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*ON A COENZYMATIC FUNCTION OF ESTRADIOL-17β**

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Alterations in the activities of numerous metabolic processes and individual enzymes induced by steroidal estrogens have been described, especially in the highly susceptible tissues of the female genital tract.[1–3] It would appear, however, that many of these diverse changes are related only indirectly to the primary site of action of estrogens, which has not been disclosed by studies of this nature. The first experimental demonstration of the direct participation of a steroid hormone in an enzymatic reaction of obvious importance in metabolic regulation was made in this laboratory, when it was shown that estradiol-17β mediated a reversible transfer of hydrogen between the oxidized and reduced forms of triphosphopyridine (TPN) and diphosphopyridine (DPN) nucleotides.[4, 5] This hormone-dependent transhydrogenation is catalyzed by a single protein, which was isolated from human placenta, and results from the following coupled reaction in which the steroids function catalytically:

$$\begin{array}{l} \text{Estradiol-17}\beta + \text{DPN}^+ \rightleftharpoons \text{Estrone} + \text{DPNH} + \text{H}^+ \\ \underline{\text{Estrone} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{Estradiol-17}\beta + \text{TPN}^+} \\ \text{Sum: TPNH} + \text{DPN}^+ \rightleftharpoons \text{DPNH} + \text{TPN}^+ \end{array}$$

If this enzyme is permitted to react with stoichiometric quantities of estradiol-17β, it effects the reduction of either DPN or TPN and thus exhibits the properties typical of a hydroxysteroid dehydrogenase.[4, 5] However, with stoichiometric amounts of pyridine nucleotides, transhydrogenation occurs in the presence of extremely small concentrations (10^{-7} *M*) of estradiol-17β.[4] The hormone is alternately oxidized and reduced during this process and can be regarded, therefore, as a hydrogen carrier or coenzyme. The ability of this mammalian hydroxysteroid dehydrogenase to catalyze hydrogen transfer between TPNH and DPN, or from

DPNH to TPN, is a reflection of the comparable reactivity of both forms of pyridine nucleotide in the dehydrogenation reaction.

Since a number of mammalian hydroxysteroid dehydrogenases, other than the placental enzyme which reacts with estradiol-17β, also possess dual pyridine nucleotide specificity,[4, 6] it was suggested that all enzymes of this class may function as transhydrogenases, with their steroid substrates acting as coenzymes. Support for this contention derives from more recent experiments,[7] which showed that the 3α-hydroxysteroid dehydrogenase of rat liver would promote hydrogen transfer between pyridine nucleotides in the presence of catalytic concentrations (10^{-6} M) of steroids which are reversibly oxidized by this enzyme.

The purpose of this paper is to show that the placental 17β-hydroxysteroid dehydrogenase which catalyzes the reduction of pyridine nucleotides by certain phenolic estrogens is the same protein as that which catalyzes the estradiol-17β–mediated transfer of hydrogen between DPN and TPN. Partial purification of this enzyme and an examination of many of its properties as a dehydrogenase have been reported previously by Langer and Engel.[8, 9] A simple purification procedure for this enzyme has been developed, and methods have been devised for the measurement of both its dehydrogenase and its transhydrogenase activities. The delicately balanced conditions required for hydrogen transfer have been investigated and related to the binding of steroids and of pyridine nucleotides by the enzyme. Knowledge of these parameters has permitted definition of the conditions under which the steroid-mediated transhydrogenation is reversible.

It has been suggested[4] that at least some of the biochemical consequences of the action of steroid hormones may have their origin in alterations of hydrogen flow between the two forms of pyridine nucleotide. On this basis it is possible to formulate a unitary theory of steroid action. A family of hydroxysteroid dehydrogenases which function as transhydrogenases is present in mammalian tissues. Such enzymes have different specificities for steroids and also are localized in certain intracellular districts.[4, 6] Differences and overlaps in the hormonal action of various steroids may be visualized in terms of the strict or partial specificities of the hydroxysteroid dehydrogenases. Variations in the response of different tissues to particular steroids may be related to differences in their content of hydroxysteroid dehydrogenases and the intracellular location thereof and also to the degree to which the transhydrogenations they catalyze are rate-limiting to growth or function.

We are attracted by the simplicity of this hypothesis of metabolic control by steroid hormones. Moreover, it has the additional merit of reconciling certain aspects of the "metabolism" and the "action" of steroid hormones as two facets of the same process, because the steroids undergo chemical transformation when they function as coenzymes. Finally, this theory ascribes a coenzymatic function to another group of trace substances with profound biological activity.

EXPERIMENTAL

THE MEASUREMENT OF DEHYDROGENASE AND TRANSHYDROGENASE ACTIVITIES

The oxidation and reduction of pyridine nucleotides were measured spectrophotometrically according to the principles first developed by Otto Warburg.[10] If the steroid-activated transfer of hydrogen between pyridine nucleotides is

catalyzed by a hydroxysteroid dehydrogenase, then the dehydrogenase and transhydrogenase activities should parallel each other during purification of the enzyme. To prove this experimentally required test systems for both functions in which the activities were strictly proportional to the amount of enzyme protein present and which permitted the measurement of maximal rates of reaction. The estimation of dehydrogenase in the presence of an excess of both steroid and pyridine nucleotide presented little difficulty. But the type of assay system for transhydrogenation used previously,[4] in which TPNH was continually generated *in situ* from low levels of TPN by the action of auxiliary TPN-specific dehydrogenases, was unsuitable for several reasons. The latter enzymes have to be present in excess and must not reduce DPN. It was found that maximal rates of transhydrogenation varied with the nature of the TPNH-generating system. Experimental details of this situation will be discussed below, as well as those relating to another factor which complicates such transhydrogenase measurements, namely, the extreme sensitivity of the reaction to the amounts of TPN added initially to the reaction mixture. These difficulties were obviated by the use of an assay system in which the over-all rate of hydrogen transfer from DPNH to the 3-acetylpyridine analog of DPN (APDPN)[11, 12] was determined. The reaction proceeds according to the following equations:

$$\begin{aligned} &H^+ + \text{DPNH} + \text{Estrone} \rightleftharpoons \text{DPN}^+ + \text{Estradiol-17}\beta \\ &\text{APDPN}^+ + \text{Estradiol-17}\beta \rightarrow H^+ + \text{APDPNH} + \text{Estrone} \\ &\text{Sum: DPNH} + \text{APDPN}^+ \rightarrow \text{DPN}^+ + \text{APDPNH} \end{aligned}$$

It can be followed spectrophotometrically at 400 mμ, at which wave length APDPNH has an appreciable extinction coefficient (ϵ = 2,500), whereas DPNH does not.[11, 12] Weber and Kaplan[13] found that a variety of flavoproteins which are present in animal tissues, catalyze hydrogen transfer from DPNH or TPNH to pyridine nucleotide analogs which have a higher oxidation-reduction potential, such as APDPN. Such transhydrogenations are uninfluenced by steroids, and enzymes which catalyze them contaminated even the most purified preparations of the placental hydroxysteroid dehydrogenase for which estradiol-17β is a substrate. Thus a correction must be introduced for this "non-specific" transhydrogenation which is independent of steroids. Another complication in the APDPN assay for transhydrogenase arose from the presence of variable amounts of estradiol-17β firmly bound to the enzyme. Estradiol-17β was added to stabilize the enzyme during the purification procedure. However, it was found that small amounts of TPN obliterated the steroid-mediated transhydrogenation but were without influence upon the "non-specific" hydrogen transfer. Detailed evidence for the latter contention will be presented below.

The reaction mixture for the measurement of *dehydrogenase activity* contained in a final volume of 3.0 ml.: 100 μmoles sodium pyrophosphate buffer of pH 9.0, 25 mg. of crystalline bovine serum albumin, 0.3 μmole of estradiol-17β in 0.04 ml. dioxane, 1.4μ mole of DPN, and suitable quantities of enzyme. The rate of DPN reduction was followed spectrophotometrically at 340 mμ at suitable time intervals against a blank cuvette containing all the ingredients except the steroid. The reaction rates were calculated from the linear slopes of the initial portion of the reaction and were found to be proportional to the amount of enzyme added over a

wide range of protein concentrations. One unit of dehydrogenase activity was defined as the amount of enzyme causing a change in absorbance of 0.001 per minute under these conditions in a cuvette of 1 cm. light path at 25°; this is equivalent to the formation of 0.483 millimicromole DPNH per minute. The measurement of estradiol-17β–dependent *transhydrogenase activity* required three vessels. All of them contained in a final volume of 3.0 ml.: 300 μmoles tris(hydroxymethyl)aminomethane (Tris) buffer of pH 7.4, 0.01 ml. dioxane, and appropriate amounts of enzyme. Cuvette 1 received no other components. Cuvettes 2 and 3 each contained both 0.5 μmole DPNH and 3.0 μmoles APDPN. Estradiol-17β (4 μg. dissolved in 0.01 ml. dioxane) was present in cuvette 2, while cuvette 3 contained 0.02 μmole TPN. The reaction was initiated by the addition of enzyme, and the absorbance at 400 mμ of cuvettes 2 and 3 was measured at suitable time intervals, cuvette 1 serving as a blank. One unit of transhydrogenase was defined as the amount of enzyme causing a change in absorbance at 400 mμ of 0.001 per minute in a cuvette of 1 cm. light path at 25°; this is equivalent to the formation of 1.2 millimicromoles APDPNH per minute. Figure 1 depicts the results of typical transhydrogenase assays on a relatively crude (Fig. 1,*A*) and a partially purified (Fig. 1, *B*) preparation of the enzyme. The rate of formation of APDPNH in the upper graphs represents the sum of the estradiol-17β–sensitive and the "non-specific" transhydrogenase activities. The rates shown in the lower graphs reflect the activities of the "non-specific" transhydrogenases. The difference between the reaction rates of the upper and lower graphs is thus a measure of the steroid-dependent transhydrogenase and is compensated for the variable amounts of estradiol-17β bound to the enzyme. In the crude enzyme preparation (Fig. 1, *A*) the estradiol-17β–sensitive transhydrogenase represented 43 per cent of the total transhydrogen-

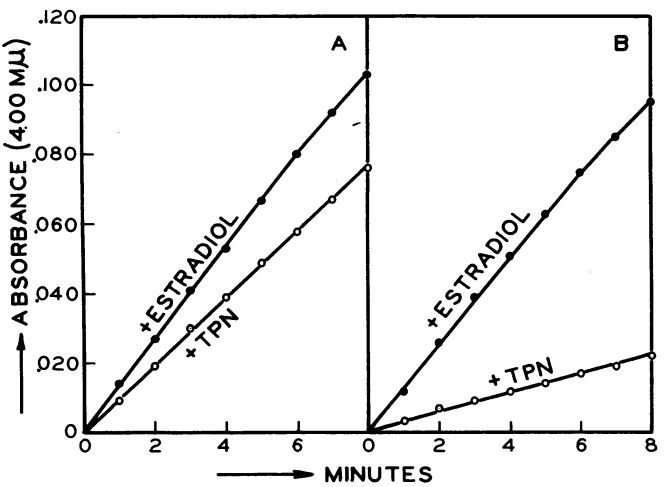

Fig. 1.—Spectrophotometric measurement of the estradiol-17β-sensitive and "non-specific" transhydrogenase activities of two preparations of the placental hydroxysteroid dehydrogenase. Hydrogen transfer from DPNH to APDPN was observed at 400 mμ as described in the text. The upper graphs represent measurements in the presence of 4 μg. of estradiol-17β, and the lower lines those in the presence of 0.02 μmole TPN, all in a final volume of 3.0 ml. The transhydrogenase activities (in units per mg. protein) were computed from the initial linear portions of the graphs. *A*, crude enzyme (2.94 mg. protein per cuvette), total = 4.62; "non-specific" = 2.65, and hence estradiol-17β-sensitive = 1.97; B, purified enzyme (0.4 mg. protein per cuvette); total = 34.3; "non-specific" = 6.7, and hence estradiol 17β-sensitive = 27.6, Temp. 25°.

ase activity, whereas in the purified preparation (Fig. 1, *B*) this fraction was 81 per cent. The initial rates of reaction were linear with respect to time and were strictly proportional to enzyme concentration (Fig. 9).

The specific activities of dehydrogenase and transhydrogenase were expressed as units of activity per mg. of protein.

PURIFICATION OF ENZYME

A human term placenta weighing 350 gm. was placed on crushed ice within 30 minutes of delivery. After thorough chilling, the surfaces were washed with cold tap water and the fetal membranes removed. The cotyledons were dissected away from connective tissue and blood vessels. All operations were carried out near 0°, unless otherwise stated. Each of six 50-gm. batches of minced tissue was homogenized with 150 ml. of medium in a slow-running Waring Blendor for 1 minute at 60 volts. The homogenization medium contained 0.001 *M* cysteine hydrochloride, 0.001 *M* disodium ethylene-diamine tetraacetate (EDTA), 0.01 *M* nicotinamide, and 0.03 *M* $NaHCO_3$. The homogenate was centrifuged at 2500 × *g*. for 20 minutes, and the fatty disk which rose to the top of the centrifuge tube was discarded. Estradiol-17β (15 mg. in 1.5 ml. acetone) was added to the supernatant fluid (950 ml.). Calcium chloride (0.1 M, 95 ml.) was then added and the turbid suspension was stirred magnetically for one hour and then stored for the same time, after which it was centrifuged for 30 minutes at 2500 × *g*. The agglutination of fine particulate material (microsomes) by calcium proved to be less tedious than ultracentrifugation (59,000 × *g* for 1 hour) of large volumes of homogenate. The clear supernate was then fractionated by the addition of solid, recrystallized ammonium sulfate; the majority of the enzyme activity was precipitated between 30 and 40 per cent saturation. All the precipitates obtained in this and the subsequent ammonium sulfate fractionation were dissolved in a solution containing 0.001 *M* EDTA, 0.001 *M* cysteine hydrochloride, 0.01 *M* Tris buffer of pH 7.4, and 5 μg./ml. of estradiol-17β. The most active fraction was transferred to a glass-stoppered Erlenmeyer flask, immersed in a water bath maintained at 57°, and heated with gentle agitation for exactly 15 minutes. After cooling rapidly on ice, the heat-treated enzyme was centrifuged for 30 minutes at 20,000 × *g*. This heat treatment was highly reproducible and resulted in a two- or threefold purification with little loss of total enzyme activity. The supernate was decanted and fractionated at once by the addition of a saturated solution of recrystallized ammonium sulfate (neutralized to pH 7.0 with NH_4OH) to give 5 per cent increments in saturation. The precipitates were collected by centrifugation and redissolved in small volumes of the suspension medium described above. The most active fractions were made 25 per cent saturated with respect to ammonium sulfate by addition of a saturated, neutral solution of this salt. These slurries retained full enzyme activity for at least 3 months when stored at 4°.

A typical purification is summarized in Table 1. The two most active fractions represented a 73 per cent recovery of the initial dehydrogenase activity with a purification of one hundred fold. The apparent twofold gain in dehydrogenase activity from step 1 to step 2 is probably related to the removal of particle-bond enzymes, which oxidize reduced pyridine nucleotides and thus lead to artificially low values for the dehydrogenase activity.

TABLE 1

PURIFICATION OF DEHYDROGENASE AND TRANSHYDROGENASE*

	Volume (ml.)	Protein (Mg/ml.)	Dehydrogenase Activity (Units per ml.)	Dehydrogenase Activity (Units per Mg. Protein)	Transhydrogenase Activity Estradiol-specific (Units per ml.)	Transhydrogenase Activity Non-specific (Units per ml.)	Ratio of Dehydrogenase Activity to Estradiol-Specific Transhydrogenase Activity
1. Supernate of original homogenate	950	15.6	35	2.21	..	..	..
2. Supernate from calcium chloride precipitation	990	14.7	66	4.5	29	39	2.3
3. First ammonium sulfate fraction (30–40 per cent)	45	21.4	1,250	58.5	220	210	5.8
4. Supernate of heat-treated No. 3	42	10.7	1,150	108	215	75	5.4
5. Second ammonium sulfate fractions:							
0–10 per cent	2	6.7	620	93	109	51	5.7
10–15 per cent	3	20.0	4,100	205	552	133	7.4
15–20 per cent	4.5	10.8	2,650	246	320	80	8.2
20–25 per cent	4.0	4.9	850	173	88	33	9.7

* The methods of assay of the activities are described in the text.

The ratios of dehydrogenase to estradiol-17β-specific transhydrogenase activity vary from 2.3 to 9.7 and lie between 5.4 and 9.7 for all fractions obtained after step 2. The relationship between dehydrogenase and transhydrogenase-specific activities is depicted graphically in Figure 2. There is proportionality between the dehydrogenase and the estradiol-17β–transhydrogenase activities, with no tendency for separation of them over a purification range of one hundred fold.

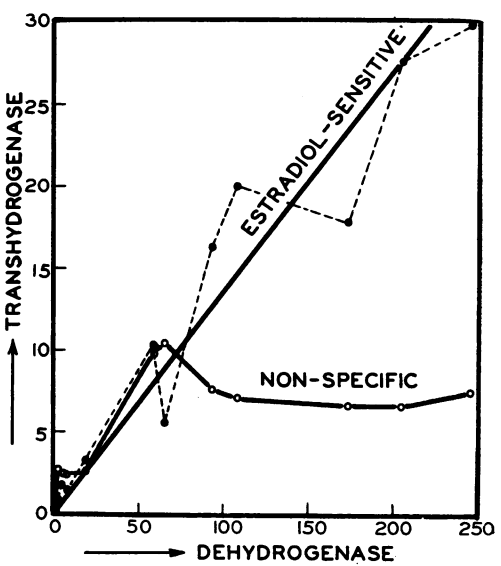

FIG. 2.—Relation between the estradiol-17β-sensitive transhydrogenase, the "non-specific" transhydrogenase, and the estradiol-17β dehydrogenase activities of various placental fractions obtained during the purification procedure. The measurements were carried out as described in the text and all the activities are expressed as units per mg. protein.

However, the rates of the latter functions bear no relationship to the activity of the "non-specific" transhydrogenase, which is uninfluenced by estradiol-17β. Thus the relative enzyme activities observed at each stage of the purification provide strong evidence for the catalysis of both the dehydrogenase and the estradiol-17β–dependent transhydrogenase reactions by the same protein.

Stability of the Enzyme.—Preliminary experiments showed that the enzyme was extremely unstable, even with the most rigorous precautions to exclude contamination with heavy metals and to preserve the presumptive sulfhydryl groups in the reduced state. The stabilization of the enzyme by low concentrations of estradiol-17β was remarkable. For example, one preparation lost 95 per cent of its dehydrogenase activity in 24 hours at 4°, while the same sample in the presence of estradiol-17β (15 μg/ml) could be heated for 15 minutes at 60° or stored for 24 hours at 25° without loss of activity. The placental enzyme binds estradiol-17β firmly, and no simple method for the complete removal of the steroid from the enzyme has been devised. The ability of small amounts of steroids to stabilize hydroxysteroid dehydrogenases has been noted previously.[8, 9, 14]

The fresh placental tissue may be processed immediately or stored at $-15°$ for at least 4 weeks. Supernates of homogenates prepared from frozen placenta exhibited little or no dehydrogenase activity, which was marked in more purified fractions. The oxidation of DPNH is rapid in crude extracts of such frozen tissue and makes the dehydrogenase assays erroneously low. In early experiments[4] each placenta was perfused with 0.9 per cent sodium chloride in order to remove hemoglobin. However, a comparison of the total and specific dehydrogenase activities of extracts prepared from perfused and unperfused portions of the same placenta revealed that they were 1.5 to 2 times higher in the unperfused segment, and hence perfusion was abandoned. It is not known whether perfusion inactivated the hydroxysteroid dehydrogenase or whether the fetal blood contributed significantly to the total enzyme activity.

TRANSHYDROGENATION REACTIONS CATALYZED BY THE HYDROXYSTEROID DEHYDROGENASE

Reduction of Pyridine Nucleotides by Estradiol-17β.—In the presence of stoichiometric amounts of estradiol-17β, the purified dehydrogenase catalyzed the reduction of DPN, TPN, APDPN, and the 3-pyridinealdehyde analog[12] of DPN. Desamino-DPN, in which hypoxanthine replaces the adenine moiety of the molecule was inactive as a hydrogen acceptor. The relative rates of reaction with various pyridine nucleotides are shown in Table 2. The negligible reactivity of desamino-DPN in the estradiol-17β dehydrogenase reaction is particularly interesting, since in the experiments of Kaplan and others[15] all of the DPN-linked enzymes studied reacted with desamino-DPN, irrespective of their reaction with other DPN analogs. Van Eys *et al.*[16] have also found that the reduction of desamino-DPN by yeast alcohol dehydrogenase was much faster in pyrophosphate than with Tris buffers. However, the placental hydroxysteroid dehydrogenase does not catalyze the reduction of desamino-DPN in either Tris buffer of pH 9.5 or pyrophosphate buffer of pH 9.0, whereas the rate of reaction with DPN was observed to be about the same under both conditions.

Transhydrogenations.—Experiments on the dehydrogenase activity of the

TABLE 2

RELATIVE RATES OF TRANSHYDROGENASE AND DEHYDROGENASE REACTIONS WITH PYRIDINE NUCLEOTIDES AND THEIR ANALOGS*

NUCLEOTIDE	DEHYDROGENASE† Rate (Millimicromoles per Hour)	DEHYDROGENASE† Relative Rates	TRANSHYDROGENASE‡ Rate (Millimicromoles per Hour)	TRANSHYDROGENASE‡ Relative Rates
DPN	410	100	147	100
TPN	174	42.5	..	..
APDPN	189	46.3	43.1	29.4
Pyridinealdehyde-DPN	232	61.0	52.7	38.5
Desamino-DPN	0	0	0	0

* Optical measurements were taken at the following wave lengths: 340 mμ for DPN, TPN, and desamino-DPN (ε for these nucleotides was assumed to be 6,220); 365 mμ for APDPN (ε = 7,800) and 355 mμ for pyridinealdehyde-DPN (ε = 7,000). Temp. 25°.

† The dehydrogenase assays were carried out in cuvettes containing, in a final volume of 3.0 ml.: 300 μmoles Tris buffer of pH 7.4; 20 μg. estradiol-17β in 0.01 ml. dioxane; 2.51 mg. enzyme protein; and the following quantities of nucleotides: DPN = 0.67 μmoles; APDPN = 0.75 μmoles; pyridinealdehyde-DPN = 0.85 μmoles; desamino-DPN = 0.78 μmoles; and TPN = 0.52 μmoles.

‡ The transhydrogenase assays were carried out in cuvettes containing, in a final volume of 3. 0 ml.: 300 μmoles Tris buffer of pH 7.4; 10 μmoles of glucose-6-phosphate; 0.02 μmole TPN; an excess of purified yeast glucose-6-phosphate dehydrogenase; 5.0 mg. of enzyme protein; and the following quantities of nucleotides: DPN = 1.34 μmoles; APDPN = 1.5 μmoles; pyridinealdehyde-DPN = 1.7 μmoles; desamino-DPN = 1.56 μmoles.

placental enzyme with various nucleotides suggested that it would catalyze the following transhydrogenation reactions in the presence of catalytic amounts of estradiol-17β:

$$\text{TPNH} + \text{DPN}^+ \rightleftharpoons \text{TPN}^+ + \text{DPNH} \quad (1)$$

$$\text{TPNH} + \text{APDPN}^+ \rightarrow \text{TPN}^+ + \text{APDPNH} \quad (2)$$

$$\text{TPNH} + \text{Pyridinealdehyde-DPN}^+ \rightarrow \text{TPN}^+ + \text{Pyridinealdehyde-DPNH} \quad (3)$$

$$\text{DPNH} + \text{APDPN}^+ \rightarrow \text{DPN}^+ + \text{APDPNH}. \quad (4)$$

All these reactions have been demonstrated as predicted. No transfer of hydrogen from TPNH to desamino-DPN was detectable (Table 2), which is consistent with the lack of reactivity of the latter nucleotide in the dehydrogenase assay.

Hydrogen Transfer from TPNH to DPN (Eq. [1]).—This reaction can be followed by measurement of an increase in absorbance at 340 mμ in the presence of an excess of DPN, if TPNH is generated continuously from very low levels of TPN by the action of TPN-specific dehydrogenases.[4] Crude preparations of the hydroxysteroid dehydrogenase contained active isocitric and glucose-6-phosphate dehydrogenases, both of which are specific for TPN. The most active placental preparations were devoid of these enzymes and had to be supplemented with TPN-linked dehydrogenases purified from other sources. Figure 3 shows that the steroid-mediated transhydrogenation could be measured when the following enzymes were used to generate TPNH: isocitric dehydrogenase of rat heart[17] and either glucose-6-phosphate[18] or the TPN-specific acetaldehyde dehydrogenases of yeast.[19] Since the factors which determine the rate of hydrogen transfer in these systems have not been investigated exhaustively, little significance can be attached to the differences in rate observed with the three TPNH-generating systems. However, limited studies indicated that maximal rates of transhydrogenation are obtained when the activity of the generating system exceeds a certain minimum, which must be considerably greater than that of the hydroxysteroid dehydrogenase. These experiments strengthen previous conclusions[4] that the manner in which TPNH is generated is immaterial for the demonstration of this transhydrogenase activity of the placental enzyme.

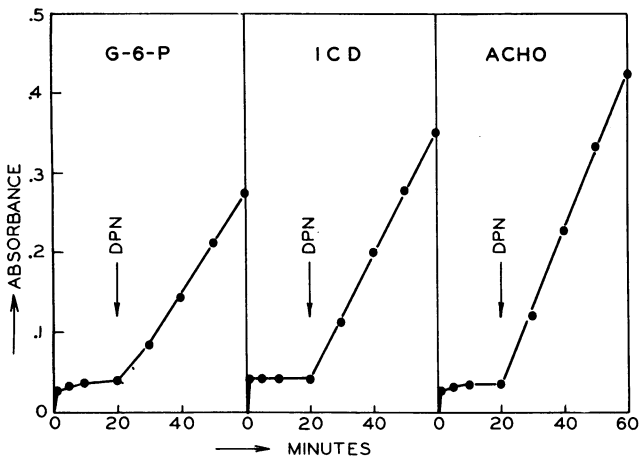

FIG. 3.—Rates of transhydrogenation from generated TPNH to DPN with various TPNH - generating systems. The reaction cuvettes contained, in final volumes of 3.0 ml.: 300 μmoles Tris buffer of pH 7.4; 0.02 μmole TPN; 16 units placental hydroxysteroid dehydrogenase; and, at time 20 minutes, 1.4 μmoles of DPN were added to all cuvettes. In addition to these components, the glucose-6-phosphate (G-6-P) system contained 10 μmoles glucose-6-phosphate; 50 μmoles $MgCl_2$; and an excess of the purified yeast glucose-6-phosphate dehydrogenase (see ref. 18). The isocitric dehydrogenase system (ICD) contained 1 μmole $MnCl_2$, 1.5 μmoles isocitrate and an excess of purified rat heart isocitric dehydrogenase (see ref. 17). The acetaldehyde dehydrogenase system (ACHO) contained 50 μmoles $MgCl_2$, 2 μmoles acetaldehyde, and an excess of purified yeast TPN-acetaldehyde dehydrogenase (see ref. 19).

Absorbance at 340 mμ was measured in each case against a control cuvette containing buffer, placental dehydrogenase, and DPN. With each generating system an additional control was included which contained all the ingredients of the complete system except TPN. The measurements are corrected for low rates of DPN reduction by the generating enzymes. The amount of each TPNH-generating enzyme required for maximum rates of transhydrogenation was determined by separate experiment. Temp. 25°.

It is unnecessary to generate TPNH from TPN, since TPNH added as such will, under suitable conditions, act as a hydrogen donor. The course of this reaction could be followed optically by the addition of acetaldehyde and crystalline yeast-alcohol dehydrogenase, which reoxidized any DPNH formed by the hydrogen transfer, as follows:[20]

$$\begin{aligned} \text{TPNH} + \text{DPN}^+ &\rightleftharpoons \text{DPNH} + \text{TPN}^+ \\ \text{H}^+ + \text{DPNH} + \text{CH}_3\text{CHO} &\rightleftharpoons \text{CH}_3\text{CH}_2\text{OH} + \text{DPN}^+ \\ \hline \text{Net: } \text{H}^+ + \text{TPNH} + \text{CH}_3\text{CHO} &\rightleftharpoons \text{CH}_3\text{CH}_2\text{OH} + \text{TPN}^+ \end{aligned}$$

Figure 4 shows an experiment of this reaction, in which appropriate controls to

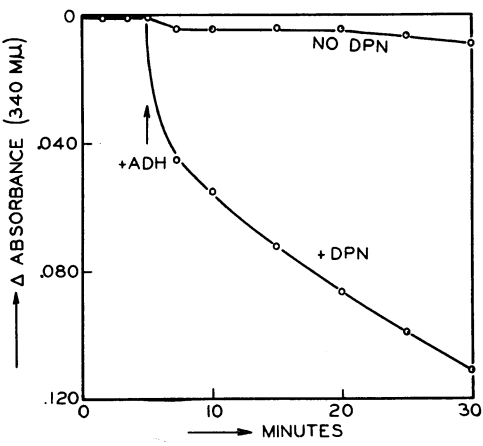

FIG. 4.—Transhydrogenation from added TPNH to DPN. The reactions were carried out in systems of 3.0 ml. final volume containing 300 μmoles Tris buffer, pH 7.4; 0.13 μmole TPNH; 4 μg. estradiol-17β in 0.01 ml. dioxane; 2 μmoles acetaldehyde; and 110 units placental dehydrogenase (specific activity 267 units per mg. protein). Two reaction vessels were employed, of which one received, in addition, 1.4 μmoles DPN. At time 5 minutes, 0.5 μg. crystalline yeast alcohol dehydrogenase (ADH) was added to both vessels. Readings of absorbance at 340 mμ were taken against a blank cuvette from which nucleotides and steroid were omitted. The decrease in absorbance with time is recorded. Temp. 25°

correct for any oxidation of TPNH in the absence of steroid and hydroxysteroid dehydrogenase were included.

Hydrogen Transfer from DPNH to TPN (Eq. [1]).—Free reversibility of transhydrogenation between TPN and DPN is to be anticipated on purely thermodynamic grounds. It was found that the demonstration of the reverse reaction depended critically upon the experimental conditions. Thus, if a small and constant level DPNH was maintained in the reaction mixture by the addition of DPN and yeast-alcohol dehydrogenase, only negligible transfer of hydrogen to TPN was observed. In order to demonstrate a steroid-sensitive transhydrogenation from DPNH to TPN, a relatively high ratio of DPN(H) to TPN(H) must be maintained throughout the course of the reaction. This was achieved by adding a relatively large amount of DPNH, a much smaller quantity of TPN, and oxidized glutathione (GSSG) and glutathione reductase to oxidize any TPNH formed by transhydrogenation:[21]

$$\begin{aligned} \text{DPNH} + \text{TPN}^+ &\rightleftharpoons \text{DPN}^+ + \text{TPNH} \\ \text{H}^+ + \text{TPNH} + \text{GSSG} &\rightarrow 2\ \text{GSH} + \text{TPN}^+ \\ \hline \text{H}^+ + \text{DPNH} + \text{GSSG} &\rightarrow 2\ \text{GSH} + \text{DPN}^+ \end{aligned}$$

Figure 5 shows an experiment of this type in which the decrease in absorbance at 340 mμ due to the oxidation of 0.25 μmole DPNH occurred in the presence of 0.005 μmole TPN, GSSG, glutathione reductase,[22] estradiol-17β, and the placental hydroxysteroid dehydrogenase. The oxidation of DPNH depended upon the presence of both the placental dehydrogenase and TPN. In other experiments it was found that in similar systems 0.002 μmole TPN was only slightly less effective than 0.005 μmole, whereas 0.02 μmole TPN was definitely inhibitory, and 0.05 μmole

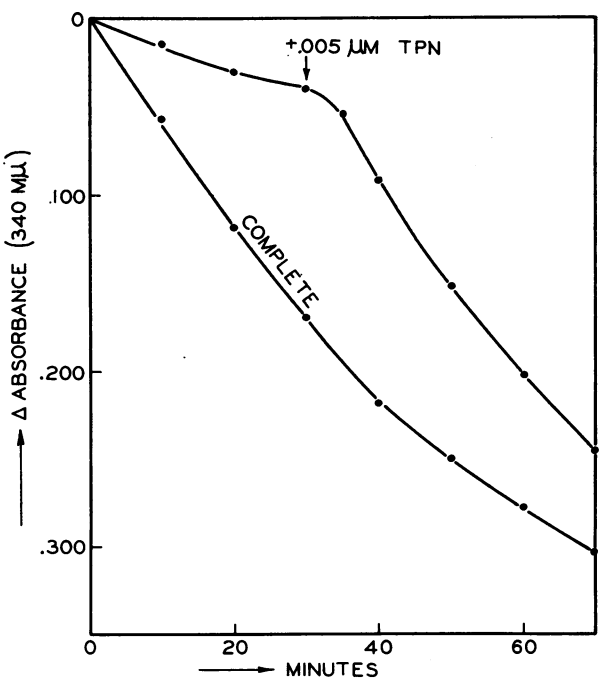

FIG. 5.—Transhydrogenation from added DPNH to TPN. The complete reaction system contained in a final volume of 3.0 ml.: 300 μmoles Tris buffer, pH 7.4; 0.24 μmole DPNH; 0.005 μmole TPN; 4 μg. estradiol-17β in 0.01 ml. dioxane; 10 μmoles oxidized glutathione; 154 units estradiol dehydrogenase (specific activity 205 units per mg. protein); and an excess of purified yeast glutathione reductase (see ref. 22). Readings of the decrease in absorbance at 340 mμ were taken with time against a control cuvette containing all ingredients except nucleotides and steroid. In a separate cuvette, the addition of TPN was delayed for 30 minutes. Temp. 25°.

TPN prevented the hydrogen transfer completely. This emphasizes the extreme sensitivity of the rate and extent of transhydrogenation to the levels of TPN(H) in the reaction mixture. The slow rate of reaction in the absence of added TPN can be ascribed to very small amounts of bound TPN in the hydroxysteroid dehydrogenase preparation, since the glutathione reductase, at the levels added to the test system, failed to catalyze any oxidation of DPNH by GSSG. It may be mentioned that the purified hydroxysteroid dehydrogenase possessed marked TPNH-glutathione reductase activity. This was an unexpected finding, since the preparations were devoid of other TPN-linked enzymes such as isocitric and glucose-6-phosphate dehydrogenases.

Hydrogen Transfer from TPNH to Analogs of DPN (Eq. [2] and [3]).—In assay systems in which TPNH was generated continuously from TPN, hydrogen transfer could be demonstrated not only to DPN, but also to certain of its analogs, such as APDPN and pyridinealdehyde-DPN. The relative rates of reduction of these nucleotides by stoichiometric amounts of estradiol-17β (dehydrogenase activity) and by continuously generated TPNH in the presence of catalytic amounts of this steroid (transhydrogenase activity) were found to be about the same, as shown in Table 2. Thus, in both systems pyridinealdehyde-DPN reacted more rapidly than APDPN and more slowly than DPN.

Hydrogen Transfer from DPNH to APDPN (Eq. [4]).—This reaction served as an assay for transhydrogenase activity during purification of the enzyme (Fig. 1) and will be discussed in detail below.

BINDING AND INTERACTION OF NUCLEOTIDES IN THE DEHYDROGENASE REACTION

The transhydrogenations for which estradiol-17β acted as a coenzyme were influenced greatly by the concentrations of hydrogen donor and acceptor pyridine nucleotides in the test systems. The nucleotides appear to compete for the same binding site(s) on the enzyme surface, which are presumably occupied alternately by donor and acceptor nucleotides. Since large differences exist in the Michaelis constants of the nucleotides, it can be readily appreciated that the relative concentrations of the nucleotides are critical in determining the rate of transhydrogenation. Clearer insight into these factors was obtained from studies of the Michaelis constants of pyridine nucleotides in the dehydrogenase reaction.

Binding of Nucleotides in the Dehydrogenase Reaction.—Determinations of the rate of oxidation of estradiol-17β in Tris buffer of pH 7.4 were performed with varying concentrations of DPN, TPN, and APDPN (Fig. 6). The majority of measurements were made in a final volume of 3.0 ml. in cuvettes of 1-cm. light path. In the case of TPN, no decline in velocity was observed with the lowest concentrations of nucleotide at which measurements could be made ($2 \times 10^{-5}\ M$), and additional determinations were performed in a total volume of 4.7 ml. in cells of 10-cm. light path. This permitted measurements to be made with concentrations of TPN as low as $2 \times 10^{-6}\ M$, but the Michaelis constant for this nucleotide was so low that even under these conditions it could not be determined accurately. In the reverse reaction the rates of oxidation of DPNH and TPNH were studied as a function of their concentrations with estrone as substrate (Fig. 6). Again, the Michaelis constant for TPNH was so low that experimental limitations

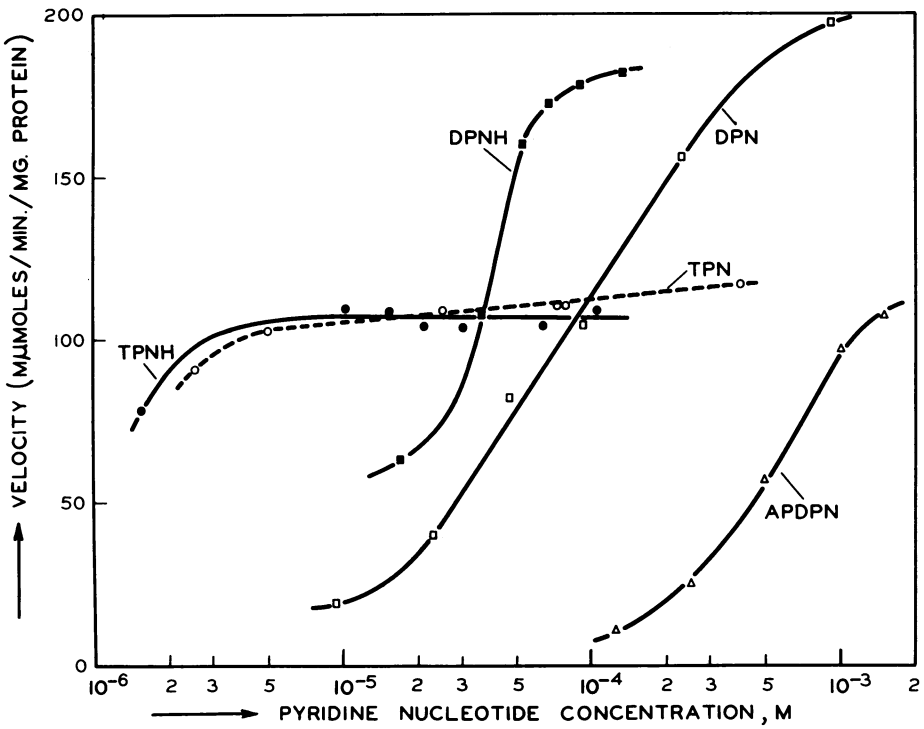

FIG. 6.—Effect of pyridine nucleotide concentration on velocity of the estradiol-17β dehydrogenase reaction. Experimental conditions are given in the text.

precluded an accurate determination. The approximate values for the maximal rates of reaction and the Michaelis constants for the various nucleotides are given in Table 3. It is clear that the Michaelis constants for TPN(H) are very much lower than those for DPN(H). In the case of DPN, it can be stated with certainty that the reduced nucleotide saturates the enzyme at lower concentrations than the oxidized form (Fig. 6 and Table 3). Moreover, the maximum velocities of the forward and reverse reactions observed under these conditions with various nucleotides differed by less than a factor of 2. The latter must be of profound importance for catalysis of transhydrogenations by the enzyme.

TABLE 3

MICHAELIS CONSTANTS (K_M) OF VARIOUS NUCLEOTIDES AND MAXIMUM VELOCITIES (V_M) IN ESTRADIOL-17β DEHYDROGENASE ASSAY

Pyridine Nucleotide	K_M (M)	V_M
APDPN	5×10^{-4}	50
DPN	8×10^{-5}	100
DPNH	3×10^{-5}	90
TPN	$<10^{-6}$	60
TPNH	$<10^{-6}$	55

Interaction of Nucleotides in the Dehydrogenase Reaction.—The influence of very low levels of TPN on the oxidation of estradiol-17β by other nucleotides is shown in Figure 7. As little as 3.3×10^{-7} M TPN depressed the rate of reduction of DPN to about half that of the control value. When TPN was added to give a final con-

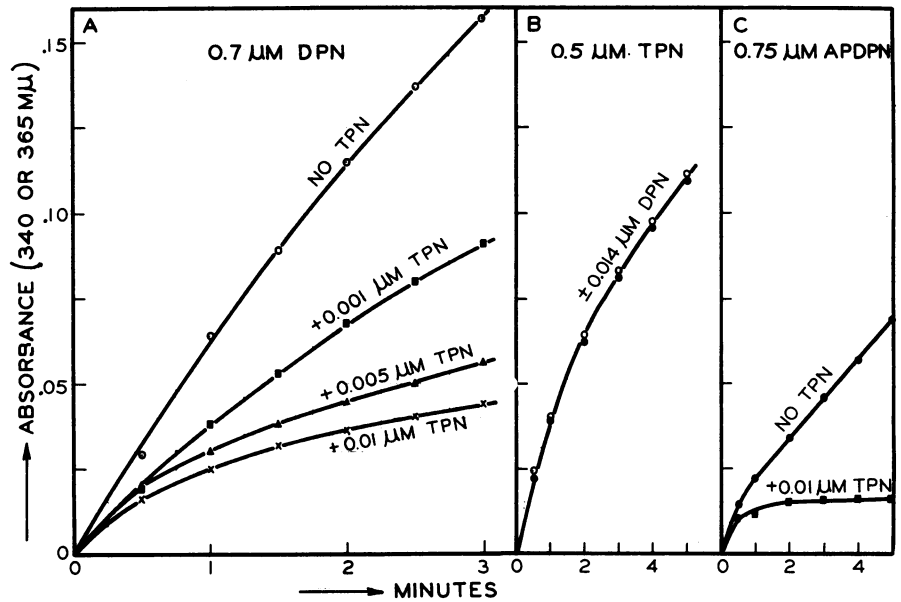

FIG. 7.—Interaction of pyridine nucleotides in the dehydrogenase reaction. The experimental systems contained, in a final volume of 3.0 ml.: 300 μmoles Tris buffer, pH 7.4; 80 μg. estradiol-17β in 0.04 ml. dioxane; 25 mg. crystalline bovine plasma albumin; 75 units placental dehydrogenase (specific activity 396 units per mg. protein); and 0.7 μmole APDPN (graph *A*) or 0.5 μmoles TPN (graph *B*) or 0.75 μmole APDPN (graph *C*). The effect of the addition of varying amounts of TPN on the rate of reduction of DPN and APDPN is shown in graphs *A* and *C*, respectively. Graph *B* illustrates the lack of influence of 0.014 μmole DPN on the rate of reduction of TPN.

All components were mixed and the reaction initiated by the addition of the enzyme in 0.02 ml. The absorbance was measured at 340 mμ (graphs *A* and *B*) and at 365 mμ (graph *C*) at intervals against a blank cuvette containing all ingredients except the steroid. Temp 25°.

centration of 3.3×10^{-6} *M*, the oxidation of estradiol-17β by DPN was practically abolished (Fig. 7, *A*). The total change in absorbance due to the reduction of even the largest amount of TPN used was negligible compared with that resulting from the reduction of DPN in the course of this experiment. Since, however, the small amounts of TPN added in these inhibition experiments are, at least in part, reduced by the steroid, the TPNH thus formed may yet be a more powerful inhibitor than TPN. This could account for the progressive nature of the inhibition by TPN of the reduction of DPN (Fig. 7, *A*).

The oxidation of estradiol-17β by APDPN was also suppressed almost to completion by 3.3×10^{-6} *M* TPN (Fig. 7, *C*). In contrast, when the dehydrogenase assay was carried out with TPN as the hydrogen acceptor, the rate of reaction was uninfluenced by the further addition of 3.3×10^{-6} *M* DPN (Fig. 7, *B*). These results are entirely consistent with the relative magnitudes of the Michaelis constants for the three nucleotides and their competition for the same binding site(s) on the enzyme surface. They indicate again that the affinity for TPN ≫ DPN > APDPN.

INTERACTION OF NUCLEOTIDES IN THE TRANSHYDROGENASE REACTIONS

Transfer of Hydrogen from TPNH to DPN.—The immense affinity of the enzyme

for TPN is of critical importance in determining the rate and direction of the steroid-mediated transfer of hydrogen from one pyridine nucleotide to another. It can be stated categorically that no hydrogen transfer between DPN and TPN has been demonstrable unless the ratio of ([DPN] + [DPNH])/([TPN] + [TPNH]) in the test system was relatively large. For instance, DPNH can act as a hydrogen donor to TPN only when the concentration of the latter nucleotide is kept relatively low (cf. Fig. 5). For similar reasons, in a 3-ml. system, negligible transfer of hydrogen from enzymatically generated DPNH (0.14 μmole) to TPN (0.5 μmole) was observed in the presence of estradiol-17β and the placental dehydrogenase.

The influence of the relative concentrations of TPNH and DPN on the rate of transhydrogenation has been examined in some detail (Fig. 8). TPNH was generated *in situ* from TPN by the action of glucose-6-phosphate dehydrogenase. The TPN concentration was varied from 1.7 to 333 $\times$ 10^{-7} M, and the rate of

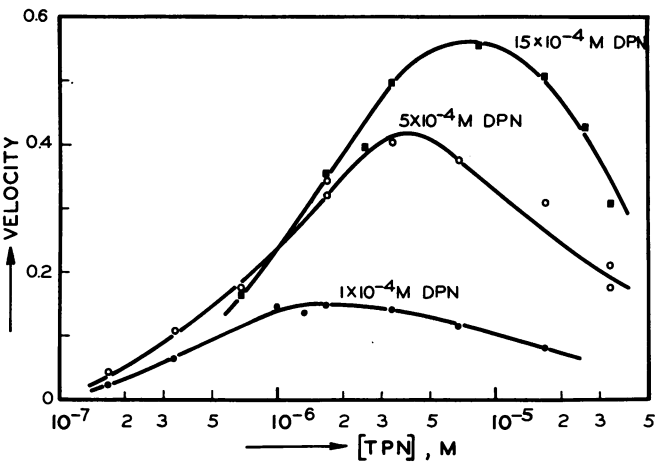

FIG. 8.—Interdependent influence of TPNH and DPN concentrations on the rate of transhydrogenation. Measurements of the rate of hydrogen transfer from generated TPNH to DPN were made for a series of TPNH concentrations (varying from 1.7 $\times$ 10^{-7} to 3.33 $\times$ 10^{-5} M) at each of three DPN concentrations (1 $\times$ 10^{-4}, 5 $\times$ 10^{-4}, and 15 $\times$ 10^{-4} M). The velocity is expressed as the change in absorbance at 340 mμ per hour. The reaction systems contained, in a final volume of 3.0 ml.: 300 μmoles Tris buffer pH 7.4; 10 μmoles glucose-6-phosphate; 4 μg. estradiol-17β in 0.01 ml. dioxane; an excess of purified yeast glucose-6-phosphate dehydrogenase; indicated amounts of DPN and TPN; and 62 units placental estradiol dehydrogenase (specific activity 205 units per mg. protein). Readings were taken against control cuvettes containing no added nucleotides. For each DPN concentration, an additional blank containing no TPN was included and the rates corrected for a slow reduction of DPN in the absence of TPN. These corrections were of the order of 0–0.01 per hour at different DPN concentrations. Temp. 25°.

reduction of DPN was measured at three concentrations of the latter nucleotide (1 $\times$ 10^{-4}, 5 $\times$ 10^{-4}, and 15 $\times$ 10^{-4} M). Thus the initial ratio of DPN to TPNH varied from a maximum of 8400 to a minimum of 3. For each concentration of DPN, an optimum TPN concentration exists, and the optimum ratio of concentrations of DPN to TPNH lies in the region of 50–200. The reaction velocity declines when this ratio lies outside these limits. It may be argued that the activity of the TPNH-generating system might have determined the rate of transhydrogenation at different TPNH concentrations in this experiment. However, the rate of transhydrogenation was not changed appreciably when the amount of glucose-6-phosphate dehydrogenase was increased several fold.

Transfer of Hydrogen from DPNH to APDPN.—When the placental hydroxysteroid dehydrogenase is isolated in the presence of estradiol-17β as described above, the purified preparations contain unknown quantities of bound steroid which

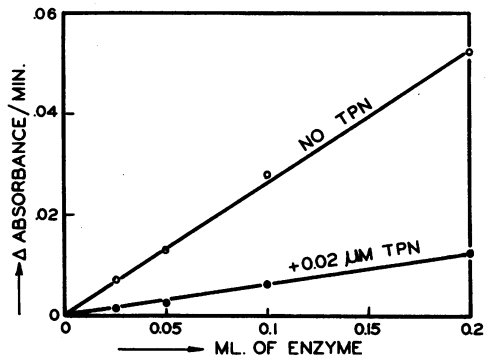

FIG. 9.—Rates of estradiol-17β-dependent and "non-specific" transhydrogenations as a function of enzyme concentration. Reduction of APDPN by DPNH was measured at 400 mμ under conditions described in the text. The upper graph represents the velocities observed in the presence of 4 μg. estradiol-17β and is a combined measure of the estradiol-17β-sensitive and "non-specific" transhydrogenases. The lower graph represents the velocities of the reaction inhibited by 0.02 μmole TPN and measures the steroid-independent transhydrogenase. The enzyme containing 1,310 units dehydrogenase per ml. and had a specific activity of 166 units per mg. protein. Temp. 25°.

partially activate this transhydrogenation. The addition of increasing amounts of estradiol-17β raised the rate of hydrogen transfer from DPNH to APDPN (measured spectrophotometrically at 400 mμ) to a maximum, beyond which the further addition of the estrogen was without effect (Table 4). About 1 μg/ml of

TABLE 4

ACTIVATION OF TRANSHYDROGENATION BETWEEN DPNH AND APDPN BY ESTRADIOL-17β*

Amount of Estradiol Added (μg/3 ml)	Rate of Reduction of APDPN, (Δ Absorbance, 400 mμ per hour)
0	0.276
0.5	0.492
2	0.588
4	0.588
8	0.600

* The measurements were carried out in a system identical with that described for the assay of transhydrogenase activity. The enzyme was purified in the presence of estradiol-17β and had a specific dehydrogenase activity of 396 units/mg protein. Temp. 25°.

estradiol-17β was sufficient for maximal activation. This is in marked contrast to the transhydrogenation from generated TPNH to DPN, which is fully activated by much lower (0.1 –0.2 μg/ml) concentrations of estradiol-17β. Thus preparations of the enzyme made in the presence of estradiol-17β showed no requirement for added steroid in the latter system. Small amounts of TPN or TPNH exerted a profound inhibitory effect on the reduction of APDPN by DPNH in the presence of estradiol-17β (Fig. 7, *C*, and Table 5). For example, as little as 3.3×10^{-7} *M*

TABLE 5

EFFECT OF TPN AND TPNH ON RATE OF HYDROGEN TRANSFER BETWEEN DPNH AND APDPN*

TPN Concentration ($\times 10^{-6}$ *M*)	Relative Rates	TPNH Concentration ($\times 10^{-6}$ *M*)	Relative Rates
0	100	0	100
0.33	61.7	0.6	42.8
1.67	35.3	3	34.1
3.33	32.4	6	36.3
16.7	27.4	30	35.2
66.7	26.5	120	35.2

* Reactions were carried out in 3.0-ml. systems containing: 300 μmoles Tris buffer, pH 7.4; 3.0 μmoles APDPN; indicated amounts of TPN or TPNH; 0.5 μmole DPNH; 4 μg. estradiol-17β in 0.01 ml. dioxane; and 75 units estradiol dehydrogenase (specific activity 396 units/mg protein). Absorbance was measured against a blank cuvette containing no nucleotides at 400 mμ. Temp. 25°.

TPN and TPNH inhibited 59 and 87 per cent, respectively. Concentrations of TPN and TPNH from 10^{-4} to 10^{-6} M suppressed the hydrogen transfer to a constant minimum, which in this enzyme preparation amounted to about one-third the total activity. This residual activity is not mediated by steroids. The rate of hydrogen transfer from DPNH to APDPN in the absence of added estradiol-17β was also depressed by TPN or TPNH to a level identical with that observed in the TPN-inhibited reaction found in the presence of the hormone.

These relations between rate of transhydrogenation and the estradiol-17β and TPN concentrations are illustrated in Figure 10, in which the rate of transhydrogenation is plotted as a function of estradiol-17β concentration. In the upper graphs the rate of hydrogen transfer is maximal with 1 μg. estradiol-17β or more. The rate is markedly depressed by 1 mμmole TPN and inhibited to about 25 per cent of maximum by 5 mμmoles TPN. The simplest explanation for these observations lies in assuming that only the steroid-sensitive transhydrogenation is inhibited by TPN and that the small inhibition by TPN observed in the absence of

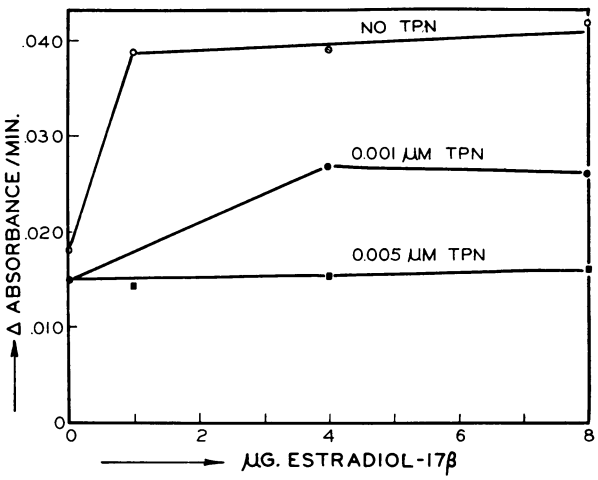

FIG. 10.—Stimulation of transhydrogenation from DPNH to APDPN by increasing concentrations of estradiol-17β and the inhibitory effect of TPN. Reaction system contains, in a final volume of 3 ml.: 300 μmoles Tris buffer, pH 7.4; 3 μmoles APDPN; 0.5 μmole DPNH; 0–8 μg. estradiol-17β dissolved in 0.04 ml. dioxane; varying amounts of TPN as indicated; and 32 units placental dehydrogenase of specific activity 173 units per mg. protein. Measurements were taken at 400 mμ against a blank containing no pyridine nucleotides. Temp. 25°.

added estradiol-17β represents a component of the transhydrogenation due to endogenous estradiol-17β. The uninhibited transhydrogenation is then that catalyzed by contaminating enzymes probably of a flavoprotein nature.[13] Figure 10 also shows that the maximum degree of inhibition produced by TPN is independent of the amount of estradiol-17β added. These considerations lend weight to the view that a true measure of the estradiol-sensitive transhydrogenase may be obtained by the difference between the total rate in the presence of extradiol-17β and that obtained in the presence of inhibitory concentrations of TPN.

HYDROGEN ION CONCENTRATION AND EQUILIBRIUM CONSTANTS

The activity of the placental estradiol-17β dehydrogenase as a function of hydrogen ion concentration has been examined by Langer,[9] who finds little variation in velocity over the range of about pH 6–9. This behavior with pH of a pyridine nucleotide-linked dehydrogenase is unusual, and in the case of certain bacterial hydroxysteroid dehydrogenases the velocities of forward and reverse reactions are

extremely pH-dependent.[23] In limited experiments with the purified placental enzyme we have confirmed the results of Langer.[9] For instance, the rate of oxidation of estradiol-17β by the placental enzyme with TPN is almost identical in Tris buffer pH 7.4 and pyrophosphate buffer pH 9.0.

The rates of transhydrogenation of DPN were measured from TPNH generated by purified yeast glucose-6-phosphate dehydrogenase and were found to be practically identical in the following buffers, varying in final pH from 7.1 to 7.4 :0.03 and 0.1 *M* Tris, 0.03 *M* phosphate, 0.03 and 0.1 *M* glycylglycine. The rate of hydrogen transfer was, however, very sensitive to pH and had a sharp maximum at pH 6.8 (Fig. 11). It has been previously pointed out[4] that this pH optimum probably represents the over-all operation of a number of complex factors in addition to the ionization of the catalytic protein. The equilibrium constant for the interconver-

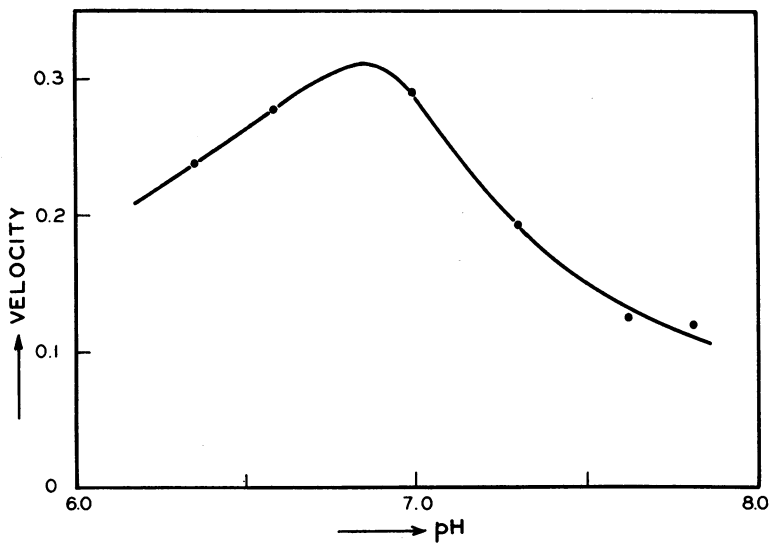

FIG. 11.—Effect of pH on rate of transhydrogenation. The reaction systems contained, in 3 ml. volume: 100 μmoles Sorensen's phosphate buffer of indicated final pH; 0.02 μmole TPN; 1.4 μmoles DPN; 10 μmoles glucose-6-phosphate; an excess of purified yeast glucose-6-phosphate dehydrogenase; 4 μg. estradiol-17β in 0.01 ml. dioxane; and 46 units of estradiol dehydrogenase. Measurements of absorbance at 340 mμ were made at intervals against a control cuvette containing no glucose-6-phosphate dehydrogenase and no TPN. At each pH, a separate control without TPN was also included and the rates corrected for the slow reduction of DPN by the glucose-6-phosphate dehydrogenase. The velocity is expressed as the change in absorbance per hour. Temp. 25°.

sion of estrone and estradiol-17β has been determined enzymatically by Langer,[9] who gives for

$$K_H = \frac{[\text{Estrone}][\text{DPNH}][\text{H}^+]}{[\text{Estradiol-17}\beta][\text{DPN}^+]}$$

the value $1.8 \pm 0.5 \times 10^{-8}$ *M*. This is not greatly different from the equilibrium constant for the interconversion of testosterone and 4-androstene-3,17-dione ($K_H = 3.8 \times 10^{-8}$ *M* at 298° K.).[24] On the basis of the K_H given by Langer, the ratio of concentrations of estrone to estradiol-17β may be computed under the conditions of the experiment of Figure 11. Since the oxidation-reduction potential

of DPNH and TPNH are virtually the same,[25] the sum of the concentrations of the reduced nucleotides may be substituted for DPNH in the above equilibrium expression. Similarly, in place of [DPN$^+$], one may substitute [DPN$^+$] + [TPN$^+$], but in practice this is unnecessary, since [TPN$^+$] is negligible under conditions of these experiments. Hence, during the first hour of the experiment, the ratio of concentrations of estrone to estradiol-17β varied from about 1 to 10 at pH 7.0.

DISCUSSION

Catalysis of dehydrogenation and transhydrogenation by the same enzyme.—The interaction between steroids and pyridine nucleotides catalyzed by the placental hydroxysteroid dehydrogenase can occur in two different ways, depending upon the experimental conditions. When relatively large amounts of steroid are added, a *stoichiometric* reaction between estradiol-17β and either DPN or TPN, or between estrone and the reduced forms of these nucleotides, can be demonstrated by optical means. Under these conditions, the enzyme behaves as a typical, freely reversible, pyridine nucleotide–linked dehydrogenase. On the other hand, minute quantities of the hormone can also exercise a *catalytic* function in transhydrogenase reactions. In the latter case the steroid, by undergoing alternate oxidation and reduction, acts as an intermediary hydrogen carrier (coenzyme) for the transfer of hydrogen from one pyridine nucleotide to another.

The environmental conditions require for maximal dehydrogenase and transhydrogenase activities are not the same. Thus the net reduction of pyridine nucleotides by estradiol-17β shows a rather broad pH optimum,[9] whereas the hormone-mediated hydrogen transfer from TPNH to DPN occurs only within a relatively narrow range of hydrogen ion concentrations. Moreover, the levels of pyridine nucleotides and steroids required for the two reaction sequences are quite different. Nevertheless, there is strong experimental evidence for the identity of the hydroxysteroid dehydrogenase with the protein which catalyzes transhydrogenation. This evidence, part of which has been presented elsewhere,[4, 5] may be summarized as follows: (1) Provided that the two activities are assayed properly, no separation of the two functions occurs during fractionation procedures which result in purification of more than one hundred fold; (2) only those steroids which are substrates for the hydroxysteroid dehydrogenase can act as coenzymes for the transhydrogenase reactions;[4] (3) the specificity toward natural pyridine nucleotides, and analogs thereof, is the same for both types of reaction; (4) as shown previously,[4] it is impossible to differentiate the two activities by the use of inhibitors; (5) the rates of oxidation or reduction of pyridine nucleotides in the dehydrogenase assay are compatible with the rates of transhydrogenation between pyridine nucleotides in which hydrogen is transported by the steroid; (6) the affinity for TPN(H) is much greater than for DPN(H) in both reactions, and a similar competition between nucleotides is demonstrable in the two situations.

Conditions for the Demonstration of Transhydrogenation.—Because of the great differences in binding constants between pyridine nucleotides, the ratio of the hydrogen donor and acceptor nucleotides cannot lie outside certain well-defined limits in the test system if transhydrogenation is to be demonstrated at all. Optimal values for this relationship will vary with the forms of pyridine nucleotide used as substrates and with the direction of hydrogen flow under study. Similar factors

have been found by Kaplan *et al.*[26, 27] to apply to the kinetics and reversibility of the reactions catalyzed by the soluble transhydrogenase of *Pseudomonas fluorescens*, which does not appear to employ a steroid as a coenzyme.

Equilibrium constants for the interconversion of several hydroxy- and ketosteroids, with pyridine nucleotides acting as hydrogen acceptors, have been shown to vary with the hydrogen ion concentration.[23] It would appear that equilibrium factors favor the catalysis of transhydrogenation by hydroxysteroid dehydrogenases and are responsible in part for the differences in the hydrogen ion concentrations at which maximal rates of transhydrogenation occur with the placental enzyme here described and the 3α-hydroxysteroid dehydrogenase of rat liver.[7] Furthermore, the velocities with DPN(H) and TPN(H) in forward and reverse reactions are of the same magnitude, thus also favoring reversibility. The immense affinity of hydroxysteroid dehydrogenases for steroids[4, 6, 23, 24] is probably also concerned with the ability of this class of enzymes to promote hydrogen transfer between pyridine nucleotides.

It cannot be overemphasized that, in any attempt to study steroid-mediated hydrogen transfers, strict attention must be paid to the relative concentrations of pyridine nucleotides and steroids in the reaction mixture, the pH thereof, and the possible presence of enzyme-bound reactants. It is obvious that, for a given hydroxysteroid dehydrogenase, the amounts of steroids and pyridine nucleotides required for maximal rates of transhydrogenation will vary with the purity of the enzyme preparation. Such considerations have been examined extensively in relation to the 3α-hydroxysteroid dehydrogenase of rat liver.[7] Side reactions which transform pyridine nucleotides by other enzymatic pathways will also interfere with the measurement of steroid-mediated transhydrogenation, especially in crude tissue extracts. Important examples of the latter are hydrolytic enzymes, which rupture various bonds in pyridine nucleotides,[28] or phosphokinases, which phosphorylate the 2′-position of the ribose attached to the adenine moiety of DPN(H).[29, 30] Reactions of this type were quantitatively unimportant in the present experiments, even when the most crude placental extracts were used. However, destruction of pyridine nucleotides and the dephosphorylation of TPN(H) to DPN(H) are exceptionally rapid in hormone-sensitive tissues such as the prostate gland and seminal vesicle[31] and interfere with spectrophotometric demonstrations of steroid-mediated hydrogen transfers in these tissues.

Transhydrogenation as a Possible Basis for Hormonal Action.—Following the discovery that certain *naturally occurring* steroid hormones serve as coenzymes for transhydrogenation, it was postulated[4] that this function constituted, at least in part, the chemical basis for the manifold morphological and functional changes which these hormones induce in various types of cells. Both the specificity and the overlap in action of the steroid hormones secreted by the gonads, the adrenal cortex, and the placenta, considered from the standpoint of both their chemical constitution and the tissues which they influence, could be understood if the following facts are taken into account. There exists a class of mammalian hydroxysteroid dehydrogenases which differ in respect to (*a*) their specificity and affinity for steroids, (*b*) the tissues in which they occur, and (*c*) their intracellular localization (the majority appear to be either soluble or microsome-bound). A common property of these proteins is that they react with DPN and TPN at comparable

rates.[4, 6] The original prediction that enzymes of this class, other than the placental enzyme described here, should act as transhydrogenases has been borne out fully in experiments with the soluble 3α-hydroxysteroid dehydrogenase of rat liver.[7]

Assume that a steroid-mediated transhydrogenation is responsible for the effect of a steroid hormone in a given physiological situation. Then differential responses of various tissues to different steroids could depend upon variations in the content of hydroxysteroid dehydrogenases among them. The extent to which the growth and function of a tissue are limited by steroid-dependent transhydrogenations at one or more intracellular locations would also relate to such tissue specificity. The classical "target organs" of gonadal steroids (such as the uterus and mammary gland, the prostate gland and the seminal vesicle) would be examples of tissues which are most sensitive in these respects. Minor structural alterations in the steroid molecule can alter not only the magnitude of their biological activity but also the quanitative nature of the response they elicit. The determinant of this specificity could reside in the affinity of various hydroxysteroid dehydrogenases for different steroids. This would also explain the inhibition of the activity of one steroid hormone by another[1, 2] if the inhibitory steroid displaced the active one from the surface of the dehydrogenase. Synergism between two different types of hormone and the "permissive" action of steroids in some types of biological response[32] might have their origin in transhydrogenations occurring in different parts of the cell, one of which would have to be operative before the other could promote alterations in structure or function. An example of the latter situation would be the action of estrogens and of gestagens on the uterus or the mammary gland.

The loss of hormonal dependence (Huggins[33]) which sometimes occurs during the life-history of certain neoplasms could be visualized in similar terms. A hormone-dependent tumor would be one whose growth is limited by steroid-mediated transhydrogenations. A certain proportion of the cells, however, might possess alternate, steroid-independent, enzymatic mechanisms which would bypass the rate-limiting function of the hydroxysteroid dehydrogenases. By a process of selection, these cells might survive preferentially, resulting in a hormone-independent tumor.

In addition to the interconversion of hydroxyl and ketone groups attached to the steroid skeleton, there are a number of other types of metabolic changes which are undergone by steroid hormones, e.g., hydrogenation of ring A, hydroxylations, conjugation with sulfate or glucuronic acid, scission of side chains, etc.[6] However, it would appear less likely that the latter types of reaction are related to the mode of action of steroid hormones. Experiments with eviscerate animals[34] and also with cells grown in culture[35] have shown that the conversion of hydroxy- to ketosteroids takes place in a wide variety of tissues, whereas other metabolic pathways are confined to the liver, kidney, adrenal, gonads, and placenta. Furthermore, the oxidation of hydroxysteroids with pyridine nucleotides as hydrogen acceptors is the only one of these metabolic reactions which is freely reversible at physiological pH.

Regulatory Role of Transhydrogenations.—In the biological oxidation of many metabolic intermediates bearing alcohol or aldehyde functions, hydrogen is transferred to the active group (nicotinamide) of either DPN or TPN. In most instances, the particular dehydrogenases react with one form of pyridine nucleotide either exclusively or at much greater rates than with the other. Krebs,[36] Krebs and Korn-

berg,[37] Kaplan *et al.*,[38] Horecker and Hiatt,[39] and we[4] have argued that this specificity for one of the two forms of pyridine nucleotide with the same group (oxidation-reduction) potential, permits regulation of interactions between the enzyme systems in which they participate. This regulation appears to be important in two main types of metabolic process: (*a*) energy capture from oxidations of reduced pyridine nucleotides and (*b*) participation of pyridine nucleotides in biosynthetic reactions. The first of these reactions concerns the oxidation of DPNH by mitochondria which is coupled to phosphorylation, whereas the oxidation of TPNH is not, unless hydrogen is first transferred to DPN.[36, 38] The pyridine nucleotide transhydrogenases of mammalian mitochondria[20] appear to have properties quite distinct from the steroid-requiring transhydrogenases. These mitochondrial enzymes can permit TPNH, formed by the action of TPN-specific dehydrogenases, to be a substrate for oxidative phosphorylation. However, since the majority of the TPN-reducing enzymes are present in the soluble portion of the cytoplasm and also because the amounts of DPN(H) therein are much greater than those of TPN(H), the extramitochondrial, steroid-mediated transhydrogenations can control the hydrogen flow from TPNH to DPN and thereby permit TPNH to act as a substrate for oxidative phosphorylation. It is well established that DPNH formed outside the mitochondria can, under suitable conditions, diffuse onto the surface of these particles and become oxidized, with the concomitant formation of adenosine triphosphate (ATP). These considerations point to the potentially important role of steroid-dependent transhydrogenases in regulating energy capture from the oxidation of TPNH formed in the cytoplasm.

A second area in which steroid-mediated transhydrogenations may exert a controlling influence involves biosynthetic reactions. There is increasing awareness that the key reductions in many biosyntheses occur at the expense of TPNH[36,37,39] and that the enzymes catalyzing most of these reactions are located outside the mitochondria. In many of these pathways, a reduction of an intermediate by TPNH is followed by the oxidation of the product by a DPN-specific dehydrogenase. Such reaction sequences are involved, for example, in the synthesis of (*a*) ascorbic acid,[41] (*b*) fructose by male accessory sexual tissues,[42, 43] (*c*) phosphopyruvate from pyruvate by the combined action of "malic" enzyme, malic dehydrogenase, and oxalacetic carboxylase,[36, 37, 39] and also (*d*) in the enzymatic isomerization of L-xylulose to D-xylulose,[44] which, like *a*, is an integral part of the "uronic acid pathway."[39] Transfer of hydrogen from TPNH to DPN results from the action of all these multienzyme systems. In the living cell, the extent to which such over-all reactions operate from left to right will depend, *inter alia*, upon the factors which determine the ratio of oxidized to reduced forms of TPN and DPN in the cellular compartments in which they occur.[45] Depending upon the latter conditions, steroid-mediated transhydrogenations could shift the equilibria of these biosynthetic sequences in either direction.

One would expect that, in *model* systems consisting of purified enzymes, a hydroxysteroid dehydrogenase and a suitable steroid would allow this type of couple reaction, in which no *net* oxidation or reduction takes place, to proceed in the presence of *catalytic* amounts of TPNH and DPN. . This is illustrated by the following equations:

$$\begin{aligned} S + TPNH + H^+ &\rightleftharpoons SH_2 + TPN^+ \\ SH_2 + DPN^+ &\rightleftharpoons S' + DPNH + H^+ \\ DPNH + TPN^+ &\rightleftharpoons DPN + TPNH \\ \hline S &\longleftrightarrow S' \end{aligned}$$

where S would be D-glucuronate, glucose, pyruvate + CO_2, and L-xylulose; SH_2 would be L-gulonate, sorbitol, malate and xylitol; and S′ would be the hypothetical intermediate 3-keto-L-gulonate, fructose, oxalacetate and D-xylulose in pathways (*a*), (*b*), (*c*), and (*d*), respectively.

It is also worthy of mention that in such systems, the net result of transhydrogenase action could be simulated by the action of enzymes which dephosphorylate TPN to DPN and which could rephosphorylate DPNH to TPNH at the expense of ATP as follows:

$$\begin{aligned} S + TPNH + H^+ &\rightleftharpoons SH_2 + TPN^+ \\ TPN^+ &\rightarrow DPN^+ + \text{inorganic phosphate} \\ SH_2 + DPN^+ &\rightleftharpoons S' + DPNH + H^+ \\ DPNH + ATP &\rightarrow TPNH + ADP \\ \hline S + ATP &\rightarrow S' + ADP + \text{inorganic phosphate} \end{aligned}$$

The steroid acting catalytically as a coenzyme for transhydrogenation can thus accomplish a transformation which would otherwise require stoichiometric quantities of ATP.

Much more experimental evidence is required to establish the truth or falsity of these speculations. In particular, there is great need for information concerning the quantitative significance of steroid-mediated hydrogen transfers in different types of cells. It is also possible that steroid hormones, by undergoing alternate oxidation and reduction, can function catalytically in the transfer of hydrogen between molecules other than pyridine nucleotides.

SUMMARY

Purified estradiol-17β-hydroxysteroid dehydrogenase has been isolated from human placenta. In the presence of *stoichiometric* quantities of estradiol-17β, this enzyme reduces DPN, TPN, APDPN, pyridinealdehyde-DPN, but not desamino-DPN. However, in the presence of stoichiometric amounts of pyridine nucleotides and *catalytic* concentrations of steroid hormones, the same enzyme promotes a reversible hydrogen transfer between the pyridine nucleotides. The hormone is alternately oxidized and reduced during this process and acts therefore in the manner of a hydrogen carrier or coenzyme. Evidence is presented for the identity of the protein catalyzing dehydrogenase and transhydrogenase functions.

Detailed measurements have been made of the binding constants of the enzyme for the naturally occurring pyridine nucleotides and several synthetic analogs. These measurements have given insight into the delicately balanced conditions required for the demonstration of steroid-mediated transhydrogenations. Many of the biochemical consequences of the action of steroid hormones may be related to their action as coenzymes of transhydrogenation. The implications of this hypothesis have been examined in some detail.

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