[6] M. Gibbs and O. Kandler, these PROCEEDINGS, **43**, 446, 1957.
[7] S. Korkes, *Ann. Rev. Biochem.*, **25**, 701, 1956.
[8] I. A. Rose and S. V. Rieder, *J. Am. Chem. Soc.*, **77**, 5764, 1955.
[9] I. A. Rose and S. V. Rieder, in preparation.
[10] The following abbreviations are used: G3P, D-glyceraldehyde-3-phosphate; D-G, fructose-1,6-diphosphate; EDTA, ethylenediaminetetraacetate; DPN, diphosphopyridine nucleotide; and DPNH, reduced DPN.
[11] C. Neuberg and H. Lustig, *J. Am. Chem. Soc.*, **64**, 2722, 1942.
[12] K. Lohmann and O. Meyerhof, *Biochem. Z.*, **273**, 413, 1934.
[13] I. Berenblum and E. Chain, *Biochem. J.*, **32**, 295, 1938.
[14] H. J. Deuticke and S. Hollmann, *Z. physiol. Chem.*, **258**, 160, 1934.
[15] A. C. Palladini and L. F. Leloir, *Biochem. J.*, **51**, 426, 1952.
[16] D. Herbert, H. Gordon, V. Subrahmanyan, and D. E. Green, *Biochem. J.*, **34**, 1108, 1940.
[17] H. G. Wood, *Physiol. Rev.*, **35**, 841, 1955.

---

*ACTIVATION OF HYDROGEN TRANSFER BETWEEN PYRIDINE NUCLEOTIDES BY STEROID HORMONES**

BY PAUL TALALAY† AND H. G. WILLIAMS-ASHMAN

BEN MAY LABORATORY FOR CANCER RESEARCH AND DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CHICAGO

Communicated by Charles Huggins, November 7, 1957

It will be shown in this paper that a soluble enzyme from human placenta promotes the transfer of hydrogen between two pyridine nucleotide coenzymes and that this reaction is activated by minute quantities of certain steroid hormones. This enzyme system catalyzes the over-all reaction:

$$\text{TPNH} + \text{DPN}^+ \rightarrow \text{TPN}^+ + \text{DPNH},$$

where DPN and TPN refer to the oxidized forms of di- and triphosphopyridine nucleotides and DPNH and TPNH designate the respective reduced nucleotides. Consideration will be given to the pivotal role of this reaction in the control of metabolic processes. The implications of the control of pyridine nucleotide balance by steroids will be discussed in relation to the physiological actions of these hormones.

Recent experiments of Villee *et al.*[1, 2, 3] showed that, in the presence of isocitrate the reduction of DPN catalyzed by crude extracts of placenta was accelerated by certain steroids. These findings were interpreted in terms of a steroid activation of a DPN-specific isocitric dehydrogenase. The observations of Villee were readily confirmed in this laboratory. However, it was found that the response of crude placental extracts to steroids was variable and that the remarkable stimulation of the reduction of DPN by certain hormones disappeared upon fractionation of the extracts. We have observed that the addition of *catalytic* quantities of TPN to the reaction mixture increased markedly the ability of different placental preparations to respond to steroids and restored this effect in preparations which were inactive in this respect. It will be shown that the apparent stimulation of a DPN-linked isocitric dehydrogenase can be accounted for in terms of a coupling of the

TPN-specific isocitric dehydrogenase of placenta with the action of a soluble transhydrogenating system here described:

$$\text{Isocitrate} + \text{TPN}^+ \rightleftarrows \alpha\text{-ketoglutarate} + CO_2 + \text{TPNH} + H^+ \quad (a)$$
$$\text{TPNH} + \text{DPN}^+ \rightleftarrows \text{TPN}^+ + \text{DPNH} \quad (b)$$
$$\text{Isocitrate} + \text{DPN}^+ \rightleftarrows \alpha\text{-ketoglutarate} + CO_2 + \text{DPNH} + H^+$$

Only step *b*, the transhydrogenase system, is activated by steroids. Isocitric dehydrogenase is not the only TPN-specific enzyme which can act as a generator for TPNH in this coupled reaction. It can, for instance, be replaced equally well by glucose-6-phosphate dehydrogenase.

Partially purified fractions from placenta which catalyze hydrogen transfer between the two forms of pyridine nucleotide also catalyze the oxidation of steroids by DPN and TPN. Of the steroids examined, only those which can undergo oxidoreduction by pyridine nucleotide-linked hydroxysteroid dehydrogenases[4] are capable of activating the transhydrogenase mechanism. The experiments are consistent with the view that the proteins catalyzing dehydrogenation and transhydrogenation are identical.

METHODS

Preparation of Placental Enzymes.—Crude extracts were prepared by the method of Villee and Gordon.[2, 3] Human placenta was obtained immediately after birth and placed on ice. As much blood as possible was removed by perfusion with approximately 1 liter of isotonic sodium chloride. The tissue was homogenized with 4–5 volumes of ice-cold 0.25 *M* sucrose, either in a glass homogenizer equipped with a Teflon pestle or in a Waring Blendor. All further operations were carried out at less than 2°. The homogenates were centrifuged at 2,000 × *g* for 15 minutes, and the resulting supernatant fluid was centrifuged at 59,000 × *g* for 1 hour in a Spinco preparative ultracentrifuge. The supernatant fluid obtained from the second centrifugation was stored either in an ice-bath or in the frozen state and will be referred to as the "crude placental extract." Fractionation was carried out with ammonium sulfate at 0°. The precipitates which formed upon the addition of various amounts of ammonium sulfate were collected by centrifugation and were dissolved in 0.01 *M* tris(hydroxymethyl)aminoethane (Tris) buffer of pH 7.4. These fractions were stored at −10°.

Determinations.—Spectrophotometric measurements were made with a Beckman model DU spectrophotometer using either Pyrex or silica cells of 1 cm. light path. The formation of reduced pyridine nucleotides was followed by measurement of change in absorbance at 340 mμ. The molar extinction coefficient of reduced pyridine nucleotides at 340 mμ was assumed to be 6,220. The TPN was estimated with purified isocitric dehydrogenase of rat heart in Tris buffer at pH 9.0 in the presence of an excess of isocitrate and 0.0003 *M* $MnCl_2$. The isocitrate was determined in the same system with an excess of TPN. Glucose-6-phosphate and DPN were estimated with the aid of yeast glucose-6-phosphate and alcohol dehydrogenases, respectively. Protein was determined by the method of Warburg and Christian.[5]

Materials.—DPN and TPN were commercial products of greater than 85 per cent purity and were uncontaminated by each other. Isocitric dehydrogenase,

purified from rat heart by a slight modification of the method of Siebert *et al.*,[6] was generously donated by S. K. Wolfson, Jr. This purified enzyme did not reduce DPN upon the addition of isocitrate and $MnCl_2$ in the presence or absence of estradiol 17β. It had a specific activity of 0.6 μmole of isocitrate oxidized per minute per milligram protein under the conditions of the experiments to be described. Twice recrystallized yeast alcohol dehydrogenase was obtained as a paste suspended in 60 per cent saturated ammonium sulfate solution from the Sigma Chemical Company, St. Louis, Missouri. It was diluted with ice-cold deionized water just prior to use. The samples of estradiol 17β and of testosterone were of greater than 98 per cent purity as determined by enzymatic assay.[7] The other steroids and diethylstilbestrol were of high purity. All other preparations were of commercial origin.

RESULTS

Response of Crude Extracts of Placenta to Estradiol 17β.—The experiments were carried out under conditions slightly different from those described by Gordon and Villee.[2, 3] Each vessel contained 300 μmoles Tris buffer of pH 7.4, 1 μmole of DPN, and 1.0 ml. of a crude extract of placenta equivalent to approximately 0.2 gm. of fresh tissue. The total volume was made up to 3.0 ml. with water, and estradiol 17β was added in 0.01 ml. of dioxane, which was also added to the control vessels.

The reduction of DPN by some crude placental extracts was accelerated markedly by estradiol 17β at a final concentration of 5×10^{-6} M, with or without the addition of isocitrate (1×10^{-3} M) or glucose-6-phosphate (1×10^{-3} M) to the reaction mixture. Under similar conditions, other placental preparations did not respond to estradiol 17β, but it was possible to restore their sensitivity to this hormone by the addition of *catalytic* amounts (0.01–0.05 μmole) of TPN.

Further experiments with crude placental extracts revealed that (*a*) these preparations reduced TPN very rapidly in the absence or presence of added substrates (the reduction of TPN was unaffected by estradiol 17β); (*b*) the magnitude of the increase in rate of DPN reduction induced by estradiol 17β was enhanced by the addition of TPN-reducing substrates, such as isocitrate or glucose-6-phosphate; and (*c*) even with those extracts of placenta in which the steroids accelerated the reduction of DPN in the absence of exogenous TPN, the addition of catalytic amounts of the latter nucleotide magnified the stimulation induced by estradiol 17β.

These phenomena are explicable in terms of a coupled reaction between a TPN-specific isocitric dehydrogenase or a TPN-specific glucose-6-phosphate dehydrogenase and an enzymatic mechanism which could transfer hydrogen from TPNH to DPN (see eqs. [*a*] and [*b*]). Presumably those extracts which were stimulated by estradiol 17β without the further addition of catalytic amounts of TPN contained sufficient quantities of the latter nucleotide to allow the coupled reaction to proceed. The amount of TPN required was found to be very small, since as little as 0.01 μmole of TPN restored the ability of inactive placental extracts to respond to estradiol 17β. Definitive evidence in support of this hypothesis required enzyme preparations free from endogenous substrates and TPN. Extracts of acetone powders of the placental enzymes were not suitable, but fractionation with ammonium sulfate provided a simple method for removing interfering substances.

Experiments with Fractionated Enzymes.—The material precipitated from crude placental extracts by the addition of ammonium sulfate to 40 per cent saturation contained active, TPN-linked isocitric and glucose-6-phosphate dehydrogenases, and a steroid-sensitive pyridine nucleotide transhydrogenating system. It was devoid of endogenous substrates and pyridine nucleotides. The experiment depicted in Figure 1 shows that, over a 45-minute period without added substrate,

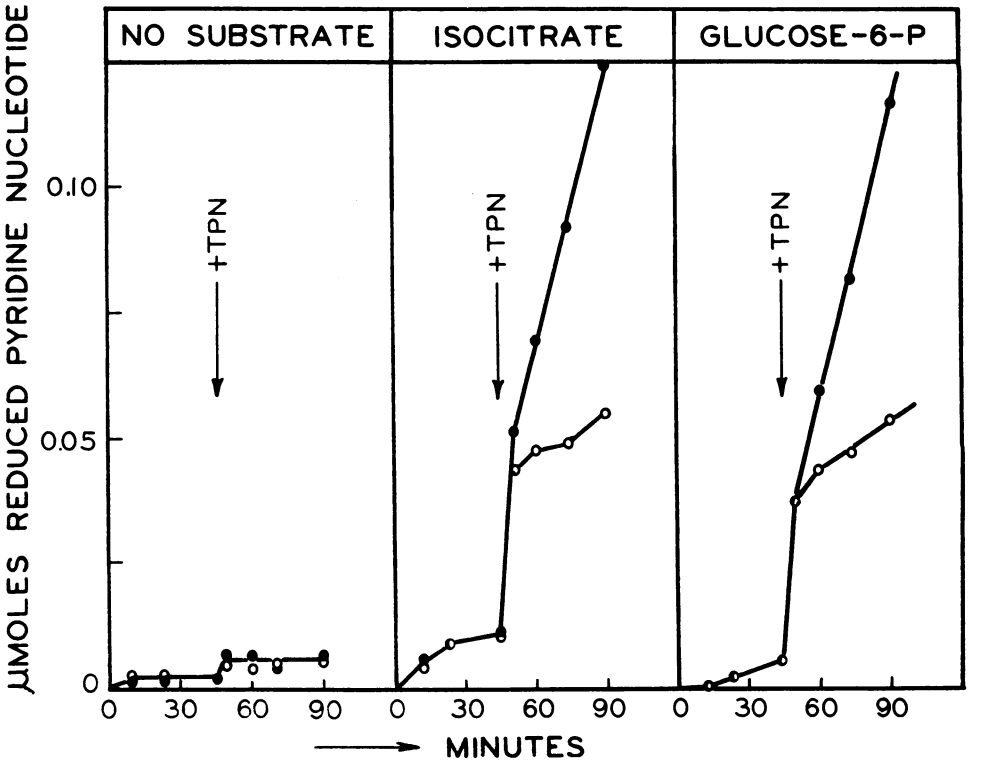

FIG. 1.—Estradiol-stimulated formation of reduced pyridine nucleotides. The reaction systems contained in 3.0 ml. the following ingredients: 300 μmoles Tris pH 7.4; 1 μmole DPN^+; 1 μmole $MnCl_2$; and 4.2 mg. protein of a 30–40 per cent saturated ammonium sulfate fraction of placenta. Paired cuvettes were run with (● ● ●) or without (○ ○ ○) 4 μg. estradiol 17β in 0.01 ml. dioxane. Parallel experiments were carried out without added substrate (*left*), with 1.5 μmoles isocitrate (*center*), and with 3 μmoles glucose-6-phosphate (*right*). At time 45 minutes, 0.04 μmole TPN was added to all cuvettes. Optical measurements at 340 mμ in cuvettes of 1 cm. light path. Temp. 25°.

this preparation did not reduce DPN, whether estradiol 17β (5×10^{-6} *M*) was present or not. Furthermore, the addition of 0.04 μmole of TPN after 45 minutes of incubation did not induce a reduction of DPN during the subsequent 45 minutes. When isocitrate was included in the incubation mixture, DPN was not reduced with or without the further inclusion of estradiol 17β. Later addition of TPN led to an instantaneous and complete reduction of the nucleotide by the highly active TPN-linked isocitric dehydrogenase in the enzyme preparation. In the absence of hormone, the subsequent change in absorbance at 340 mμ was very small over the following 45 minutes. However, with both isocitrate and extradiol 17β present, DPNH gradually accumulated after all the TPN had been converted to TPNH.

This experiment is in full accord with a stimulation by estradiol 17β of a mechanism which transfers hydrogen from TPNH to DPN. A corollary of this hypothesis would be that any other substrate which could reduce TPN in this system should substitute for isocitrate. This proved to be the case with glucose-6-phosphate. Figure 1 shows that DPN was not reduced by glucose-6-phosphate unless catalytic amounts of TPN were added, in which case the further reduction of DPN was stimulated by estradiol 17β.

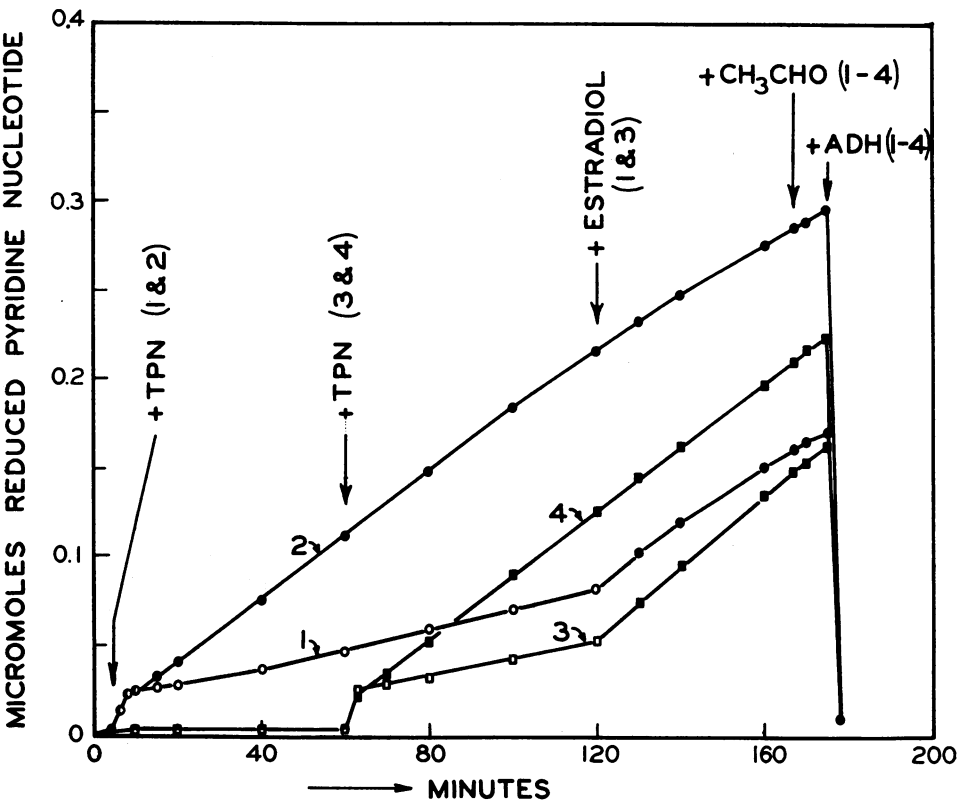

FIG. 2.—Time course of the formation of reduced pyridine nucleotides. The reactions were carried out in 3.0 ml. systems containing 300 μmoles Tris pH 7.4; 1 μmole DPN; 1 μmole $MnCl_2$; 1.5 μmole sodium isocitrate; 107 μg. purified rate heart isocitric dehydrogenase; and 1.92 mg. protein of a 0–35 per cent saturated ammonium sulfate fraction of a placental extract. Cuvettes *2* and *4* contained 4 μg. estradiol 17β in 0.01 ml. dioxane initially. TPN (0.023 μmole) was added at 5 minutes to cuvettes *1* and *2*. 4 μg. estradiol 17β was added to cuvettes *1* and *3* at 120 minutes. All cuvettes received 10 μmoles acetaldehyde in 0.01 ml. at 167 minutes and an excess of yeast alcohol dehydrogenase at 175 minutes. Optical measurements at 340 mμ against a control containing enzyme and buffer. Temp. 25°.

These considerations are amplified further by the findings summarized in Figure 2. The activity of the TPN-specific isocitric dehydrogenase in the enzyme preparation used in this experiment was rather feeble, so an excess of this TPN-reducing enzyme was added. All the cells contained isocitrate, DPN, and purified isocitric dehydrogenase. The DPN was not reduced under any circumstances unless TPN (0.02 μmole) was also present. If the TPN was added 5 minutes after initiation of the reaction, it was reduced instantaneously. Without estradiol 17β, very little

reduced pyridine nucleotide accumulated above the expected amount of TPNH, whereas a rapid formation of reduced nucleotide took place in cells containing the hormone. Similar changes were manifest if the TPN was added 60 minutes after the beginning of the reaction. The delayed addition of estradiol 17β also increased greatly the accumulation of reduced pyridine nucleotide in cells to which TPN had been added after 5 or 60 minutes of incubation. Finally, it will be seen that when acetaldehyde was added at the end of the experimental period, the rate of change in absorbance in cells containing TPN and estradiol 17β was unimpeded. But the further addition of yeast alcohol dehydrogenase caused a virtually instantaneous disappearance of all the reduced pyridine nucleotide. This is convincing evidence that the increase in absorbance at 340 mμ which took place after all the added TPN had been reduced reflected a gradual accumulation of DPNH and not of some other ultraviolet-absorbing product (e.g., reduced nicotinamide mononucleotide).

It is noteworthy that the steroid-sensitive transhydrogenating system can be readily fractionated with ammonium sulfate and that it is relatively stable, since its activity remains unimpaired after storage at 2° for several days and is not destroyed by repeated freezing and thawing.

TABLE 1*

EFFECT OF VARYING CONCENTRATIONS OF ESTRADIOL 17β ON STIMULATION OF PYRIDINE NUCLEOTIDE TRANSHYDROGENATION

Estradiol 17β (M)	Total Reduced Pyridine Nucleotide Formed (μmoles)
0	0.034
1.2×10^{-8}	0.042
1.2×10^{-7}	0.114
1.2×10^{-6}	0.158
4.9×10^{-6}	0.134

* Each cuvette contained 300 μmoles Tris buffer of pH 7.4; 1 μmole $MnCl_2$; 1.5 μmole sodium isocitrate; 1 μmole DPN; 0.023 μmole TPN; 107 μg. of purified rat heart isocitric dehydrogenase and ammonium sulfate fraction from placenta (0–35 per cent saturation) containing 1.92 mg. protein, in a final volume of 3.01 ml. Estradiol 17β was added in 0.01 ml. dioxane. The reaction was initiated by the addition of the placental fraction. Time 100 minutes. Temperature 25°. The rate of formation of reduced pyridine nucleotide was approximately linear with time over the period studied.

Steroid Specificity.—Table 1 shows that maximal stimulation of the transhydrogenating system occurred with estradiol 17β at a final concentration of 1×10^{-6} M. Marked activity of estradiol 17β was observed at a concentration of 1×10^{-7} M, and a small but detectable effect of this hormone could be demonstrated at 1×10^{-8} M.

The reactivity of various ammonium sulfate fractions toward other steroids differed somewhat from one preparation to another. At steroid concentrations of about 5×10^{-6} M, the transhydrogenating mechanism was stimulated by estradiol 17β and estrone, but not by estradiol 17α or by diethylstilbestrol. At a steroid concentration of 3×10^{-5} M, stimulation of the reaction was observed with testosterone, but not by 17α-methyltestosterone or by cortisone. The transhydrogenating mechanism stimulated by testosterone appeared to be more labile than that which was activated by estradiol 17β.

Reduction of Pyridine Nucleotides by Steroids.—Fractions of placenta obtained by precipitation between 0 and 40 per cent saturation of ammonium sulfate reduced DPN in the presence of *substrate amounts* of estradiol 17β, in confirmation of the studies of Langer and Engel.[8] The relatively crude enzyme was found to react

almost equally well with DPN and TPN. Table 2 shows that, under conditions similar to those used to study the transhydrogenating system and at a steroid concentration of 2.5×10^{-5} *M*, 10–20 mμmoles of estradiol 17β were oxidized per hour per milligram of protein. Although, by necessity, these measurements of the hydroxysteroid dehydrogenase activity were carried out at higher steroid concentrations than were used in the transhydrogenase experiments, it has been observed that the rates of the estradiol 17β-activated transhydrogenation and of the reduction of pyridine nucleotides by estradiol 17β are of the same order of magnitude. In a specific instance, 1 mg. of protein of an ammonium sulfate fraction of placenta at pH 7.4 catalyzed the oxidation of estradiol 17β (2.5×10^{-5} *M*) at a rate of 59 mμmoles per hour with DPN (3.3×10^{-4} *M*). The transhydrogenase activity of the same preparation at pH 7.4 (3.3×10^{-4} *M* DPN; 1.3×10^{-5} *M* TPN; and 4.9×10^{-6} *M* estradiol 17β), with isocitric dehydrogenase as the reducing system, was 10.9 mμmoles per hour per milligram protein. The differences in steroid concentrations involved in this comparison are probably of no great influence on the reaction rates, since at least the bacterial 17β-hydroxysteroid dehydrogenase has a Michaelis constant for estradiol 17β far less than 1×10^{-6} *M*.[9]

TABLE 2*

OXIDATION OF ESTRADIOL 17β BY AMMONIUM SULFATE OF PLACENTAL FRACTIONS

Ammonium Sulfate Fraction (Per Cent Saturation)	Millimicromoles Reduced Pyridine Nucleotide Formed per Hour per Mg. Protein		Relative Rates
	DPN	TPN	DPN/TPN
0–30	15.2	8.2	1.85
30–40	21.5	12.5	1.72

* The activities were measured by observing the reduction of pyridine nucleotide at 340 mμ in systems of 3.0 ml. volume containing 300 μmoles Tris pH 7.4; 1 μmole DPN or TPN; 0.074 μmole estradiol 17β in 0.01 ml. dioxane, and appropriate amounts of enzyme. Readings were made at intervals against control cuvettes containing all ingredients except estradiol 17β. Temperature 25°.

Inhibition by Mercuric Ions.—Low concentrations of mercuric ions inhibited the enzymatic reduction of TPN and DPN by estradiol 17β. The degree of inhibition of the transhydrogenation reaction by Hg^{++} was found to be of the same order of magnitude. The latter reaction was carried out with a sufficient excess of added isocitric dehydrogenase. Whereas 2×10^{-5} *M* $HgCl_2$ had little effect on both reactions, brief preincubation of the enzyme with 4×10^{-5} *M* $HgCl_2$ inhibited both the hydroxysteroid dehydrogenase and the transhydrogenating reactions by 80–90 per cent. The placental and certain other hydroxysteroid dehydrogenases are known to be inactivated by heavy-metal ions and have been presumed to be sulfhydryl group containing proteins.[4, 8, 10]

DISCUSSION

It is evident from these experiments that the increased rate of reduction of DPN promoted by steroid hormones in crude extracts of placenta is explicable in terms of an activation of the transfer of hydrogen from TPNH to DPN by steroids. Thus it is not necessary, as others have,[2, 3] to postulate the separate existence of a DPN-specific isocitric dehydrogenase as responsible for these hormonal effects.

There is considerable evidence that DPN and TPN serve different metabolic functions and that the natural occurrence of these two different pyridine nucleotides is of profound functional significance. TPNH can act as a reducing agent in a

number of synthetic reactions which take place outside the mitochondria and in which DPNH cannot participate. Examples of such biosynthetic pathways are (*a*) the synthesis of fatty acids, where TPNH acts as a specific reductant in fatty acyl dehydrogenase reactions,[11, 12] and (*b*) the entry of one-carbon fragments into serine[13] and into purines[14] catalyzed by a series of folic acid-dependent enzyme systems which utilize TPNH as a specific hydrogen donor.[15]

It is indeed remarkable that studies on the metabolic concomitants of the action of steroid hormones upon accessory sexual tissues[16] have shown that these same extra-mitochondrial synthetic reactions, which specifically require TPNH, are extremely sensitive to the action of steroids. Thus Mueller[16] found that, shortly after the administration of estradiol 17β to the ovariectomized rat, the incorporation of one-carbon fragments (derived from a variety of precursors) into serine and into the purines of nucleic acids in the uterus was increased immensely. In similar experiments, estradiol 17β stimulated the conversion of acetate to fatty acids and to cholesterol but did not influence the oxidation of acetate.

Furthermore, a number of biochemical changes in the accessory organs of reproduction of the male induced by testosterone could have, as their common denominator, a change in the balance between TPNH and DPNH. Androgenic steroids initiate and support the accumulation and secretion of fructose and citric acid in some male accessory sexual tissues.[17] The synthesis of fructose by these organs involves the reduction of glucose to sorbitol by TPNH, followed by the DPN-linked oxidation of sorbitol to fructose.[18, 19] The over-all conversion of glucose to fructose thus simulates the action of pyridine nucleotide transhydrogenase insofar as there is a stoichiometric transfer of hydrogen from TPNH to DPN in this process. Those lobes of the prostate gland which accumulate and secrete citrate in response to steroids do, in fact, possess the enzymatic machinery for the oxidation of citrate by the tricarboxylic acid cycle, involving the action of a TPN-specific isocitric dehydrogenase.[20] Citrate—and no other organic acid—probably accumulates in response to steroids because the unfavorable TPNH/TPN ratio acts to brake the isocitric dehydrogenase reaction and prevents the rate of oxidation of citrate from keeping pace with its rate of synthesis.[21] Again, the synthesis of fatty acids from acetate in the prostate gland is most sensitive to testosterone.[22]

In all mammalian tissues examined, including accessory sexual organs, TPNH is present in far higher concentrations than TPN, whereas the steady-state level of DPN is usually greater than that of DPNH.[23] Hence the ratio

$$\frac{[\text{TPNH}]\,[\text{DPN}]}{[\text{TPN}]\,[\text{DPNH}]}$$

is always very high. This quotient is the equilibrium constant of the pyridine nucleotide transhydrogenase reaction. The vast majority of pyridine nucleotide-linked dehydrogenases employ only one type of hydrogen acceptor; they do not function equally well with TPN and DPN. Any disturbance in the balance between TPNH and DPNH by a transhydrogenating mechanism would alter the nice equilibrium between synthesis and degradation required for the orderly growth and function of cells. Since there is not a uniform intracellular distribution of pyridine nucleotides and of enzymes with which they react, the factors which determine the steady-state levels of the reduced and oxidized forms of these coenzymes will

vary in different regions of the cell.[11] Similar considerations concerning the regulation of the balance between hydrogenation and phosphorylation have been advanced by Hoch and Lipmann.[24]

The existence of enzyme systems which catalyze the transfer of hydrogen between the two natural forms of pyridine nucleotide is well documented. Kaplan *et al.*[25] have purified a soluble transhydrogenase, apparently a discrete protein, from *Pseudomonas fluorescens*. In mammalian species, the transfer of hydrogen between the oxidized and reduced forms of TPN and DPN, between certain unnatural pyridine nucleotides, and even from DPNH to DPN has been observed heretofore only with mitochondrial preparations from some, but not all, tissues.[26, 27] The mitochondrial transhydrogenase system has not been purified extensively. It is by no means clear whether the hydrogen transfer from one pyridine nucleotide to another carried out by mitochondria is catalyzed by a single enzyme or reflects the operation of a coupled reaction, whereby an enzyme with dual nucleotide specificity alternately oxidizes and reduces an intermediate, present in minute amounts and thereby permits a rapid net transfer of hydrogen from one pyridine nucleotide to another.

Kaplan[28] has suggested that the mitochondrial pyridine nucleotide transhydrogenase system plays an important role in regulating the balance between various synthetic and energy-yielding reactions. His experiments suggest that, while the oxidation of DPNH by liver mitochondria serves as a source of energy for the synthesis of adenosine triphosphate, the oxidation of TPNH is not coupled with phosphorylation unless hydrogen is first donated from TPNH to DPN by the mitochondrial transhydrogenase reaction.

In our studies, those steroid hormones which accelerate the transfer of hydrogen between two forms of pyridine nucleotide also reduce both DPN and TPN in the presence of the same soluble placental enzymes. The enzyme activities are associated in certain protein fractions and absent from others. Both activities are inhibited by similar concentrations of Hg^{++} to approximately the same extent. Furthermore, the activities are of comparable magnitude in the preparations studied. The simplest interpretation of these experimental findings is that the reversible oxidation and reduction of the steroids themselves constitute the steroid-activated transhydrogenating mechanism. Consider, for example, the case of estradiol 17β. The rapid conversion of this steroid to estrone with either TPN or DPN as a hydrogen acceptor would establish an equilibrium mixture of estrone and estradiol 17β. We visualize the transfer of hydrogen from TPNH to DPN as taking place according to the following equations:

$$\begin{array}{l} H^+ + \text{TPNH} + \text{estrone} \rightleftarrows \text{TPN}^+ + \text{estradiol } 17\beta \\ \underline{\text{Estradiol } 17\beta + \text{DPN} \rightleftarrows \text{estrone} + \text{DPNH} + H^+} \\ \text{TPNH} + \text{DPN}^+ \rightleftarrows \text{TPN}^+ + \text{DPNH} \end{array}$$

This reaction is made possible by the dual nucleotide specificity of the dehydrogenating enzyme.

The placental hydroxysteroid dehydrogenase which oxidizes estradiol 17β is not alone in this class of enzymes in having dual nucleotide specificity. A soluble 3α-hydroxysteroid dehydrogenase isolated from liver reacts with both DPN and

TPN.[10] Recent experiments[29] have shown that liver microsomes of various species contain a firmly bound 3α-hydroxysteroid dehydrogenase. These particles also interconvert hydrocortisone and cortisone by the action of a specific 11β-hydroxysteroid dehydrogenase. Both microsomal enzymes react at comparable rates with DPN and with TPN.

Detailed studies of the specificities and substrate affinities of highly purified, adaptive hydroxysteroid dehydrogenases of bacteria have revealed that these enzymes bind certain steroids tenaciously.[9,30] The magnitudes of the Michaelis constants correspond with the concentrations at which many steroids exert their physiological actions. Moreover, the binding of steroids by these enzymes is very sensitive to even minor structural alterations of the steroid molecule. A number of striking similarities have been shown to exist between the molecular features necessary for physiological activity and those which favor efficient binding of the steroid to the enzyme surfaces.[9, 30] The observation that these enzymes exhibit both the high affinity and the specificity for steroids demanded by hormonal activity led to the suggestion that hydroxysteroid dehydrogenases are intimately concerned with the hormonal action of steroids.[9, 30] The importance of the state of oxidation of steroids had already been pointed out by Huggins and his co-workers[31] from considerations of the relationship of molecular structure to the growth-promoting activity of these hormones.

The present experiments strongly suggest that steroids can participate in transhydrogenation between pyridine nucleotides. On the assumption that the enzyme in placenta which catalyzes the reduction of TPN and DPN by estradiol 17β is the same as that which promotes the steroid-activated transfer of hydrogen from TPNH to DPN, it is possible to regard the pyridine nucleotides as substrates for the transhydrogenation, and the steroid as a coenzyme for the latter reaction. In this way it becomes possible to assign a functional role to the mammalian hydroxysteroid dehydrogenases which possess dual nucleotide specificity. In the presence of truly minute amounts of steroids, they act as mediators of hydrogen transfer between pyridine nucleotides.

It would appear that the thermodynamic properties of steroid hormones are well suited for the promotion of hydrogen transfer in such systems. Equilibrium constants for the interconversion of hydroxy- and ketosteroids have recently become available from measurements made with highly purified bacterial hydroxysteroid dehydrogenases.[30] The free-energy changes involved in these reactions are such as to favor the existence of significant amounts of both oxidized and reduced forms of steroid at physiological hydrogen ion concentrations. Thus, at pH 7 and at 298° K., the equilibrium ratio of 4-androstene-3,17-dione to testosterone is in the vicinity of 0.4, assuming that the oxidized and reduced pyridine nucleotides are present in equimolar amounts. The calculated change in free energy for the interconversion of these two steroids is thus $\Delta F^\circ = 5.2$ Kcal/mole. For several 3-hydroxysteroids, the equilibrium ratio of ketone to alcohol varies from 0.01 to 0.07 ($\Delta F^\circ = 6.0$–7.2 Kcal/mole) under the same conditions. The oxidation of a steroid hydroxyl group to a ketone with a pyridine nucleotide as a hydrogen acceptor involves the stoichiometric liberation of hydrogen ion; hence the equilibrium is strictly dependent upon pH. It follows that the ability of steroids to effect transhydrogenation by their reversible oxido-reduction would vary in a predictable manner with the hydrogen

ion concentration. For example, at pH 9.0 and in the presence of the appropriate hydroxysteroid dehydrogenase and equimolar concentration of oxidized and reduced pyridine nucleotide, 17β hydroxysteroids would exist in the ketone form to an extent of 97 per cent. Thus, at pH 9.0, one would not expect the steroid-hydroxysteroid dehydrogenase system to function as an efficient mediator of transhydrogenation. Similar considerations would hold if the hydrogen ion concentration was too far removed from neutrality on the acid side. If the present interpretation of our findings is correct, these deductions are in agreement with the finding of Gordon and Villee[2] that the activation of the reduction of DPN in crude extracts of placenta by estradiol 17β is maximal at pH 7.3.

It may be emphasized that the oxidation of the 17β-hydroxyl group of steroids gives, at neutrality, an equilibrium ratio of alcohol to ketone closer to 1 than that of any other steroid oxido-reduction which has been studied.[30] It is, perhaps, not altogether fortuitous that the 17β-hydroxyl function is of paramount importance for the biological activity of estrogenic and androgenic hormones.

The few known mammalian pyridine nucleotide-linked enzymes which exhibit dual nucleotide specificity with non-steroidal substrates, e.g., glutamic dehydrogenase[32] and glucose dehydrogenase,[33] may not be expected to function as efficiently as hydroxysteroid dehydrogenases to effect a transfer of hydrogen from TPNH to DPN, or vice versa, at pH 7. The equilibria which they catalyze are unfavorable, and the affinities for their substrates are too low in this respect.

All types of *naturally occurring* steroid hormones undergo a variety of oxido-reductions in animal tissues, and the enzymes catalyzing these transformations are widely distributed in nature.[4] A number of these enzymes possess the properties requisite for them to act as transhydrogenating systems between pyridine nucleotides and, possibly, between other coenzymes engaged in hydrogen transport. From the limited evidence at hand, it appears that these enzymes differ considerably in their affinities and specificities for steroids and in their intracellular and tissue distribution. Thus some of the enzymes which permit the oxidation or reduction of oxygen substituents on steroids may have significance not only as mechanisms for the inactivation of hormones[4] but also as catalysts for the interconversion of the oxidized and reduced forms of hydrogen-transporting coenzymes. For this reason, further studies on the properties and distribution of the family of enzymes which catalyze the oxido-reduction of steroids in animal tissues are of the utmost importance. The fact that certain steroid hormones exert a profound action in more than one physiological domain may well relate to the lack of rigid specificities of various hydroxysteroid dehydrogenases.

SUMMARY

Soluble enzyme preparations of human placenta promote the transfer of hydrogen from TPNH to DPN in the presence of minute amounts of certain steroid hormones. The same enzyme preparations catalyze the oxidation of these steroids by both forms of pyridine nucleotide. Evidence is presented for the identity of a placental hydroxysteroid dehydrogenase with the transhydrogenase activity.

It is proposed that the metabolic function of hydroxysteroid dehydrogenases with dual pyridine nucleotide specificity is to act as pyridine nucleotide transhydrogenases. Properties of some hydroxysteroid dehydrogenases which favor transhydro-

genation are: (1) their high affinity and specificity for particular steroids; (2) their ability to react with both DPN and TPN; and (3) the suitable equilibria between steroid alcohols and ketones which obtain at physiological hydrogen ion concentrations.

* Supported by grants from the American Cancer Society.

† Scholar in Cancer Research of the American Cancer Society.

[1] C. A. Villee, *J. Biol. Chem.*, **215**, 171, 1955.

[2] E. E. Gordon and C. A. Villee, *J. Biol. Chem.*, **216**, 215, 1955.

[3] C. A. Villee and E. E. Gordon, *J. Biol. Chem.*, **216**, 203, 1955.

[4] P. Talalay, *Physiol. Revs.*, **37**, 362, 1957.

[5] O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384, 1941.

[6] G. Siebert, J. Dubuc, R. C. Warner, and G. W. E. Plaut, *J. Biol. Chem.*, **226**, 965, 1957.

[7] B. Hurlock and P. Talalay, *J. Biol. Chem.*, **227**, 37, 1957.

[8] L. Langer and L. L. Engel, *Federation Proc.*, **15**, 296, 1956.

[9] P. I. Marcus and P. Talalay, *Proc. Roy. Soc. London, B*, **144**, 116, 1955.

[10] G. M. Tomkins, *J. Biol. Chem.*, **218**, 437, 1956.

[11] R. G. Langdon, *J. Biol. Chem.*, **226**, 615, 1957.

[12] F. Lynen and K. Decker, *Ergeb. Physiol.*, **49**, 327, 1957.

[13] F. M. Huennekens, Y. Hatefi, and L. D. Kay, *J. Biol. Chem.*, **224**, 435, 1957.

[14] J. G. Flaks, L. Warren, and J. M. Buchanan, *J. Biol. Chem.*, **228**, 215, 1957.

[15] Y. Hatefi, M. J. Osborn, L. D. Kay, and F. M. Huennekens, *J. Biol. Chem.*, **227**, 637, 1957.

[16] G. C. Mueller, *Cancer Research*, **17**, 490, 1957.

[17] T. Mann, *The Biochemistry of Semen* (London: Methuen, 1954).

[18] H. G. Hers, *Biochim. et Biophys. Acta*, **22**, 203, 1956.

[19] H. G. Williams-Ashman, J. Banks, and S. K. Wolfson, Jr., *Arch. Biochem. and Biophys.* (in press).

[20] H. G. Williams-Ashman, *Abstracts of Communications, Third International Congress of Biochemistry, Brussels, 1955* (New York: Academic Press, 1956), p. 64.

[21] H. G. Williams-Ashman and S. Liao (in preparation).

[22] S. J. Nyden and H. G. Williams-Ashman, *Am. J. Physiol.*, **172**, 588, 1953.

[23] G. Glock and P. McLean, *Biochem. J.*, **61**, 388, 1955.

[24] F. L. Hoch and F. Lipmann, these PROCEEDINGS, **40**, 909, 1954.

[25] N. O. Kaplan, S. P. Colowick, and E. F. Neufeld, *J. Biol. Chem.*, **195**, 107, 1952.

[26] N. O. Kaplan, S. P. Colowick, and E. F. Neufeld, *J. Biol. Chem.*, **205**, 1, 1953.

[27] G. F. Humphrey, *Biochem. J.*, **65**, 546, 1957.

[28] N. O. Kaplan, M. N. Schwartz, M. E. Frech, and M. M. Ciotti, these PROCEEDINGS, **42**, 481, 1956.

[29] B. Hurlock and P. Talalay (in preparation).

[30] P. Talalay, *Record Chem. Progr.*, **18**, 31, 1957.

[31] C. Huggins, E. V. Jensen, and A. S. Cleveland, *J. Exptl. Med.*, **100**, 225, 1954.

[32] J. A. Olson and C. B. Anfinsen, *J. Biol. Chem.*, **202**, 841, 1953.

[33] H. J. Strecker and S. Korkes, *J. Biol. Chem.*, **196**, 769, 1952.