The Conversion of Pregnenolone to Progesterone by Small Particles from Rat Adrenal*

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Received March 16, 1964

The DPN^+-dependent transformation of pregnenolone to progesterone by a small-particle preparation from the rat adrenal has been investigated. Inhibition by DPNH was found to be competitive with DPN^+ and of such a magnitude as to make reasonable its possible physiological significance in steroidogenesis. TPN^+ also was found to function in the reaction and TPNH to inhibit it, but both were much less effective than the corresponding diphosphopyridine nucleotide. Evidence was obtained for the presence of an inhibitor of the reaction in many commercial preparations of TPN^+. The enzyme preparation was found to act on a variety of steroids possessing a 3β-hydroxyl group. The evidence presented is consistent with the postulation of a single steroid-3β-ol-dehydrogenase. No oxidation of hydroxyl groups in the 17α or 17β positions of androstene derivatives or of 20α or 20β positions of pregnene derivatives could be detected.

The inhibition by DPNH of corticoid synthesis, from both endogenous and exogenous precursors, in rat adrenal preparations has been reported (Koritz, 1962b, 1963). It has been established that the transformation of pregnenolone to progesterone is the locus of the inhibition and that the inhibition can be reversed by conditions which result in the oxidation of DPNH. Among these is an ascorbic acid-dependent DPNH oxidase, suggesting a possible function for ascorbic acid in adrenal metabolism. Thus the pregnenolone-to-progesterone system can be regarded as consisting of two parts. One is the steroid transformation resulting in the formation of progesterone and of DPNH, an inhibitor of this transformation. The other may be the ascorbate-dependent DPNH oxidase which is capable of removing this inhibition.

In this paper some of the properties of the pregnenolone-to-progesterone transformation have been further explored, especially with respect to pyridine nucleotide specificity, the inhibition by reduced pyridine nucleotides, and the question of the number of steroid dehydrogenases present in the preparation used. For this work it has been found convenient to use a small-particle preparation from rat adrenals. This type of preparation contains negligible endogenous substrate or cofactors, and it has been shown by Beyer and Samuels (1956) that the small-particle fraction from the beef adrenal cortex contains most of the activity of the cell for the reaction under study.

Experimental Procedures

Adrenal glands were removed from male Sprague-Dawley rats killed by decapitation. The glands were homogenized in 0.154 M KCl, usually at a concentration of about 50 mg wet wt of tissue per ml, and the homogenate was fractionated as described elsewhere (Péron and Koritz, 1960). The supernatant from pellet 2 was further fractionated by centrifugation at 100,000 $\times g$ for 60 minutes. The supernatant from this centrifugation was discarded, the sides of the centrifuge tube were carefully wiped with paper tissue, and the sides of the tube and the firmly packed sediment of small particles (pellet 3) were carefully rinsed with 0.154 M KCl. Pellet 3 was then resuspended by homogenization in 0.154 M KCl. In a typical experiment, the pellet 3 obtained from 600 mg, wet wt, of rat adrenals was suspended in 6 ml of 0.154 M KCl. The protein content of such a preparation usually ranged from 0.5 to 0.6 mg/ml as determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as a standard.

The contents of the incubation beaker were analyzed for the formation of Δ^4-3-ketones in the steroid substrate and DPNH in the following manner. A suitable aliquot of the incubation mixture (usually 1.5 ml from an incubation mixture of 2.0 ml) was transferred to a glass-stoppered tube containing 1.5 ml of Spectrograde dichloromethane. The tube was shaken vigorously for 1 minute and then centrifuged to separate clearly the two layers. The aqueous layer was read at 340 mμ to give a measure of DPNH formation and the dichloromethane layer was read at 240 mμ to give a measure of the formation of Δ^4-3-ketones. Adequate controls, usually with a boiled pellet 3 preparation, were routinely run. All incubations were carried out in duplicate at 37° in air in a Dubnoff metabolic incubator.

Pyridine nucleotides were obtained from the Sigma Chemical Co. and solutions were freshly prepared for each experiment.

Results

The time course of the transformation of pregnenolone to progesterone is shown in Figure 1A, and the effect of enzyme concentration is shown in Figure 1B. It is to be noted that for each μmole of progesterone formed a μmole of DPNH is formed. This is to be expected from the stoichiometry of the reaction, and holds throughout the times measured and the enzyme concentrations used. Under the conditions used the reaction proceeds in a linear manner up to about 15 minutes. In the presence of pyruvate and lactic acid dehydrogenase the linear portion is extended, as would be expected on the basis of a DPNH inhibition of the reaction (Koritz, 1962b, 1963). It can be seen that a 10-minute incubation with 0.15 ml of pellet 3 results in values which fall on the linear part of the curves and these conditions were used in most experiments. In Figure 2 the *p*H curve for the reaction is presented. There was no buffer effect on the rate of reaction since the same rates were obtained using the different buffers at overlapping *p*H values.

Regardless of the excellent stoichiometry between DPNH formation as measured by the absorbancy at 340 mμ and progesterone formation as measured by the absorbancy at 240 mμ, it was considered necessary to

* This work was supported by a grant (A-3676 END) from the National Institutes of Health, U. S. Public Health Service.

TABLE I
CHROMATOGRAPHIC EVIDENCE FOR THE FORMATION OF PROGESTERONE FROM PREGNENOLONE[a]

Result	Incubation Condition I	II
Pregnenolone found[b] (μg)	105	20.8
Progesterone found[c] (μg)	0	88.4
	Chromatography System A	
Pregnenolone put on paper (μg)	44.5	10.4
Pregnenolone found (μg)	45.4	9.3
Progesterone put on paper (μg)	0	41.0
Progesterone found (μg)	0	37.1
	Chromatography System B	
Pregnenolone put on column (μg)	24.6	5.6
Pregnenolone found (μg)	21.0	3.1
Progesterone put on column (μg)	0	11.7
Progesterone found (μg)	0.5	12.8

[a] Incubation condition I: each beaker contained 0.3 ml P-3 (0.21 mg protein), 60 μmoles of Tris buffer, *p*H 8.6, 60 μg of pregnenolone, and 0.154 M KCl to a final volume of 2.0 ml. Incubation II: additions were the same as for condition I plus 1.1 μmoles of DPN^+. All incubations were for 30 minutes. The contents of three beakers for each group were pooled and extracted three times with dichloromethane. After removal of aliquots for analysis, the extract was chromatographed on paper for 7 hours using a ligroin–propylene glycol system (Savard, 1954) (System A). The pregnenolone areas were detected by an antimony trichloride reagent (Rosenkrantz, 1954) and the progesterone areas by an ultraviolet lamp. After elution of the areas corresponding to these two steroids, and removal of aliquots for analysis, the eluates were examined by gas chromatography (System B) in an F & M chromatograph, Model 609, on a 183 × 0.64-cm (6 ft × 0.25 in.) column containing 3% SE-52 (a phenyl silicone obtained from General Electric Silicone Div., Waterford, N. Y.) on Gas-Chrom P (obtained from Allied Scientific Corp., State College, Pa.) as a support. The column temperature was 220° with argon as the carrier gas and a hydrogen-flame detector. The retention times of the appropriate experimental samples were identical with authentic pregnenolone or progesterone. Concentrations were calculated on the basis of areas under the appropriate curves. All values have been corrected for a control incubation containing DPN^+ but no pregnenolone. The values for the paper chromatography have been corrected for recoveries of 80% using pregnenolone and of 72% using progesterone run along with the experimental samples in this chromatographic system. [b] Determined colorimetrically (Koritz, 1962a). [c] Determined by absorbancy at 240 mμ.

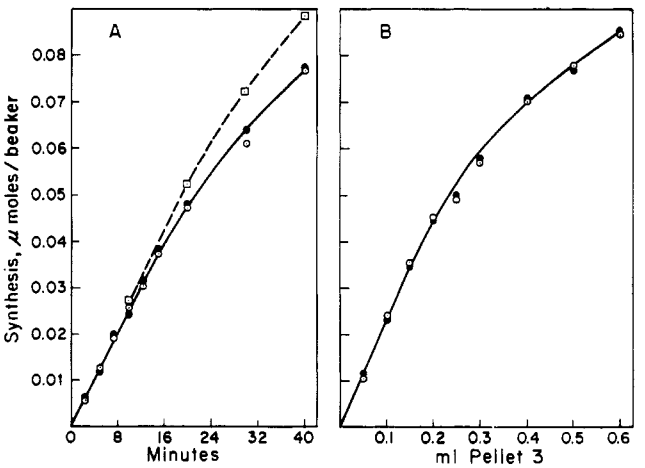

FIG. 1.—Transformation of pregnenolone to progesterone. (A) Time course of the transformation. The medium contained 0.15 ml of pellet 3, 0.2 ml of 0.3 M Tris buffer (*p*H 8.6), 1.1 μmoles of DPN^+, 0.19 μmole of pregnenolone in 0.02 ml ethanol, and 0.154 M KCl to a final volume of 2.0 ml. Ethanol, 0.02 ml, was also added to the appropriate control incubations. Incubations were at 37° in air for the indicated times. ●, DPNH formed; ⊙, progesterone formed; ⊡ - - - ⊡, progesterone formed in the presence of 4 μmoles of pyruvate and 2-times-crystallized lactic acid dehydrogenase obtained from Worthington Biochemical Corp. The amount of lactic acid dehydrogenase used oxidized more than the theoretical amount of DPNH formed in the incubation in less than 30 seconds. (B) The effect of pellet 3 concentration on the transformation of pregnenolone to progesterone. The medium additions were as described in (A). Incubation was for 10 minutes at 37° in air. ●, DPNH formed; ⊙, progesterone formed.

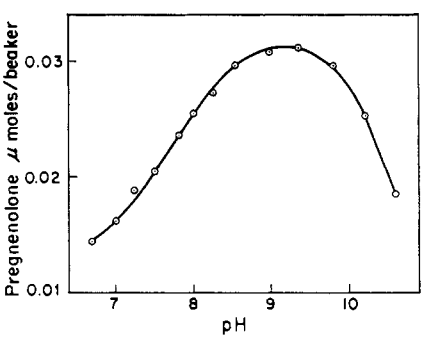

FIG. 2.—The effect of *p*H on the transformation of pregnenolone to progesterone. The medium additions were as described in Fig. 1 except that 0.2 ml of 0.3 M solutions of the following buffers were used: *p*H 6.7 and 7.0 potassium phosphate; *p*H 7.0–9.0 Tris; *p*H 9.0–10.6 glycine.

further establish that the dichloromethane-soluble material absorbing at 240 mμ was indeed progesterone. This was done by examining, in two chromatographic systems, the steroids found in incubations with pregnenolone in the absence and presence of DPN^+ (Table I). It may be seen that progesterone is indeed the product and that there is good stoichiometry between the disappearance of pregnenolone and the formation of progesterone.

Data obtained previously (Koritz, 1963) suggested that the DPNH inhibition of the transformation of pregnenolone to progesterone is competitive in nature, but precise information on this point could not be obtained at that time. However, with the present system it has been possible to carry out adequate kinetic studies. A representative set of data showing that the DPNH inhibition is competitive with respect to DPN^+ is given in Figure 3. The apparent K_m for DPN^+ in this reaction has been found to be 4.5×10^{-5} M (seven determinations with a range of 3.2 to 5.5 $\times 10^{-5}$ M). The apparent K_i in the presence of 3.8×10^{-5} M DPNH is 2.2×10^{-4} M. The K_i for the inhibition has been determined both from the double-reciprocal plot and by the method of Dixon (1953), and has been found to be 1.4×10^{-5} M (four determinations with a range of 1.1 to 1.8×10^{-5} M). Thus, the apparent K_i for DPNH with respect to DPN^+ is 3.2 times smaller than the apparent K_m for DPN^+. The apparent K_m for pregnenolone in this reaction has been found to be 6.2×10^{-5} M (four determinations with a range of 4.9 to 8.3×10^{-5} M). This is essentially the same as that for DPN^+, indicating that both substrates are bound equally well (assuming $K_m = K_s$). The nature of the DPNH inhibition with respect to pregnenolone could not be reliably determined. As may be seen from Figure 4, there is no inhibition by DPNH at low pregnenolone concentration and as the pregnenolone concentration is increased the DPNH inhibition reaches a plateau value very quickly. This has made it difficult to ob-

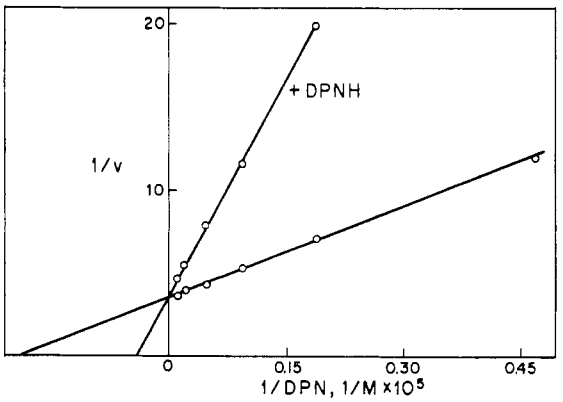

FIG. 3.—The inhibition by DPNH, with respect to DPN+, of the transformation of pregnenolone to progesterone. The medium additions were as described for Fig. 1 except as indicated. DPNH was present at 3.8×10^{-5} M final concentration. Velocity is expressed as μmoles of progesterone formed per 0.5 mg protein per 10 minutes' incubation at 37° in air.

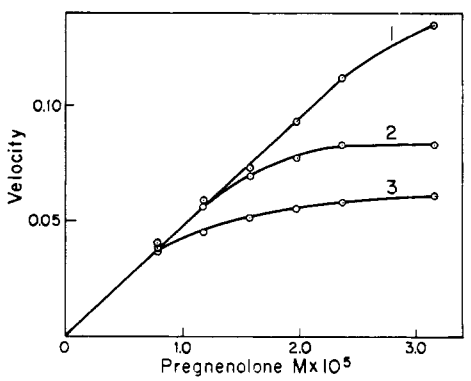

FIG. 4.—The inhibition by DPNH, with respect to pregnenolone, of the transformation of pregnenolone to progesterone. The medium additions were as described for Fig. 1 except as indicated. Velocity is expressed as μmoles progesterone formed per 0.5 mg protein per 10 minutes' incubation at 37° in air. Curve 1, no DPNH present; curve 2, DPNH at 1.28×10^{-4} M final concentration; curve 3, DPNH at 2.56×10^{-4} M final concentration.

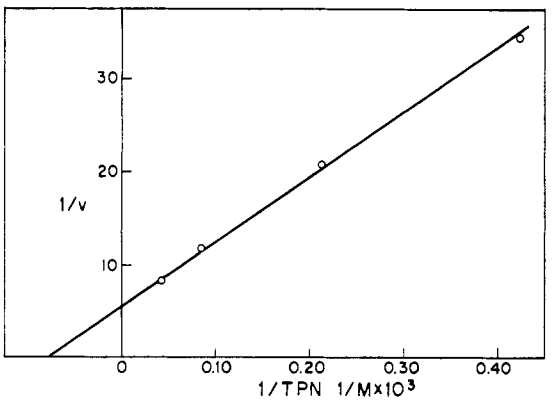

FIG. 5.—The effect of TPN+ concentration on the transformation of pregnenolone to progesterone. The medium additions were as described for Fig. 1 except that DPN+ was omitted and TPN+ was included as indicated. Velocity is expressed as μmoles of progesterone formed per 0.5 mg protein per 10 minutes' incubation at 37° in air.

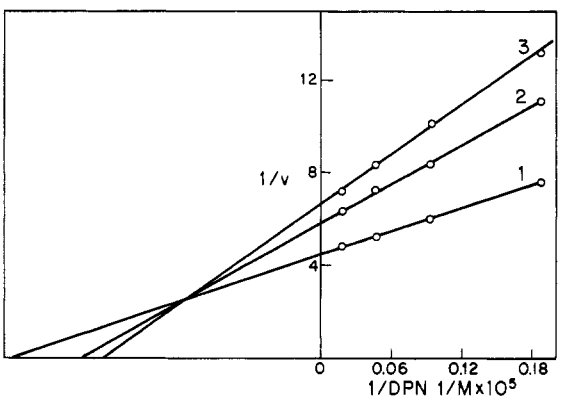

FIG. 6.—The inhibition by TPNH, with respect to DPN+, of the transformation of pregnenolone to progesterone. The medium additions were as described for Fig. 1 except as indicated. Velocity is expressed as μmoles of progesterone formed per 0.5 mg protein per 10 minutes' incubation at 37° in air. Curve 1, no TPNH present; curve 2, TPNH at 8.4×10^{-4} M final concentration; curve 3, TPNH at 1.7×10^{-3} M final concentration.

tain data amenable to a double-reciprocal plot analysis. Reasonable data of this sort have been obtained in a few experiments and the inhibition by DPNH appears to be noncompetitive with respect to pregnenolone with an apparent K_i of 4.1×10^{-4} M (two values of 4.5 and 3.7×10^{-4} M). If these values have some validity, it is seen that the apparent K_i of DPNH with respect to pregnenolone is more than six times greater than the apparent K_m for pregnenolone. This indicates that the DPNH inhibition is of much greater significance with respect to DPN+ than it is with respect to pregnenolone.

Although Samuels (1953) has reported that the reaction proceeds only with DPN+, the specificity of the reaction with respect to the pyridine nucleotide was investigated in the present system. When TPN+ from the Sigma Chemical Co. was used the results presented in Figure 5 were obtained. The apparent K_m for TPN+ is 1.3×10^{-2} M. Since the K_m for DPN+ is 4.5×10^{-5} M, it can be considered that TPN+ is about 290 times less effective than DPN+ in this reaction. When TPN+ from commercial sources other than the Sigma Chemical Co. was used, little synthesis of progesterone took place. In addition, these samples of TPN+ inhibited the synthesis of progesterone in the presence of DPN+. The Sigma TPN+ was not inhibitory. It would appear that these samples of TPN+ contain an inhibitor of this DPN+-dependent reaction. A Lineweaver-Burk plot of data on the inhibition indicated a mixed type of inhibition (Dixon and Webb, 1958). A possible contaminant in TPN+ preparations is ADPR, and this substance has been found to inhibit the reaction 70% at 10^{-3} M final concentration. However, no further work has been done on the nature of the inhibitory contaminant. From Figure 6 it may be seen that TPNH, unlike DPNH, inhibits the reaction, with respect to DPN+, as a mixed-type inhibitor. The relative effectiveness of DPNH and TPNH as inhibitors for this reaction has been approached by determining the concentrations of DPNH and TPNH needed to effect 50% inhibition of the reaction. It has been found that 50% inhibition was brought about by 2.0×10^{-4} M DPNH and by 6.6×10^{-3} TPNH. Thus by this criterion TPNH is some thirty-three times less effective than DPNH in inhibiting this reaction. In addition, it has been shown that 3.8×10^{-5} M DPNH will increase the apparent K_m for DPN+ 5-fold, while it can be seen from Figure 6 that a much higher concentration of TPNH, 1.7×10^{-3} M, increases the K_m for DPN+ only about one and one-half times. Considering that DPNH is a product of the reaction and that the level of DPNH in the adrenal is higher than that of TPNH, it would appear that TPNH

TABLE II
STEROID SUBSTRATE SPECIFICITY OF THE HYDROXYSTEROID DEHYDROGENASE IN THE PELLET 3 PREPARATION[a]

Substrate	Relative Velocity Δ^4-3-Keto Formation	Relative Velocity DPNH Formation
Pregnenolone	100	100
Cholesterol	0	0
20α-Hydroxycholesterol	0	0
16-Dehydropregnenolone	106	99
Δ^5-Pregnene-3β,20α-diol	97	98
Δ^4-Pregnene-3β,20β-diol	100	100
5β-Pregnan-3β-ol-20-one	0	128
5α-Pregnan-3β-ol-20-one	0	103
Δ^5-Androsten-3β-ol-17-one	125	129
Δ^4-Androstene-3β,17β-diol	115	110
Δ^5-Androstene-3β,17α-diol	118	118
5α-Androstan-3β-ol-17-one	0	120
5β-Androstan-3β-ol-17-one	0	126

[a] The medium additions were as described for Figure 1 except as indicated. Incubation was for 10 minutes. These assays were run at low substrate concentrations so that excess enzyme was present and the reaction was linear with respect to substrate concentration.

is of little consequence, compared to DPNH, in the transformation of pregnenolone to progesterone.

The data of Table II describe the specificity of the rat adrenal preparation with respect to the steroid substrate. Some specificity studies of this nature have been reported for a variety of tissues by Samuels (1953) and by Berliner *et al.* (1962). In confirmation of Samuels' results, substrates with the 3α-hydroxyl group are inactive. In addition, neither cholesterol nor 20α-hydroxycholesterol is active, reflecting the presence of the eight-carbon side chain. The effect of a side chain at position 17 is also apparent from the observation that, in general, compounds derived from the androstane nucleus are better substrates than those derived from the pregnane nucleus. The position of unsaturation in ring A or ring B is of little consequence. Saturation of the double bond in the androstane series has little effect. However, the 5β conformation in the pregnane series results in an enhancement of activity. These data also show that the enzyme preparation used is devoid of pregnane 20α- and 20β-hydroxyl and androstane 17α- and 17β-hydroxyl dehydrogenase activities. This is indicated by the equivalence of Δ^4-3 keto formation to DPNH formation when the appropriate compounds are used as substrates.

The question of the presence of one or several 3β-hydroxysteroid dehydrogenases in the enzyme preparation was raised by the broad substrate specificity demonstrated by the data of Table II. To help partially resolve this question advantage was taken of the fact that substrates with saturated rings A and B will produced only DPNH while substrates with a $\Delta^{4,5}$ or $\Delta^{5,6}$ point of unsaturation will produce both DPNH and Δ^4-3-ketones in equimolar amounts. Thus it is possible to assay two substrates in the presence of each other. The results presented in Table III indicate that a single enzyme is involved in the oxidation of the 3β-hydroxyl group of pregnenolone, 5α-pregnan-3β-ol-20-one, and 5α-androstan-3β-ol-17-one, and, hence, probably of the other substrates tested (Table II). It can be seen that in the region of low substrate concentration, where the enzyme is not yet saturated, neither substrate interferes with the oxidation of the other substrate. As the substrate concentration increases there is a mutual interference with the oxidation of one substrate by the other. These results are consistent with two substrates competing for the same enzyme. If several enzymes were involved one substrate should not interfere with another unless a substrate for one enzyme acts as an inhibitor for the other enzyme. However, in this case one would expect the inhibition to be overcome by high concentration of the substrate of the inhibited enzyme. This is not seen. The results are not owing to inhibition by DPNH since the appropriate parts of the experiments (Δ^4-3 keto formation) could be reproduced in the presence of pyruvate plus lactic acid dehydrogenase. It should also be noted that 5α-androstan-3β-ol-17-one, which is a better substrate than both pregnenolone and 5α-pregnan-3β-ol-20-one, interferes with pregnenolone oxidation to a greater extent than does 5α-pregnan-3β-ol-20-one. Correspondingly, pregnenolone interferes with the oxidation of 5α-androstan-3β-ol-17-one to a lesser extent than 5α-androstan-3β-ol-17-one interferes with the oxidation of pregnenolone.

DISCUSSION

It has been suggested that the inhibition by DPNH of the conversion of pregnenolone to progesterone may be of consequence in the control of steroidogenesis in the adrenal (Koritz, 1962b, 1963). The observation that the apparent K_i for DPNH with respect to DPN^+ is more than three times smaller than the K_m for DPN^+ indicates that DPNH is a potent inhibitor. This is also seen from the 5-fold increase in the apparent K_m for DPN^+ by 3.8×10^{-5} M DPNH. Since the inhibitor, DPNH, is a product of the reaction and hence present at the site of the reaction, the significance of the inhibition may be increased. It is of interest that a consequence of ACTH administration is an oxidation of the reduced pyridine nucleotides of the adrenal (Greenberg and Glick, 1960; Chance *et al.*, 1962) and it has further been suggested that the pyridine nucleotide oxidized is primarily DPNH (Chance *et al.*, 1962). The possible role of product inhibition in biological control mechanisms has been briefly discussed by Pardee (1959) and by Walter and Frieden (1963). The inhibition of the reaction by TPNH does not appear to be of significance, since TPNH is at least thirty times less effective than is DPNH. In addition, the concentration of TPNH in the rat and rabbit adrenal is less than that of DPNH (Glock and McLean 1955).

The presence in moistened, aged preparations of DPNH of a substance inhibitory for some dehydrogenases has been reported (Fawcett *et al.*, 1961). The inhibitor appears to be competitive with DPNH. Dalziel (1961) has also reported on the existence of an inhibitor of liver-alcohol dehydrogenase in DPNH preparations. These inhibitors do not appear to be involved in this study, since the inhibition applies to DPN^+, freshly prepared DPNH solutions were routinely used, the extent of inhibition by DPNH did not vary with the batch of DPNH used, and the inhibition is also seen when DPNH is generated from DPN^+ (Koritz, 1962b). The observation, reported here, that many commercial samples of TPN^+ contain an inhibitor of the DPN^+-mediated 3β-hydroxysteroid dehydrogenase is probably significant for other DPN^+-dependent dehydrogenases as well. The inhibitor will be of consequence only when TPN^+ is not the preferred electron acceptor and hence high concentrations of TPN^+ must be used for a measurable reaction to take place. These results would suggest that other DPN^+-linked dehydrogenase reactions which have been reported to be inactive with TPN^+ could bear reexamination.

TABLE III
EVIDENCE THAT ONLY ONE 3β-HYDROXYSTEROID DEHYDROGENASE IS PRESENT IN THE PELLET 3 PREPARATION[a]

Experiment 1	Progesterone from Pregnenolone (μmoles per beaker)						
		Plus 5α-Pregnan-3β-ol-20-one					
		5 μg	Inhibition (%)	10 μg	Inhibition (%)	30 μg	Inhibition (%)
Pregnenolone							
5 μg	0.0105	0.0106	0	0.0100	0	0.0081	23
10 μg	0.0205	0.0186	9	0.0175	15	0.0120	41
30 μg	0.0367	0.0316	14	0.0282	23	0.0211	43
	DPNH from 5α-Pregnan-3β-ol-20-one (μmoles per beaker)						
		Plus Pregnenolone					
		5 μg	Inhibition (%)	10 μg	Inhibition (%)	30 μg	Inhibition (%)
5α-Pregnan-3β-ol-20-one							
5 μg	0.0097	0.0097	0	0.0086	11	0.0043	55
10 μg	0.0186	0.0162	13	0.0159	14	0.0089	52
30 μg	0.0371	0.0289	22	0.0265	29	0.0165	55
	Progesterone from Pregnenolone (μmoles per beaker)						
		Plus 5α-Androstan-3β-ol-17-one					
Experiment 2		5 μg	Inhibition (%)	10 μg	Inhibition (%)	30 μg	Inhibition (%)
Pregnenolone							
5 μg	0.0138	0.0137	0	0.0132	5	0.0055	60
10 μg	0.0270	0.0222	18	0.0191	29	0.0067	75
30 μg	0.0362	0.0267	26	0.0204	44	0.0076	79
	DPNH from 5α-Androstan-3β-ol-17-one (μmoles per beaker)						
		Plus Pregnenolone					
		5 μg	Inhibition (%)	10 μg	Inhibition (%)	30 μg	Inhibition (%)
5α-Androstan-3β-ol-17-one							
5 μg	0.0185	0.0195	0	0.0191	0	0.0193	0
10 μg	0.0338	0.0347	0	0.0332	0	0.0313	7
30 μg	0.0842	0.0711	15	0.0716	15	0.0707	16

[a] The medium additions were as described for Figure 1 except as indicated. Incubation was for 10 minutes.

The data of Table III indicate that, in spite of the number of steroids which can act as substrates, a single 3β-hydroxysteroid dehydrogenase is present in the pellet 3 preparation. On the basis of evidence obtained with slices from a case of human hyperplastic cortical tissue, Weliky and Engel (1963) have suggested that 3β-hydroxysteroid dehydrogenases with more restrictive substrate specificities exist. In this study, the small particles from the rat adrenal appear to contain one dehydrogenase with a fairly broad substrate specificity.

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