked repeatedly in the light of each fundamental advance in our knowledge of nucleic acid and protein synthesis, carbohydrate metabolism, lipide systems, steroid biochemistry, and other areas of cell physiology.

In attempting to assess present knowledge on the mechanism of action of steroid hormones, one is confronted with a vast sea of observations which range from the molecular level to the behavioral realm. It is the purpose of this review to consider criteria for establishing relevance among these varied findings. In a general section, general aspects of hormone action will be discussed; steroid hormones will be presented as a molecular class of compounds, and some of the characteristics of the biological responses will be related for representative hormones. Turning to units of cellular structure and function, a discussion of basic control mechanisms will be presented with respect to their possible implication in hormone action. In a more experimental section an analysis of studies on the mechanism of action of estrogenic hormones will be undertaken; in fact, the estrogens will serve as a model system throughout the paper, since (in the opinion of the author) more is known concerning their mechanism of action than for any other steroid. Relevant findings with other steroids will be reported in less detail.

A number of unanswered questions will be raised in the attempt to point out major deficiencies in our knowledge; it is hoped that these will provoke discussion and experimentation. In the present state of knowledge one can hardly consider hormone action without resorting to speculation; if in the course of this activity certain “straw men” are set up, perhaps they will kindle the fires of research.

Before proceeding, the thoughtful review by Hechter (37) on the general subject of hormone action is recommended to the reader’s attention. Similarly, the manuscripts of Roberts and Szego (75) and Lieberman and Teich (52) contribute nobly to the documentation of the steroid biochemistry.

I. THE HORMONAL AGENT
Steroid hormones belong in a class of compounds having a common cyclopentanoperhydrophenanthrene nucleus. Biosynthetically they are all derivable from cholesterol or a common intermediate by a series of reactions involving the re-
removal of all or part of the side chain, selective dehydrogenation, and strategic oxidation of certain positions on the nucleus (18). Some of these reactions are highly developed processes in the adrenals, ovaries, testes, and placenta and are themselves regulated by the action of nonsteroid hormones referred to as tropins. Other steps in the interconversion of biologically active steroids may be carried out in a variety of tissues (18, 61).

A certain degree of correlation exists between the chemical structure of the steroid and the ability to elicit a biological response; this permits a loose classification, which is shown in Table 1. Biological responses are mentioned which permit assay or reveal characteristic effects. However, it is to be emphasized that there is considerable overlap in this regard, which may result from metabolic interconversion, tolerance by the receptor system of multiple sites of potential specificity in the steroid, or metabolic sparing of a natural analog. High biological activity in general is associated with fusion of rings A and B in the trans position relative to the angular methyl groups; this affords a more planar molecule from the rear aspect. A side chain at position 17 in progesterone and in the adrenal cortical steroids is invariably cis to the angular methyl groups. In those steroids without a side chain, the β hydroxyl group at position 17 is associated with higher biological activity than the ketone; and an α hydroxyl group drastically reduces activity. A similar relationship exists for adrenal steroids with reference to oxygenation at carbon 11. In general, the more polar functions are found protruding from the β aspect of the molecule.

The steroids, as hydrocarbons, are lipophilic, but this property is diminished by hydroxyl substitution. The hydroxyl groups in turn are reversibly convertible to the ketonic forms by specific steroid dehydrogenases (59); the ketones are considerably less polar.

One of the most striking properties of steroids is their ability to associate with proteins; this propensity appears to play a part in the transport of steroids in the body fluids (7, 59, 83). Aqueous solutions of protein, particularly albumin, are able to solubilize remarkable quantities of steroid (6, 20); in this latter case, however, the association appears to be nonspecific, and the steroid can be readily recovered by extraction with organic solvents. A more rigid binding with protein in which a covalent linkage appears to be established enzymatically has been described by Riegel and Mueller (72).

Steroids possessing oxygen functions are also substrates for the glucuronide- and sulfate ester-synthesizing systems (51). Such compounds possess detergent-like properties; however, most of them have not been well characterized or investigated from a functional point of view. Information on the reactivity of the carbonyl group of steroids in biological systems is rather limited to reduction; whether or not Schiff base formation (such as involved in transamination), aldol condensations, enol-ester or mercaptal formation occurs will require further research. It seems that, at this stage of our knowledge, the participation of a steroid as a substrate in all these types of metabolic reactions should be surveyed, since the biologically active form of these agents cannot be defined as yet. It is quite possible that the steroids may express biological action through entering into such reaction mechanisms.

II. UNITS OF STRUCTURE AND FUNCTION

For this discussion it is appropriate to consider some of the factors involved in the expression of biological function; this is of interest, since we seek ultimately to explain how steroid hormones modify function. It must be recognized first that biological function is composed at all the different levels of organization; i.e., the animal, organ, cell, subcellular particulates, and the multiple enzyme systems. The contributions to function at each level may be measured by the spectrum of products and the rate at which they are formed. That is to say that, for any cell, the type of products and the quantity contributed to the environment in a finite period of time is characteristic of that cell in a specific environment; and, similarly, the type and rate of production of products of a subcellular particulate are characteristic of that unit in its environment; and so it continues down through the successively lower levels of organization to the fundamental unit of catalytic activity, the enzyme.

At each level we have a condition of semi-isolation of varying degree wherein a definite barrier to communication exists. Some of these barriers are recognized cytologically as the cell wall, nuclear surface, mitochondrial membrane, endoplasmic reticulum, and Palade granules; many others remain to be studied. In this sense it seems appropriate to visualize the cell as being made up of a tremendous number of compartments, each of which contains a specific association of enzymes. Thus, to mention a few, we have the tricarboxylic acid cycle-electron transport system concentrated in the mitochondria; diphosphopyridine nucleotide synthesis and pyrophosphorolysis of uridine coenzymes concentrated in nuclei; protein and cholesterol synthesis concentrated in the endo-
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<th>CLASS AND REPRESENTATIVE COMPOUNDS</th>
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<th>BIOLOGICAL RESPONSES WHICH PERMIT ASSAY</th>
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<td>Progestational:</td>
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plasmic reticulum-Palade granule fractions; and glycolysis, amino acid activation, and many others localized in the operationally defined soluble fraction. Each of these is probably made up of many smaller compartments, the smallest of which may be single enzyme molecules loosely localized on a surface (interface) or may constitute a catalytic surface amid the structure of a contractile, protein, or a semipermeable membrane.

With this state of semi-isolation, products formed in one area have limited access to enzyme systems localized in another. As soon as utilization in the second area exceeds the diffusion or transport of products from the first, a gradient is established which is maintained dynamically in accordance with the function of both areas. This type of interaction is of course not confined to only two areas, but exists simultaneously between all.

In each fundamental compartment the enzymes of a multiple enzyme system oscillate within a steady state. The particular steady state attained is determined by the amounts and varieties of specific enzymes and the concentrations of substrates and products which are maintained over the period of measurement.

1. The catalytic unit: the enzyme.—Before considering some of the properties of multiple enzyme systems let us take a look at a single enzyme, since this is the fundamental machine permitting the flow of energy involved in product formation (Chart 1). An enzyme is a protein carrier of a specific catalytic surface. Through specificity studies it has been established that this surface is complementary with the structure and configuration of a particular substrate molecule. Through the formation of enzyme-substrate complexes upon these surfaces (involving a three-point contact), reactions can be catalyzed which are thermodynamically possible. The enzyme acts by lowering the amount of activation energy required for the conversion.

The over-all velocity of an enzyme-catalyzed reaction is determined chiefly by the inherent properties of the enzyme, the amount of the enzyme, and the substrate and product concentrations. Thus, at low substrate levels the velocity may be limited by the number of active molecules that collide with the enzyme surface. With increasing substrate concentration, the frequency of collision is higher and the velocity increases in a manner characteristic of the relationship between rates of association and dissociation of the product from the enzyme. From the standpoint of rapid alterations of velocity in a biological system, variation of substrate concentration is probably the most important. However, in certain systems the enzyme activity may be released from an "inhibited" form or position. Cori has indicated that this may be the case with phosphorylase which is brought into high activity with muscle contraction (15). The experimental findings are strongly suggestive that contractile protein, possibly adherent to a membrane or surface of multiple enzyme compartment, may mechanically bring these enzymes into the environment of their substrates. If this is true in muscle, one might ask whether a vestige of such a control mechanism might also be operative in other tissues.

Accessory factors such as pH, equilibrium constant, oxidation-reduction potential, and free energy are also implicitly involved. Similarly, if the enzyme is missing a cofactor or activating ion, a profound decrease in catalytic activity will be observed and finally (endogenous) inhibitors may also play a role.

2. Some properties of multiple enzyme systems.—The fact that two enzymes attacking the same substrate may have different Michaelis constants (substrate concentration necessary for half maximum velocity) and different turnover numbers allows the substrate concentration to determine the degree of competition between the two alternative pathways. Thus, one enzyme may be saturated at a substrate concentration which permits only a low reaction velocity of the second enzyme, even though the latter under optimal substrate concentration has a higher turnover rate. In such a system the steady state is determined by the level at which the substrate is maintained. With this in mind, let us turn to a diagram, Chart 2, and a discussion of a multiple enzyme system presented by Greenberg (33). In this scheme substrates A, B, O,
K, and R are beginning substrates which have diffused into the environment of our multiple enzyme system from other compartments. These are introduced into a major synthetic chain along which A is converted sequentially to C \( \rightarrow \) F \( \rightarrow \) J \( \rightarrow \) P. The sequence O \( \rightarrow \) P \( \rightarrow \) E represents the activation of substrate O such as known for fatty acids, amino acids, and nucleotides. \( \text{GH}_1 \rightarrow \text{G} \) is a reversible oxidation-reduction carrier system which is reoxidized by substrate R. Y is a side product. Each number represents an enzyme-catalyzed reaction. Reaction (5) occupies a strategic position. If in the intact system only traces of intermediates C, F, and J are present during the formation of P, it follows that each of the enzymes may be acting at a low and different level of saturation; thus a steady state exists, limited by reaction (1) involving substrates A and B. In this case either the enzyme or substrate may be limiting. If, however, the rate of reaction (5) is decreased below a critical level, the sequence of reactions from (1) to (4) is disturbed. The system then seeks a new steady state in accordance with the fundamental properties of each enzyme and the reaction it catalyzes. Intermediate F may increase while product P diminishes. If reactions (1) to (4) are reversible, the new steady state will be characterized by a difference in the saturation levels for each enzyme. Of greatest importance is that, in the establishment of the new steady state, the ratios between the formation of products F, P, and Y are changed. They accumulate, accordingly, to form different gradients through which they diffuse to other multiple enzyme systems in other compartments. Here they contribute to the substrate environments which help determine, in turn, the steady state for a different group of enzymes and a different spectrum of products.

Thus, the compartmental nature of the cell increases the versatility to respond to certain stimuli (substrates) and provides for maintenance of order among the multitudes of enzyme reactions. Feedback mechanisms are implicit among such systems. Communication between these semi-isolated organized systems operating in different steady states requires time; therein lies a considerable stability of the systems with respect to time.

3. Some additional comments on organization.— An integral aspect of the compartmental structure is that a local concentration of enzymes and substrate can be obtained. For enzymes operating in a sequence it would appear that the efficiency might be increased if they were organized spatially in close proximity to one another. Such appears to be the case for mitochondria. This particle contains a group of enzymes which will oxidize acetate to carbon dioxide and water, and trap the energy in adenosinetriphosphate. Attempts to fractionate this group of enzymes have demonstrated that electron transport and oxidative phosphorylation are intimately coupled (12, 51, 50). In fact, Green and associates have isolated submitochondrial particles which appear to be organized aggregates of enzymes with lipide backbones (56, 57, 88). In their “electron transport particle” (Chart 3), the enzymes behave as if they were linked sequentially with their prosthetic groups in sufficiently close apposition so that electron transfer proceeds through the chain with little opportunity for random interaction. High efficiency is observed. The same situation seems to hold for the other substrates and products. Disruption of the organized structure by deoxycholate allows the escape of cytochrome C (56) with simultaneous loss of ability to catalyze the oxidation of reduced diphosphopyridine nucleotide by oxygen. This type of organization may be an example of maintaining maximal substrate concentration at or near the catalytic surfaces of enzymes acting in a sequence.

In this respect one begins to wonder about the matrix in which enzymes lie: the high phospholipide content of the mitochondria, the high cholesterol content of the endoplasmic reticulum, the inositol lipides in the nucleus, and the glycoproteins characteristic of certain cell surfaces. What role do these materials, which have the properties of forming membranes, play in regulating cell...
function? Certainly they are suspect sites of steroid hormone action.

III. SOME GENERAL ASPECTS OF HORMONE ACTION

Any explanation of the mode of action of steroid hormones must answer two basic questions (37) arising out of the nature of many hormonal responses:

A. How can trace amounts of hormone produce profound biological responses without contributing significant amounts of energy or mass to the system?

B. How can the apparent specificity of hormone action be explained?

1. Consideration of Question A.—One must accept that in the interaction of the hormone with the biological system the hormone acts as a particle of mass, that primarily it can only alter the state of another particle of mass through molecular interaction. Therefore, in order to produce the profound biological responses observed, the hormone must affect a component of an amplification system; such loci would appear to lie within the operation of multiple enzyme systems. Within these parameters a hormone might act initially by altering either the effective concentration of an enzyme or the effective concentration of a substrate.

The level of active enzyme could be altered in a number of ways without invoking new enzyme synthesis. If a hormone reacts with an enzyme to either increase or decrease the Michaelis constant it would be reflected in the velocity of the catalyzed reaction with a given substrate concentration. It also follows that, if the hormone combined with incomplete enzyme as a coenzyme or prosthetic group converting inactive to active enzyme, the initial over-all conversion of substrate to product would be increased accordingly; the opposite effect on the total process would be observed if the hormone were an inhibitor.1

Considering the compartmental nature of the cell, with enzymes fixed on or within membranous structures, a hormone might act by increasing the accessibility of pre-existing enzyme to substrate. Acting as a surface-active material or detergent, it could induce permeability changes in a membrane or compartment around an enzyme and thus indirectly alter the concentrations of those substrates diffusing in or out of the compartment. Such an interaction must, however, account for the specific relationships of hormone structure and biological activity. The low concentration of hormone required for activity (about $2 \times 10^{-9}$ M for estradiol) also points to the existence of specific receptors rather than any general detergent-like action. More probably the hormone would introduce electrostatic changes in a membrane analogous to the changes which take place in neural membranes or contractile proteins. In addition, it must be kept in mind that cell structures are being continually renewed. Thus, the hormone might interfere with either the synthesis or destruction of such a structure; however, this again would refer the initial site of action of a hormone to a specific enzyme. So far, practically no evidence can be brought to bear either pro or con, although the area appears highly promising.

On the other hand, the synthesis of new enzymes is certainly involved in the over-all hypertrophic effect of certain hormones. Whether this is a primary or secondary effect of the hormone can only be speculated upon at this time. While little is known of the factors which regulate de novo synthesis of enzymes in mammalian cells, there is reason to believe, from the studies of Knox et al. (45, 46, 47) on tryptophan peroxidase, that the principles of induced biosynthesis of enzymes, established in microbiology, also apply to mammalian systems. These principles, largely derived from the work of Monod, Cohn, Lwoff, Spiegelman, and Stanier, have been summarized by Monod (63).

1 In 1941, D. E. Green (30) first formally presented the concept that hormones might act as coenzymes or inhibitors of specific enzymes.
When supplied with adequate precursors for protein synthesis and energy, genetically competent E. coli can be induced to synthesize β-galactosidase by the simple addition of a β-galactoside to the culture medium. The inducer may be a substrate for the enzyme, an inhibitor, or have little affinity at all. The minimum requirement is a steric similarity to the actual substrate. In addition, the inducer is not used up in the process of inducing the de novo synthesis of new enzymes.

As a new active enzyme is formed it begins to act on available substrate, thus giving rise to higher concentrations of product; the latter in turn acts as inducer for the synthesis of a second enzyme, and so on. This process of sequential induction has been shown to apply in galactose and tryptophan metabolism in bacteria (80) and in the cytochrome system of yeast (78). The induction process is controlled in two ways: through the availability of inducer and a type of feedback inhibition exerted by products of the induced enzymic reaction. In addition, competition can take place between the induction of separate enzyme systems. The important principle is that changes in relative substrate concentration, however brought about, can set in motion a sequential induction of enzyme synthesis. The balance of power between interacting enzymes within the cell is then changed, giving rise to an alteration in biological function.

It should be kept in mind that this process involves actual protein synthesis and, very possibly, concomitant ribonucleic acid synthesis (39). Also, as far as it is known the presence of an inducer is required; this is normally the substrate. Thus, it is probable that hormonally induced enzyme synthesis would be associated with a primary increase in the substrate concentration. Once again, the site of action of the hormone would appear to be on an enzyme or its compartment. However, the possibility cannot be excluded that the hormone acts directly on the nucleic acid. In fact, it has been reported that estradiol (5–10 µg/ml) facilitates the action of deoxyribonuclease on the decomposition of deoxyribonucleoproteins (1).

2. Consideration of question B.—The target organ concept has arisen in endocrinology because the biological response in certain tissues completely overshadows happenings in others. For example, the response of the uterus to estrogens is dramatic; but when one looks beyond, he sees that estrogens stimulate mitoses in the skin (10), suppress the production of gonadotrophins by the pituitary (54), cause development of pituitary tumors (62), stimulate fat synthesis in the liver (70), promote protein synthesis in the connective tissue of the pubic symphysis (28), and change the histologic appearance of the salivary glands (27). These are only a few of the responses obtained in different tissues. While many of them may be complicated by other humoral factors, it does appear that the specificity of hormones for certain tissues may be more apparent than real.

It is not possible to go into the subject of differentiation and morphogenesis in this discussion; it is only necessary to recognize that the end result of such processes is a segregation of potentialities for function in certain cells. Thus, it is possible that a hormone acts similarly in all cells, but that the composition of the enzyme population, being quantitatively different for various cells, gives rise to a different response in light of the foregoing discussion. Of course, if such differences in enzyme population involve the initial receptors or the "hormone signal," the target organ concept would be upheld. Attempts thus far to show selective localization of exogenous steroids in target organs have not been very successful; however, selective localization may not be needed.

The recent findings of Huggins and Jensen indicate the complexity of the hormone specificity (41, 42). The authors carried out a very fine study on the ability of various steroids to depress estrone-induced uterine growth. It was observed that 6-keto and 16-hydroxyl derivatives of 3-hydroxyestratriene ("impeded" estrogens) inhibited the response in the uterus but not in the vagina. From this, it would appear that the hormones and the depressor steroid are eliciting responses through different mechanisms in each of these tissues. However, until such problems can be attacked in isolated systems, the question of hormone specificity must be pondered. From the present position, the apparent specificity seems to reflect the effect of quantitative differences of the spectrum of enzymes available for the response.

IV. EXPERIMENTAL APPROACHES TO THE MECHANISM OF STEROID ACTION

In the foregoing discussion an attempt has been made to set up a framework for understanding the general nature of any biological function; obviously, this is incomplete. However, it was done so that one might begin to establish some relevance among the multitudinous observations which have been made on the responses of each steroid hormone.

In this regard, it can be stated that any visible response (i.e., changes in macroscopic or microscopic appearance, weight, mechanical activity, and behavior) is likely to be many scientific miles removed from the initial action of the hormone.
This is particularly true when the observations have been made in tissues where cell reproduction or destruction has taken place over a considerable period of time. Accordingly, interpretation of the hormonal response under such circumstances should take into account the varied composition of the tissue. In fact, it is frequently difficult to know what constitutes an adequate control tissue in such cases. Nonetheless, these observations establish the important cumulative effects of hormonal stimulation and thus provide one parameter of the response.

At this time there do not seem to be any shortcuts to deciphering the mechanism of action of steroid hormones. For those hormones concerning which no clues are evident as to the area of metabolism they affect (i.e., glucose metabolism in the case of insulin), it is necessary first to establish it. This may be approached by surveying segments of metabolism at the earliest time period following the hormonal administration that one can be reasonably certain a response exists. Preferably this should be within a few hours (within 6 hours).

By utilizing isotopically labeled precursors, representative metabolic pathways can be studied in the animal or in tissue slices, and the presence or absence of a hormonal influence on a specific segment of metabolism can be assessed. Once an effect has been established, the problem becomes one of interpreting the finding. Surveys of other metabolic processes add perspective to the original result. However, one is very dependent on the state of fundamental knowledge of the processes under consideration. In the light of available knowledge, the most sensitive reaction must next be studied in cell-free systems with the properties of enzymes and multiple enzyme systems in mind. The problem then becomes one of determining whether the observed response is due to substrate effects or alteration of effective enzyme concentration. This should be considered in light of the discussion in Section III.

Another approach is to study the hormone itself: the metabolic conversions it undergoes, the enzymes that attack it, and the possible function of such systems in metabolism. In essence, this constitutes an approach opposite to the first. Both approaches are to be recommended, since in the final analysis an explanation of steroid hormone action requires both a knowledge of the initial interaction of the hormone with certain tissue constituents and knowledge on the dynamic chain of events which take place in the expression of biological response.

V. STUDIES OF THE MECHANISM OF ACTION OF ESTROGENS

In light of the foregoing discussion, let us now consider studies on the mechanism of action of estrogenic hormones. This class of hormones, while perhaps not completely representative of the nonaromatic steroids, does constitute a group of highly active modifiers of biological function. One of their chief attributes is the rapidity and magnitude of the biological response. In addition, the extremely low level of the hormone required for effect points to a specific receptor mechanism.

A. The biological response.—A synopsis of the physiological effects induced by estrogens has been given by Pincus (69). The metabolic interaction with other steroids in the reproductive tract has been summarized in the inclusive reviews by Szego and Roberts (73, 83).
Among all the physiological effects of estrogens (a few listed in Section IV), the most striking is its action upon the uterus. In response to a single physiological dose of a natural estrogen, the atrophic uterus of the ovariectomized female rat is rapidly converted to an actively growing tissue; this mobilization of latent growth processes is characterized in the first 4 hours by a gross hyperemia and imbibition of water in the uterine tissue (3); later, increased mitotic activity (2) and concomitant hypertrophy of the uterus, with increases in ribonucleic acid and protein (3, 84); and, finally, new cell production as reflected by increased deoxyribonucleic acid content per uterus (84). To better orient these responses, a chart has been prepared from the data of Telfer (84) (Table 2), and additional data from our laboratory are shown in Chart 4 on the early time response in terms of protein and ribonucleic acid.

The whole process takes about 72 hours and presents a sequential mobilization of cell machinery from growth. The first 6 hours may be regarded as the induction phase; while there has been a dramatic increase in the water content of the tissue (wet weight may double), little or no accumulation of protein or ribonucleic acid occurs during this period (Chart 6). The period from 6 to 24 hours may be referred to as the RNA (ribonucleic acid) accumulation phase. Protein accumulation also follows in the wake of the rising RNA content per uterus. If the hormonal influence is extended, a DNA (deoxyribonucleic acid) synthesis phase occurs sometime between 40 and 78 hours. The latter transition requires further definition as to time and duration of estrogen dosage required to achieve this point.

The water imbibition process during the induction phase constitutes a major problem in estrogen research. Cole has concluded that the water imbibition is due to an increase of osmotically active units from within the tissue (14). Since the evidence supported a nearly equal increase in negative and positive charges, it was suspected that protein was the primary source. However, no clear identification of the osmotically active units has been arrived at. Kalman (44) has demonstrated that uptake of Na\(^{2+}\) was greater than could be explained by the water imbibition and that an increased adsorption of I\(^{131}\)-labeled albumin was suggestive of greater permeability. Our own studies showed that potassium uptake paralleled the water imbibition.

Szego and Roberts have demonstrated that the addition of hydrocortisone acetate intravenously (83). Even though the initial imbibition of water was largely prevented by hydrocortisone acetate, these authors noted that significant growth of the uterus took place subsequently. The anterior pituitary was not necessary for the initial response of the subsequent growth.

While these findings on water imbibition have not been resolved and await basic research on water and electrolyte transport, they do assure us that by this time some very fundamental changes have taken place which may yield to biochemical dissection.

**B. Alterations in uterine metabolism induced by estradiol.**—In this section a special effort will be made to present evidence of early alterations in certain areas of uterine metabolism by estrogenic hormones. For the most part the data have been derived from experiments with isotopic precursors designed to show the sequence of metabolic changes with respect to time of estrogen treatment. In the procedure utilized in this laboratory, ovariectomized rats are injected with a single dose of estradiol via the tail vein. After varying periods the rats are sacrificed and the uterine horns removed and cut into small segments. These segments are incubated at 37°C in a glucose-balanced salt solution containing a radioactive precursor in an atmosphere of oxygen. After incubation the enzymatic reactions are stopped with acid and the tissue fractionated into the following fractions: acid-soluble (small molecules), lipide, nucleic acid, and protein. Specific isolations are carried out on these main fractions. The respired carbon dioxide is collected when indicated.

1. Influence of pretreatment with estrogen on the incorporation of amino acids into protein: The effect of a single injection of estradiol (10 μg.) on the rate of incorporation of glycine-2-C\(^{14}\) into the protein of surviving uterine segments is shown in Chart 5 (84). It should be noted that with estradiol the rate of incorporation of glycine is accelerated in a linear manner over the first 12 hours; thereafter, gradually, this stimulatory process begins to subside so as to reach a peak at 20 hours. An estradiol deficiency appears to arise at this time, because if a second injection of estradiol is given at 16 hours the acceleration continues in a linear manner well beyond this point. If the second injection is made on the descending limb of the curve, a complete reversion is obtained, with a primary response similar to that obtained with the initial dose of estradiol. The failure to respond more rap-
idly at this point would argue against the accumulation of any apo-enzyme which awaits activation by estradiol.

The stimulation of amino acid incorporation occurred with all amino acids tested, i.e., glycine, alanine, serine, lysine, and tryptophan; thus, it seems to be a part of a general protein anabolic response. Since this is one of the earliest metabolic changes with estrogen treatment (discernible as early as 1 hour) it should be highly interesting to determine whether the estrogen effect concerns the activation of amino acids by the soluble fraction of the cell or incorporation of the activated forms into the ribonucleoprotein granules of the microsome fraction (53). For reasons to be discussed later, the activation process is suspected.

2. Dose-response curve: With a sensitive indicator of estradiol action it was possible to determine dose-response relationships with glycine incorporation as the end-point. The results of such an experiment are given in Chart 6 (64). Only 0.1 μg of estradiol/200-gm rat is required to produce a maximal response when the measurements are made at 6 hours; however, in order for the activation to continue beyond 6 hours, larger doses of hormone are needed. It should be noted that with 0.1 μg, the increase obtained between 6 and 20 hours was very small; thus, it would appear that some receptor was titrated progressively as long as active estrogen was available. Beyond that point a steady state characteristic of the amount of receptor titrated was maintained for a number of hours.

Chart 200

CHART 5.—The effect of a single dose of estradiol on the rate of incorporation of glycine-2-C\textsuperscript{14} into protein of surviving uterine segments.

Ten μg estradiol injected intravenously at zero time. At the indicated times uterine segments were incubated with glycine-2-C\textsuperscript{14} for 2 hours. Data are presented in counts/min/mg protein (64).

Can this involve hormone utilization? Only careful studies on the amount and form of the estrogen in the uterus with respect to time and dosage will provide an answer. To do this, estradiol with very high specific activity will be required as well as good micro-isolation technics. It is to be expected that this study may aid in the identification of the receptor mechanism.

3. Early alterations in carbohydrate and lipide metabolism induced by estradiol: Szego and Roberts (74, 83) have studied extensively the changes in aerobic and anaerobic metabolism of carbohydrates in rat uteri following estrogen treatment. Within 4 hours after hormone injection, both aerobic and anaerobic glycolysis were elevated significantly. This increase was not accompanied by a concomitant enhancement of respiration; the extra glucose uptake was reflected in lactic acid accumulation. By 20 hours, when the uterus was well into the RNA accumulation phase, the respiration was substantially increased, as well as glycolysis. Data from this laboratory on glucose-1-C\textsuperscript{14}, pyruvate-2-C\textsuperscript{14}, and acetate-1-C\textsuperscript{14} are in essential agreement with these findings (65); it was noted that the combustion to carbon dioxide was not significantly affected. Thus, it appears that the early estrogen effects in the uteri do not involve alterations in the operation of the tricarboxylic cycle or, therefore, the energy yield from such reactions.

On the other hand, the conversion of the isotope into the nonvolatile lipide fraction is stimulated as much as 250 per cent by 6 hours. This finding is of interest in view of recent studies indicating that
fatty acid synthesis takes place in the soluble fraction of the cell (48) and that cholesterol synthesis is concentrated in the large microsomes (9). The unusual sensitivity of cholesterol synthesis to estradiol treatment has been shown by Emmelot and Bosch, who observed more than a 50-fold stimulation of acetate incorporation into cholesterol in mouse uterus at 20 hours after hormone administration (32). These workers have used this system ingeniously to study the endogenous production of estrogens by ovarian tumors (8).

In view of the discussion of the compartmental nature of metabolism (Section II), these findings on lipide metabolism are highly interesting. The remarkable estrogenic stimulation of cholesterol and fatty acid synthesis, which are indicated to be soluble and microsomal systems in other tissues, and the lack of stimulation of oxidative pathways, which are located in mitochondria in other tissues, suggest a high selectivity in the site of the estrogenic attack. Before one can be sure, however, a great amount of work will have to be done toward isolation of the systems in a cell-free state.

4. Early estrogen effects on pathways of ribonucleic acid synthesis: In the process of atrophy following ovariectomy, the uterus of the rat decreases to a very low level of RNA per unit of DNA (84). Following a single administration of estradiol one observes little or no change in RNA content for 6 hours, after which interval it rapidly accumulates, as shown in Table 2 and Chart 4 (84). When incorporation studies were done with radioactive carbon dioxide during this period, the rate of labeling of the nucleic acid adenine was found to be increased at least 150 per cent by 6 hours (Chart 7). The rate of incorporation fell off rapidly after 12 hours, even though the RNA was still accumulating at this time. Apparently, the estrogen had set in motion a process in which the initial precipitating effects were wearing off and were becoming obscured by subsequent metabolic changes. It would be very interesting to know the character of this response with varying duration of estrogenic treatment (i.e., small doses over extended periods).

Results with radioactive formate and glycine (Chart 8) parallel the response obtained with radioactive carbon dioxide at 6 hours and, in addition, have revealed a high sensitivity of “one-carbon” metabolism to estrogen influence (67). This is particularly well illustrated with serine-S-C\(^{14}\) as the precursor (Chart 9) of the “one-carbon” fragment (38). With 6 hours’ pretreatment the in vitro labeling of the nucleic acids purines was 4.4 times the control value. The inclusion of a non-radioactive pool of formate in the reaction mixture acted as a trap for the one-carbon intermediate and decreased the incorporated radioactivity in the nucleic acid purines strikingly (38). On examination of the pool it was clear that one estrogen effect occurs at or near the activation step for
serine; this, however, does not account for the large effect observed on the total pathway; there must be other steps which are highly susceptible to the action of estradiol. Similar results have been obtained with glycine-2-C\(^{14}\) (67).

Another finding in these experiments with labeled serine, glycine, and formate was that various pathways for a single precursor are not altered to the same extent by estrogen pretreatment. This appears to point to a selective sensitivity of competing enzymes to the action of the hormone; however, if two enzymes with widely different Michaelis constants were competing under conditions of limiting substrate concentration, an alteration of the substrate concentration might well account for the observed results. One can only proceed to analyze the situation stepwise in line with the discussion in Sections II and III, keeping strongly in mind the compartmental nature of the cell and the properties of multiple enzyme systems.

The segmental nature of estrogen influence on metabolism is also highly apparent when one compares the effects on carbon dioxide incorporation into pyrimidines (Chart 10) with the results just reported for purines. Prior to 6 hours, little or no effect was seen on the total radioactivity incorporated into pyrimidines. Thus, pyrimidine synthesis, which from studies on liver appears to originate in the mitochondria, does not reflect the initial estrogen influence when carbon dioxide is the precursor.

5. Effect of estradiol pretreatment on serine aldolase activity of the uterus: The high sensitivity of one-carbon metabolic pathways in the uterine segment to estrogen action prompted a study of serine synthesis in uterine homogenates. Attempts to convert radioactive formate to serine failed, owing to difficulty in the activation of formate. However, homogenates fortified with tetrahydrofolic acid, nonradioactive formaldehyde, and glycine-2-C\(^{14}\) synthesized serine readily. The process was linear with time and proportional to tissue concentration; accordingly, uterine homogenates from rats pretreated with a single dose of estradiol were assayed for serine aldolase activity. As demonstrated in Chart 11, serine aldolase activity increases linearly with time of estrogen pretreatment.\(^4\)

Thus, the estrogen-induced alterations in one-

\(^4\) A. Herranen and G. C. Mueller, manuscript in preparation.
carbon metabolism may in part be explained by increased serine aldolase activity. This introduces further questions whether the observed results are due to activation of pre-existing enzyme (unmasking) or due to de novo synthesis of a new enzyme. Answers to these questions should place us closer to an understanding of estrogen action.

C. In vitro effects with estrogens on intact tissues. —A major obstacle to progress in steroid hormone research has been the inability to obtain physiological effects when a hormone is added to in vitro systems. As a result, the question of hormone activation and possible dependency on other tissues has arisen. However, in the case of estrogen, Biggers, Claringbold, and Hardy have demonstrated that estrone produces a typical keratinization of mouse vagina in tissue culture; thus they have excluded or de-emphasized a number of theories of estrogen action which involve vascular changes or hormonal activation by other tissues (5). A similar situation was reported by Kahn, who also observed an antagonistic effect of vitamin A on the estrogen action (49). Further study of this important finding promises to be interesting.

In studying factors which modify mitotic activity, Bullough has reported that the addition of relatively high levels of estrogens to mouse skin incubated in saline containing glucose yields more mitoses in a 4-hour period (10). He concludes that this effect results from a stimulation of the glucokinase activity of this tissue which normally limits the glucose uptake in skin and the availability of energy for mitoses. The estrogen effect can be bypassed by using fructose instead of glucose; apparently, fructokinase is not limiting or estrogen-sensitive.

The addition of estradiol, estrone, or estriol to surviving uterine segments failed to influence the uptake of labeled formate into protein, whereas the 2-hydroxy and the 4-hydroxy derivatives of estradiol gave a significant "estrogen-like" stimulation to this process (66). It remains to be established whether or not the other metabolic pathways for "one-carbon" fragments are stimulated in a manner typical of estradiol in vivo. Of even greater interest is the question of whether or not these hydroxylated derivatives of estradiol will effect an in vitro activation of serine aldolase.

D. In vitro effect of estrogens on enzyme systems. —Working with human endometrium tissue, Hagerman and Villee demonstrated that natural estrogens stimulated the metabolism of glucose and pyruvate toward more complete oxidation (35). This very fine study was extended to the placenta, where a soluble diphosphopyridine nucleotide-linked isocitric dehydrogenase has been found which is stimulated by the addition of estradiol, estrone, equilin, and equilenin (86). It was also stimulated to a much lesser extent by estriol, progesterone, diethylstilbestrol, and testosterone. The relatively high specificity for natural estrogen has permitted the assay of estradiol-17β and estrone (29). While the actual manner in which the hormone participates in this system is unclear, it is possible that it may provide a model system of enzyme activation by a hormone.

The very fundamental work of Talalay and associates, using purified enzymes of bacterial origin, has established the molecular specificity for the combination of steroids with β-hydroxysteroid dehydrogenase (59). It was shown that planar molecules of the androstene and 5 a-androstane series had intermediate affinities, while the natural estrogens had a very high affinity for the enzyme surface. This work appears to be related to that of Villee in that both cases involve dehydrogenation reactions.

The possibility that the estrogens might exert their biological effects in this manner is of course intriguing. However, at this time no evidence is available on the operation of such mechanisms within the tissue. In Villee's studies, the relatively high specificity for natural estrogens argues against this reaction as being a primary step in the mechanism of estrogen action, since certain synthetic
compounds of high biological activity were not active in his system.

Further work in the very important area of hormone-enzyme interaction should be pursued, since it is difficult to escape the conclusion that the effective action of the estrogen must be exerted on an enzyme acting in a multiple enzyme system.

VI. COMMENTS ON THE MECHANISM OF ACTION OF OTHER STEROID HORMONES

While many descriptions of biological responses have been made for other steroid hormones, very few reports concerning their fundamental mechanisms of action have appeared. Accordingly, it is possible at this time only to point out a few problems where metabolic attacks are urgently needed. The tremendous importance of early biochemical studies in responsive tissues can scarcely be overemphasized. Of equal importance is the establishment of dose-response relationships in terms of the metabolic processes with respect to time.

A. Androgenic hormones.—An inclusive review of the metabolic effects of androgens has been made by Roberts and Szego (78), and the more recent aspects of the relationship of androgens to reproduction have been emphasized by Mann (58). For a very comprehensive account of the whole subject of androgens the reader is referred to the compendium prepared by Dorfman and Shipley (17).

While many toxicity studies with androgens on enzyme systems have been carried out (summarized, 78) it is not possible at this time to establish the relevance of these experiments to the problem of androgenicity. It appears that it will be necessary first to establish the metabolic parameters of the androgenic response.

Three sites of rapid and relatively specific response are the chick (or capon) comb, and the seminal vesicles and prostate in the rat. The chick comb appears to offer some advantage for biochemical attack, since it has been demonstrated that the glucosamine and mucopolysaccharide contents are increased strikingly in response to androgens (54, 77). This is an invitation to begin the metabolic dissection at this point in terms of the reactions involved in polysaccharide synthesis. Considerable knowledge on the participation of uridine coenzymes in these reactions already exists. In addition, the isotopic precursors and isolation technics are available.

Attention is again drawn to this area by the rise of glucuronidase in the kidney following androgen treatment (55). Possibly the rise of this enzyme in the kidney is an induced enzyme synthesis in response to higher glucuronide levels in the kidney. Whatever may be the answer, this area appears vulnerable to attack with our present tools and technics.

In this same light, the finding of fructose, citric acid, and ergothionine in the fluids of the accessory organs of reproduction (58) offers other leads for the study of the mechanism of androgen action. The recent observation by Hers that fructose formation in the seminal vesicles involves a direct reduction of glucose to sorbitol with a reoxidation to fructose without involving the phosphorylated sugars suggests another opportunity for biochemical attack on androgen action (59).

The demonstration by Lasnitzki that ventral prostate proliferates in tissue culture under the influence of added testosterone is a move in the direction of a controlled environment for a hormonal response (49). To be of biochemical value, however, such preparations have to be expanded to large-scale cultures.

B. Progestational hormones.—Studies on the mechanism of action of progesterone are complicated by a relative dependency on estrogen priming; however, under standardized conditions the traumatic deciduomata (4) provides a rapid and spectacular biological response. This tissue should lend itself to a sequential analysis. The correlation between carbonic anhydrase level and progesterone treatment indicates again a segmental effect on metabolism (55).

The rather striking activation of mitochondrial adenosinetriphosphatase from liver (87) is an interesting lead; however, at this time the relationship to progestational effects is unclear. It is of interest that progesterone also depresses glycine incorporation in surviving uterine segments when added to the reaction flask (64). The inhibited tissue relaxes or becomes flaccid under its influence.

C. Adrenal cortical hormones.—For an excellent summary of the literature on adrenal cortical physiology, the reader is referred to a review by Noble (68). Despite the multitude of publications on adrenal cortical steroids, the fundamental mechanism of action has resisted elucidation. While the 11-oxygenated corticosteroids have effects on carbohydrate and protein metabolism, no common denominator has emerged.

The striking involution of the thymus gland under hydrocortisone treatment now appears to result mainly from blockage of new cell formation. Thus, the incorporation of P32 into deoxyribonucleic acid is highly inhibited, while the labeling of ribonucleic acid proceeds at a nearly normal rate (18). Since there is also increased cell fragmentation, one cannot rule out completely cell destruction as an important factor in thymus maintenance. With low doses of cortisone the incorporation of labeled amino acids into thymus is also in-
hibited; in contrast, the incorporation into the liver is simultaneously stimulated.*

The studies of Dougherty and associates on inflammation have revealed that hydrocortisone is concentrated in the cytoplasm of fibroblasts within a few hours after topical application. These cells round up and exhibit increased cytoplasmic basophilia and begin to look more epithelioid (19). Under the influence of the hydrocortisone they resist damage from cytotoxic substances in areas of inflammation (19); the authors suggest that the cells become impermeable to such factors.

Thus, while the adrenal steroids exert striking effects on “connective tissue” elements, particularly in so-called connective tissue diseases, very little insight has been gained as to the fundamental site of action. It appears that a really good test system will have to be established before much progress can be made. We are especially limited by our lack of knowledge concerning the formation of collagen and the materials included in the “ground substance” lying between cells.

Our knowledge of the mechanism of action of aldosterone and deoxycorticosterone in regulating electrolyte balance is similarly deficient. Here it appears that fundamental knowledge on ion transport will have to be established before much progress can be expected. These hormones should constitute excellent tools for this study.

The studies of Stolkowski and Reinberg (71, 81, 82) demonstrate an effect of deoxycorticosterone on potassium transport in isolated systems. In their studies the corticosteroids inhibited the entrance of potassium, whereas the hydrogen ion production from glucose metabolism acts to displace potassium from the cell. While it is too early to conclude anything concerning deoxycorticosterone action, this approach promises to lead to some fundamental discoveries.

**CONCLUDING COMMENTS**

In this discussion, the mechanism of action of steroid hormones has been considered as a problem in fundamental cell physiology. The units of structure and function are the enzymes as they exist in specific association with other enzymes in semi-isolated compartments. In this state the individual enzymes compete as members of multiple enzyme systems; the amount of each enzyme, their inherent catalytic activity, and substrate availability determine a particular steady state characteristic of a certain level or type of function.

To modify function, a steroid hormone must act within these confines so as to induce a shift in the balance of power between various cell processes.

To accomplish this, it appears necessary that the *active form* of the steroid hormone interact primarily with an enzyme to produce an activation or inhibition of this unit for the catalysis of a specific process. This alteration, in turn, is amplified and expressed through the establishment of a different steady state among the various multiple enzyme systems of the cell.

The importance of the compartmental nature of the cell for organized function is emphasized. It is suggested that communication between such loci of function may be subject to hormonal influence through altered rates of formation or destruction of limiting membranes or interfaces. It is also suggested that electrostatic effects, analogous to changes taking place in nerve cell membranes or in contractile proteins, may be responsible for activating certain enzymes or increasing the accessibility of enzymes to substrates. This area of research offers exciting opportunities for discovery.

It is proposed that attempts to explain steroid hormone action in molecular terms will profit by first searching out the segments of metabolism which are responsive to the early action of a hormone. Guided by the results of such surveys on intact tissues, one can next begin to analyze the observed effects in cell-free systems. In Section V, a summary of studies on the mechanism of action of estrogens was presented as an example of this approach. The data support the conclusion that synthetic pathways involving carboxyl activation and “one-carbon” transfer are very sensitive to estrogen action. A preliminary attempt to analyze the estrogen effect on one-carbon metabolism has demonstrated that the level of serine aldolase increases rapidly in uterine tissue in response to estradiol. The relevance of this finding to the mechanism of action of estrogen as well as the further analysis of other sensitive pathways in cell-free systems awaits study.

Despite the fact that at this time we do not know the mechanism of action for any steroid hormone, this author feels highly optimistic concerning the future. The much needed information on fundamental cell processes is being accumulated at a tremendous rate; it remains for the discerning investigator to put these findings to use along the lines discussed. In this manner, the steroid hormones can be expected to serve as useful tools in the study of important problems in health and disease.

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