INHIBITION OF THE ENZYMIC OXIDATION OF DPNH BY STEROID HORMONES

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The role of the steroid hormones in the regulation of cell metabolism, although of considerable biological importance, is not well understood. Progress has been made by two groups of investigators[1, 2] who have ascribed to some steroids the function of catalyzing the enzymic transfer of hydrogen between di- and triphosphopyridine nucleotides, although the mechanism of this reaction is controversial.[3, 4]

The present communication describes an apparently unrelated general property possessed by a number of hormonally active steroids, that is, their ability to inhibit, at low concentrations, the oxidation of DPNH catalyzed by enzymes derived from both animal and microbial sources.

Materials and Methods.—DPNH, TPNH, steroids, diethylstilbesterol, cytochrome C, α-tocopherol, and *n*-butyl stearate were commercial preparations. Samples of ubiquinone (Q 275, obtained originally from Merck and Company) were gifts from Drs. T. C. Stadtman and L. Corwin. Erlich and Sarcoma 37 ascites tumor-bearing mice were kindly supplied by Dr. Morris Belkin of the National Cancer Institute. Tumor cells were harvested 4 and 6 days, respectively, after inoculation.

Saccharomyces fragilis (ATCC #10022) was grown aerobically in a 5 per cent glucose salts solution[5] for 36 hours at room temperature. *E. coli*, strain B, and *B. subtilis* (ATCC #465) were grown overnight, with shaking, at 37° in a medium consisting of Difco nutrient broth 8 gm., sodium chloride 4 gm., dissolved in 1 liter of tap water.

Preparation of DPNH Oxidases.—Fractions from the tissues of male Sprague-Dawley rats were prepared by the procedure used by Lehman and Nason to obtain the particulate DPNH-cytochrome C reductase system.[6] Organs were homogenized in a volume of 0.1 *M* phosphate buffer, pH 7.5, equal to 10 times the wet weight of tissue. The supernatant layer, obtained after centrifugation at 3000 × *g* for 15 minutes, was dialyzed against 10 volumes of 0.01 *M* phosphate buffer, pH 7.5 for one hour. The dialyzed preparation was then centrifuged at 100,000 × *g* for 30 minutes and the resulting pellet was resuspended in one-tenth its original volume of 0.1 *M* phosphate buffer, pH 7.5. All of the foregoing operations were carried out between 0 and 5°. Microsomal and mitochondrial preparations were also made from various tissues by the technique of Hogeboom[7] using 0.25 *M* sucrose-0.001 *M* EDTA as the medium for all operations.

Tumor and yeast cells, harvested by centrifugation, were resuspended in the sucrose-EDTA solution. The suspensions were mixed with an equal volume of glass beads (Minnesota Mining & Manuf. Co. #150 75μ) and fragmented in the Nossal disintegrator[8] at −5°C. Tumor cells were treated for 30, and yeast cells for 60 seconds, in 15 second periods separated by intervals of 15 seconds. Particles corresponding to mitochondria and microsomes were then isolated by differential centrifugation.[7]

Bacterial cells, harvested by centrifugation, were washed once and resuspended in distilled water. The suspensions were subjected to high frequency sound waves

for 15 minutes in a Raytheon 9 Kc sonic oscillator maintained near 0°. The disrupted cells were then centrifuged at 13,000 × *g* for 10 minutes to remove cell debris. The supernatant layer was re-centrifuged at 100,000 × *g* for one hour and the pellet was re-suspended in a volume of distilled water equal to half the original volume centrifuged. The clear supernatant fraction was also saved and tested as described below.

Microsomal cytochrome reductase, prepared from rabbit liver according to the method of Velick and Strittmatter[9] was carried through to the lyophilized alcohol extract step. Cytochrome C was reduced by hydrogen over Pd and asbestos.[10]

Emulsions of tocopherol, ubiquinone, *n*-butyl stearate, and butter were prepared according to Nason and Lehman.[11]

Steroids and diethylstilbesterol were added to reaction mixtures as solutions in 50 per cent (by volume) propylene glycol. In experiments where the effects of tocopherol, other lipids, or steroids were tested, controls were run which contained the appropriate medium.

Enzyme Assays.—Oxidation of DPNH was estimated by measuring the decrease in optical density at 340 mμ in the Beckman model DU spectrophotometer at room temperature in 3 ml silica cells of 1 cm light path. The initial rate, determined during the first 5-minute period, was expressed as the change in optical density/minute. The rates were constant until the DPNH was virtually exhausted. The molar extinction coefficient of reduced DPN^+ was considered to be 6.22×10^3 l/mole cm.[12]

DPN cytochrome C reductase activity was measured by the method described by Lehman and Nason[6] in which substrate quantities of cytochrome C were added to the reaction mixture together with sufficient KCN (10^{-3} *M*) to prevent its oxidation. The course of the reductase reaction was followed by observing either DPNH oxidation at 340 mμ, or cytochrome C reduction at 550 mμ. Succinate cytochrome C reductase was assayed in the same way[6] by measuring the rate of reduced cytochrome C formation when succinate was added to the reaction mixture.

Microsomal cytochrome reductase activity was determined from the rate of DPNH oxidation with $Fe(CN)_6^{-3}$ as the electron acceptor.[9] This value was corrected for the nonenzymic oxidation of DPNH by ferricyanide.

Cytochrome oxidase was measured spectrophotometrically by following the oxidation of reduced cytochrome C at 550 mμ.[10]

Results.—Figure 1 shows the effect of progesterone on the enzymic oxidation of both DPNH and TPNH, catalyzed by microsomes prepared from rat kidney. Curve A depicts the oxidation of DPNH in the absence of added steroid, and Curve B illustrates that, in the presence of 2×10^{-5} *M* progesterone, this rate was drastically reduced. Curves C and D demonstrate that the same concentration of steroid did not affect TPNH oxidation. This inhibitory action of progesterone appeared to be catalytic since 0.05 micromoles of hormone produced an inhibition corresponding to 0.35 micromoles of DPNH. This suggested that the reduction of DPN by the steroid could not account for the difference between the experimental and control cuvettes. In support of this, when DPN was incubated with progesterone and enzyme under the same conditions, no reduced DPN was formed.

The effects of two steroids, progesterone and deoxycorticosterone, on the DPNH oxidase reaction from a variety of sources were examined and the results are given

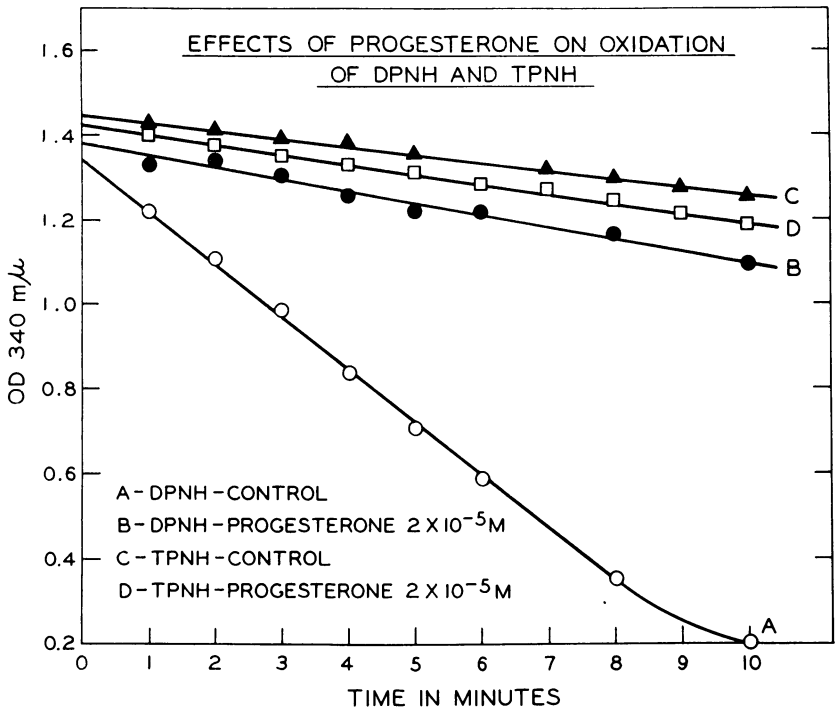

FIG. 1.—Cuvettes A and B contained 0.1 ml of a suspension of kidney microsomes equivalent to 25 mg of original wet weight of tissue, tris buffer, pH 7.25, 0.01 M, $MgCl_2$ 2×10^{-3} M, KCl 7.5×10^{-5} M, sucrose 0.12 M, DPNH 4×10^{-4} M, and 50 per cent propylene glycol 0.1 ml with or without added steroid in a volume of 2.5 ml.

Cuvettes B and C were identical except 0.2 ml of the microsomal suspension was used and TPNH was substituted for DPNH.

in Table 1. Higher steroid concentrations were used with the microbial enzymes than with the animal preparations. The reaction catalyzed by particles obtained from liver, kidney, heart, skeletal muscle, and the tumors, was strongly inhibited by both compounds at 2×10^{-5} *M*. With mammalian tissue, virtually identical results were obtained with either mitochondrial[13] or microsomal preparations, and there was no significant difference whether phosphate buffer or sucrose-EDTA was used.

The rate of DPNH oxidation, catalyzed by the particles of *S. fragilis* which sedimented at 9,000 × *g* (*Mitochondria* in Table 1), was reduced almost 70 per cent by 5×10^{-4} *M* progesterone. The sediment obtained at 100,000 × *g* was not inhibited as strongly, however.

The DPNH oxidase reactions of *E. coli* and *B. subtilis* were also studied. The soluble enzymes, i.e., those not sedimenting at 100,000 × *g* in 1 hour, were inhibited 35–50 per cent by either progesterone or DOC at 5×10^{-5} *M*.

Having thus demonstrated that progesterone and DOC have a general inhibitory effect on DPNH oxidation, it was important to ascertain both the number of steroids which possess this inhibitory capacity and the extent to which the various steroids are inhibitory. Data releveant to these points are presented in Table 2, in which the K_i values (obtained by the method of Dixon and Webb[14]) are shown

for nine steroids with enzymes derived from three tissues of the rat. These represent concentrations of inhibitors which produce half-maximal inhibition and are assumed to be proportional to the affinities of the vulnerable enzymes for the steroid

TABLE 1

EFFECTS OF PROGESTERONE AND DEOXYCORTICOSTERONE ON DPNH OXIDASES

Enzyme Source in ml	ΔO.D.$_{340}$/min. $\times 10^2$ Control	Progesterone 2×10^{-5} M	DOC 2×10^{-5} M	Inhibition Progesterone Per Cent	DOC Per Cent
Liver[1] 0.1	1.25	0.35	0.78	72	41
Kidney[1] 0.05	6.37	1.68	3.85	74	39
Heart[1] 0.05	3.85	1.60	2.76	58	28
Skeletal muscle[4] 0.02	3.78	1.62	2.40	57	36
Sarcoma 37[1] 0.05	1.53	0.36	..	77	..
Ehrlich ascites[1] 0.05	1.73	0.38	..	78	..
S. fragilis[2] 0.1	7.70	2.38*	4.20*	69	45
E. coli[3] 0.1	8.3	4.08†	4.32†	51	48
B. subtilis[3] 0.1	3.25	2.13‡	1.98‡	35	39

Experiments 1–3 and 5–9 were conducted as described under Figure 1, cuvettes A and B, except for the volume of tne enzyme used and the substitution of 2×10^{-4} M DPNH. In experiment 4, the reaction mixture contained phosphate buffer, pH 7.5, 0.09 M, cytochrome C 0.04 per cent, DPNH 2×10^{-4} M, 50 per cent propylene glycol 0.1 ml alone or with steroid. All the reaction volumes were 2.5 ml.

* Steroid concentration 5×10^{-4} M.
† Steroid concentration 5×10^{-5} M.
[1] Microsomes, 1 ml equal to 250 mg original wet weight of tissue.
[2] Mitochondria, 1 ml equal to 250 mg original wet weight of tissue.
[3] 100,000 $\times$ g supernate, 1 ml equal to 125 mg original wet weight of tissue.
[4] Fraction III of Lehman and Nason,[6] 1 ml equal to 1 gm. of original wet weight of tissue.

inhibitors. In every case progesterone and the synthetic steroid analogue, diethylstilbesterol, were the best inhibitors with K_i's in the range of 8×10^{-7} to 10^{-5} M depending on the organ. Deoxycorticosterone, testosterone, estradiol, and dihydrotestosterone were next with values in the neighborhood 5×10^{-5} M. The corticoids, cortisone, corticosterone, and the physiologically inactive dihydrocortisone, were least potent, with constants from 2×10^{-5} to 8×10^{-4} M. In general, the kidney was the most responsive of the organs tested.

TABLE 2

EFFECTIVENESS OF STEROIDS AS INHIBITORS OF DPNH OXIDATION

Steroid	K_i, M. Muscle	Liver	Kidney
Corticosterone	6.5×10^{-5}	8×10^{-5}	2.6×10^{-5}
Deoxycorticosterone	3×10^{-5}	1.3×10^{-5}	1×10^{-5}
Progesterone	1.6×10^{-5}	6.4×10^{-6}	3.2×10^{-6}
Estradiol	3.7×10^{-5}	*1.4×10^{-5}	..
Diethylstilbesterol	6.4×10^{-6}	6.5×10^{-6}	8×10^{-7}
Testosterone	3.2×10^{-5}	*4×10^{-5}	..
Dihydrotestosterone	2.8×10^{-5}	*8×10^{-5}	..
Cortisone	5×10^{-4}	*1.2×10^{-4}	..
Dihydrocortisone	8×10^{-4}	*3.8×10^{-4}	..

Reactions were carried out as described in Table 1, experiment 4, except that the concentrations of the steroids were varied. The experiments marked * were conducted as described in Table 1, experiments 1–3.

In addition to those listed in the table, the following compounds were tested with liver microsomes and found to produce substantial inhibition: cortisol, Δ^1 cortisone, 1,4-androstadiene-3,17-dione, 4-androstene-3,17-dione, androstane-3,17-dione, 5β-androstane-3,17-dione, 19-nor testosterone, 11β-hydroxyprogesterone, 11α-hydroxyprogesterone, 17α-hydroxyprogesterone, 5α-pregnan-3β-ol-20-one, and pregenolone. Several steroids, however, had no effect on DPNH oxidation. These were tetrahydrocortisone, cholesterol, ergosterol, digitoxin, and digoxin.

The DPNH oxidase reaction is composed of many individual steps: the electrons from the reduced pyridine nucleotide are passed through a flavoprotein, the cytochromes and eventually, to oxygen. It was obviously of interest to localize the steroid inhibition as precisely as possible. To this end, reduced cytochrome C was prepared and its oxidation tested to give a measure of cytochrome oxidase activity in skeletal muscle. No inhibitory effect of steroid hormones was noted.

The span between DPNH and cytochrome C in particles from skeletal muscle was then examined directly. Figure 2 illustrates the results of these experiments.

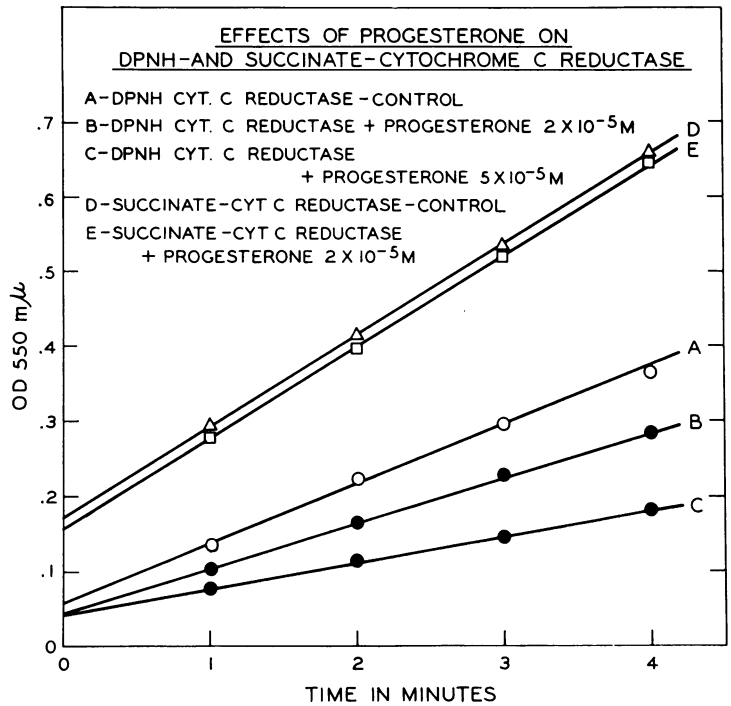

FIG. 2.—Cuvettes A, B, and C were identical to experiment 4, Table 1, with addition of 1×10^{-3} M KCN and the substitution of 0.08 per cent cytochrome C.

Cuvettes D and E contained 0.1 ml of a suspension of heart mitochondria, equivalent to 25 mg of wet weight of tissue, phosphate buffer pH 7.5, 0.09 M, KCN 1×10^{-3} M, cytochrome C 0.09 M, KCN 1×10^{-3} M, cytochrome C 0.08 per cent, sodium succinate 8×10^{-3} M, and 50 per cent propylene glycol 0.1 ml alone or with added steroid.

Here the reduction of cytochrome C, in the presence of 10^{-3} *M* cyanide, was followed at 550 millimicrons. Two and 5×10^{-5} *M* progesterone exerted a considerable inhibitory effect on the enzymic reduction of cytochrome C when DPNH was the hydrogen donor. It should be noted, however, that the degree of progesterone inhibition observed here was considerably less than when its effect on DPNH oxidation was measured in the absence of cyanide. This could be explained by the concurrent observation that the rate of DPNH oxidation was itself inhibited as much as 90 per cent when cyanide was added, even in the presence of substrate amounts of cytochrome C. This was due, perhaps to the accumulation of reduced cytochrome

C behind the cyanide block. It appeared, therefore, that a different reaction became rate-limiting in the DPNH-cytochrome C reductase sequence in the presence of cyanide. The reductase would consequently appear less sensitive to inhibition by steroid under these conditions. This interaction of steroids with the DPNH-cytochrome C reductase system could account for the observed inhibition of the DPNH oxidase reaction, at least in skeletal muscle. Since both the DPNH- and succinate-cytochrome C reductases may transfer electrons to cytochrome C via cytochrome b, it was of interest to determine whether the reduction of cytochrome C by succinate would likewise be affected by steroids. The results of an experiment

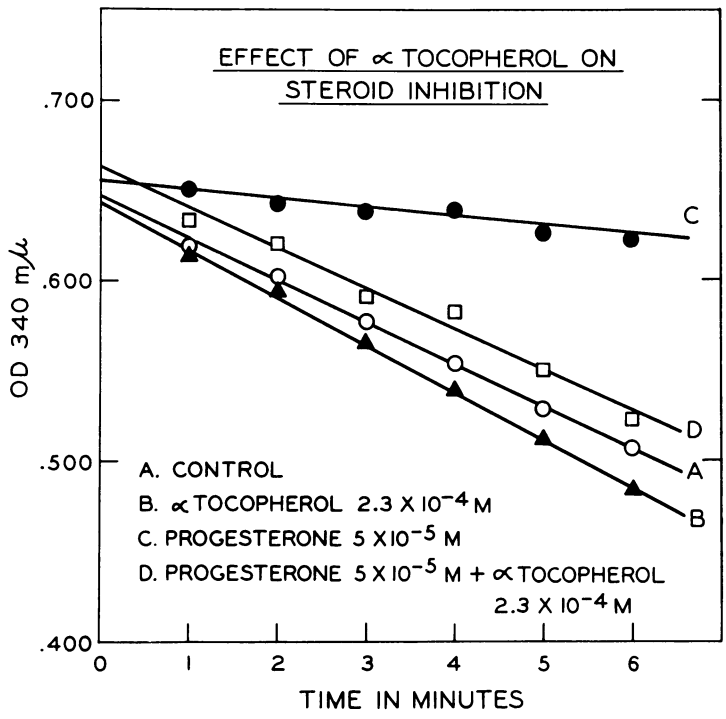

FIG. 3.—The reaction conditions were as described for experiment 4, Table 1, with additions as noted.

to test this are also illustrated in Figure 2. No inhibitory effect of progesterone on cytochrome reduction was noted when succinate was the hydrogen donor.

Recently, several investigators have been concerned with the role of lipids in the DPNH-cytochrome C reductase system.[11, 15–21] For this reason we have investigated the effect of α-tocopherol on steroid inhibition of the DPNH-cytochrome C reductase of skeletal muscle. In Curve A of Figure 3, the rate of DPNH oxidation is plotted, and in Curve B a similar experiment is shown run in the presence of 2.3×10^{-4} M α-tocopherol, which did not affect the control rate significantly. In Curve C, the inhibition due to progesterone is noted and in Curve D, this inhibition has been completely reversed by the further addition of 2.3×10^{-4} M α-tocopherol. This reversal by α-tocopherol was also demonstrated with bacterial preparations. In other experiments with the muscle enzyme, kinetic analysis has indi-

cated that inhibition by progesterone was competitively reversed by α-tocopherol.

Since Donaldson and Nason have found that, in addition to α-tocopherol, various other compounds can reactivate iso-octane-extracted preparations of DPNH-cytochrome C reductase,[21] we have tested some of these, i.e., *n*-butyl stearate, menadione, and butter. All of these substances were found to release the steroid inhibition. Ubiquinone, however, did not alter the response to steroid under the conditions of our assay.

In view of the sensitivity of liver microsomes to inhibition by the steroids, and because the major route of electron flow in these particles is thought to be through the flavoprotein, microsomal cytochrome reductase,[9] this enzyme was prepared and assayed directly. Progesterone did not reduce the rate of enzymic DPNH oxidation by ferricyanide.

Discussion.—Our results showed that steroid hormones could act as potent inhibitors of the enzymic oxidation of DPNH while the oxidation of neither TPNH nor succinate was impaired. Furthermore, this action was catalytic since low concentrations of the hormones inhibited the oxidation of much larger amounts of the reduced pyridine nucleotide. The catalytic nature of the steroid effect is clearly desirable in substances which are biologically active in low concentrations.

Recently, it has been demonstrated that some steroids can act as coenzymes for the transfer of hydrogen between DPN and TPN,[1, 2] but the actual manner in which the steroids participate is the subject of dispute.[3, 4] Also, at the present time there is insufficient evidence to decide whether this biochemical function is an important explanation for any of the biological effects of the hormones.[22] Regardless of the status of this problem, our results are almost certainly independent of a steroid-mediated transhydrogenase reaction. If transhydrogenation were involved, it could occur between the substrate, DPNH, and a small amount of endogenous TPN, which would then be oxidized. The steroids would necessarily have to function here as inhibitors rather than activators of transhydrogenation in order to explain the inhibitory effect. Furthermore, we have noted that in every preparation examined (e.g., Fig. 1), the rate of oxidation of added TPNH is considerably lower than that of DPNH, making the participation of TPN and a transhydrogenation in the DPNH oxidase reaction very unlikely.

Although equally unlikely, it might also be argued that our results were due to steroid activation of transhydrogenation between TPNH and DPN^+. The former would have to arise from the reduction of a catalytic amount of TPN by a large amount of endogenous substrate. This minute amount of TPNH could, in the presence of a steroid mediated transhydrogenation, constantly regenerate DPNH, making it appear as though the steroid were inhibiting the oxidation of DPNH. This possiblity is excluded by our findings that in the presence of DPN^+ and steroid no DPNH is formed, even in the presence of cyanide.[23] These considerations, then, make it highly improbable that the participation of steroids (or diethylstibesterol) in the present effect is as a result of inhibition or, for that matter, stimulation of the transhydrogenase reaction.

It seems very unlikely, furthermore, that metabolic alteration of the steroids is necessary for their inhibitory action, because when DPN was incubated with progesterone, no reaction was detected, although under the same conditions, steroid strongly inhibited DPNH oxidation. Also, the variety of inhibitory steroids, and

especially the affectiveness of diethylstilbesterol, makes it inconceivable that a single type of metabolic transformation could produce the observed effect.

Finally, it is significant that both the intracellular and tissue distribution of steroid metabolizing enzymes previously investigated is quite different from that of the DPNH-oxidase reaction.[24, 25]

In the present study, the detailed mechanism of steroid action was examined only in rat skeletal muscle preparations, although it seems reasonable that the same mechanism operates in other tissues as well. The data point to the DPNH-cytochrome C reductase reaction as the site of the inhibition. The latter, however, is a complicated reaction sequence and neither the components of the system nor the exact route of electron transfer from reduced pyridine nucleotide to cytochrome C is fully understood. Because of this it is impossible to say precisely where the steroid is working. It seems relevant that lipids have been implicated in electron transport, although this question is still unsettled.[15-21] The fact that α-tocopherol overcame the steroid inhibition suggests that the same step is involved which was inhibited by iso-octane extraction in the experiments of Nason and his collaborators.[11] This is not necessarily the case since iso-octane extraction inactivated the succinate-, as well as DPNH-cytochrome C reductase, and in both cases the activities could be restored by α-tocopherol.[11] Our results showed that progesterone did not affect the succinate reaction. Furthermore, there was no α-tocopherol stimulation of iso-octane-extracted DPNH oxidase from bacteria,[11] while we have found steroid inhibition of this bacterial oxidase reaction and its reversal by α-tocopherol.

One of the components of the DPNH-cytochrome C reductase reaction is a flavoprotein.[26] It is, therefore, of interest that steroids have been found to inhibit various flavoproteins,[27] but in much higher concentrations than those used in the present study. It may be especially relevant to note that the soluble, highly purified DPNH cytochrome C reductase of heart muscle can be inhibited by prolonged incubation with high concentration (3×10^{-3} M) of deoxycorticosterone,[28] but is not affected by cortisone. There is, therefore, no obvious connection between these results and our findings.

As steroids did not appear to inhibit the DPNH oxidases by virtue of alteration of the steroids themselves, we must conclude that their inhibitory effects are exerted through physical interaction with some undetermined component of the complex enzyme system. An examination of the structures of the effective steroid inhibitors does not reveal any obvious common properties. Compounds which are both saturated and unsaturated in the A ring, as well as the benzenoid female hormones, are active; and polarity, likewise, does not seem to be important. Despite this, however, there is an enormous variation in effectiveness among the various steroids, which is attested to by their K_i values which range from 8×10^{-7} for stilbesterol, to cholesterol which was completely ineffective.

It is obviously important to consider what, if any, physiological significance may be attached to the inhibition of DPNH oxidase, and various properties of this steroid-enzyme interaction suggest that this effect might indeed be biologically significant. First, the high affinity of the DPNH-cytochrome C reductases for the steroids, especially progesterone, estrogen, and stilbesterol, implies that part of the action of these hormones may be as a result of the effect reported here. It is note-

worthy that in mammalian cells the DPNH oxidase reaction is confined to particles which are rich in lipids,[29] and, as Lynn[30] has demonstrated, testis microsomes, because of their high lipid content, concentrate steroids. Therefore, even at low extracellular hormone levels, the concentration at the possible site of action might be considerably higher.

Especially interesting is the action of diethylstilbesterol. This compound, although not a steroid, is structurally very similar to estradiol[31] and has a steroid-like action *in vivo;* and the fact that it, like the steroids, also inhibits these enzyme preparations argues in favor of the biological significance of the effect reported herein.

Since organized biological systems have an amazing capacity to amplify and elaborate on a disturbance at the molecular level, it is not possible to predict what the macroscopic manifestations of such an effect would be. Be that as it may, there are several intriguing possibilities. One of these is the mechanism of progesterone action. The physiological effects of this hormone are seen principally in organs which have been previously stimulated by estrogens. Hollander has studied an enzyme which catalyzes the oxidation of DPNH by molecular oxygen and is stimulated by phenols.[32] It is virtually absent in the uteri of spayed animals, but a single dose of estrogen produces a large increase in its level. If the role of progesterone is actually to inhibit the flow of electrons from DPNH through the cytochrome system, as suggested by our data, the presence of an estrogen-induced alternate pathway for DPNH oxidation might be significant physiologically.

Another possible biological correlation pertains to the almost universal property of steroids to inhibit cell growth. Tumors,[33] tissue culture cells,[34] and microorganisms[35] are affected, and it seems reasonable to suppose that at least some of these inhibitory affects might be the result of the inhibition of DPNH oxidation.

Finally, even though the actual role of α-tocopherol in DPNH-cytochrome C reductase has been disputed,[19] and we cannot discuss with assurance the possible physiological connection between the hormones and a lipid cofactor, it might be pertinent that several of the manifestations of vitamin E deficiency, i.e., fetal resorption in the female and testicular atrophy and sterility in the male, could be related to alterations in steroid hormone action.

From the physiological standpoint, it is somewhat disturbing that all the organs tested seemed to be almost equally responsive to such hormones as stilbesterol or progesterone. It is, therefore, difficult to explain the tissue-specific physiological responses to different steroids. It may be, however, that permeability factors, etc., impose a greater specificity when intact cells are exposed to the hormones. Another important variable might be the degree to which cytochrome C reductase is rate-limiting in cell metabolism.

Summary.—Low concentrations of a large number of steroid hormones and diethylstilbesterol inhibited DPNH oxidation by enzyme preparations from both mammalian and microbial sources. Neither TPNH nor succinate were similarly affected, however.

In skeletal muscle, the site of steroid inhibition proved to be the DPNH-cytochrome C reductase reaction and α-tocopherol and other compounds could competitively reverse the steroid effect.

We should like to acknowledge the devoted expert assistance of Mrs. Janet Munday and Mr. Irwin J. Cowley.

Note added in proof: After this manuscript was submitted for publication, a letter appeared in *Nature* (P. K. Jensen, 184, No. 4684, 451) in which inhibition of DPNH oxidation in heart sarcosomes by corticosterone, cortisol, 11-deoxycorticosterone, 17-hydroxy-11-deoxycorticosterone, and cortisone was reported.

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