Cleavage of Cholesterol Side Chain by Adrenal Cortex. IV. Effect of Phosphate and Various Nucleotides on a Soluble Enzyme System*

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ABSTRACT: A soluble enzyme system, capable of oxidizing cholesterol to pregnenolone, has been shown to require reduced triphosphopyridine nucleotide (TPNH) or reduced diphosphopyridine nucleotide (DPNH) as cofactor. The optimal concentrations of the two cofactors are 0.5–1 $\times$ 10^{-5} mM and 1 $\times$ 10^{-3} mM, respectively, indicating that DPNH is probably not the active cofactor *in vivo*. The reactions with DPNH require, and those with TPNH are greatly stimulated by, arsenate or inorganic phosphate. 3′,5′-Adenosine monophosphate (AMP) has no effect. With DPNH as cofactor, triphosphopyridine nucleotide (TPN^+), 2′-AMP, and 3′-AMP are strong inhibitors. The reaction with TPNH is inhibited by excess TPNH or TPN^+ and by 2′- or 3′-AMP, but not by DPNH or diphosphopyridine nucleotide (DPN^+).

The classical work of Stone and Hechter (1954) clearly showed that ACTH[1] stimulates the conversion of cholesterol to progesterone in the adrenal cortex. In 1957, Haynes and Berthet provided evidence that the stimulatory effect of ACTH is mediated through the increase of G-6-P and of TPNH. In agreement with this hypothesis, it was found later that the cleavage of cholesterol side chain is achieved by oxygenase reactions where TPNH serves efficiently as cofactor (Solomon *et al.*, 1956; Halkerston *et al.*, 1961; Shimizu *et al.*, 1961; Constantopoulos *et al.*, 1961, 1962). There have also been numerous reports dealing with the effect of ACTH, 3′,5′-AMP, TPNH, Ca^{2+}, glucose, and puromycin on corticoid formation and other synthetic activities of adrenal tissue and homogenates (Koritz *et al.*, 1957–1959, 1962; Haynes *et al.*, 1959; Peron, 1961; Peron and Koritz, 1960; Hechter and Lester, 1960; Schonbaum *et al.*, 1956; Birmingham *et al.*, 1958, 1960; McKerns, 1962; Hilton *et al.*, 1961; Greenberg and Glick, 1961; Fiala and Glinsmann, 1961; Chance *et al.*, 1962; Ferguson, 1963; Scriba and Reddy, 1963; Harding and Nelson, 1963). Some of these supported, while others questioned, the hypothesis of Haynes and Berthet that the *in vivo* effect of ACTH is mediated *via* the stimulation of TPNH production.

For the past several years, we have been engaged in studying the enzymatic reactions involved in the conversion of cholesterol to pregnenolone. In a previous communication (Constantopoulos and Tchen, 1961) we have stated that the soluble enzyme system extracted from adrenal cortex mitochondria can utilize either TPNH or DPNH. In view of the controversy on the Haynes–Berthet hypothesis, we wish to report here a detailed analysis of the effectiveness of TPNH or DPNH for this process and the effect of several phosphorylated compounds.

Materials and Methods

These are as described in previous communications (Constantopoulos *et al.*, 1961, 1962). The assay is based on the conversion of cholesterol-4-^{14}C to pregnenolone and/or progesterone, which can be followed by pregnenolone formation or cholesterol disappearance or both. In most of the experiments described in this communication, both product formation and substrate disappearance were determined. The only exception is the experiments dealing with the inhibiting effect of AMP. In this case, the reaction was followed by product formation only, since preliminary experiments had shown that the presence of the three adenylic acids did not alter the products of the reaction.

The reactions proceed at a linear rate until the conversion of *ca.* 30–40% of the added cholesterol. Thereafter, the rate of conversion declines and a residual 15–20% of the added cholesterol remains even after several hours of incubation. In the current experiments, an incubation time of 1 hr was chosen. This is not ideal for all the reaction mixtures. Indeed, with optimal concentrations of cofactors, more than half of the added cholesterol has been converted in this time. Rather, this period of incubation was chosen as a compromise so that the low conversion values, obtained under unfavorable conditions, would be readily measurable

* From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received November 22, 1965; revised February 15, 1966.* Supported by grants (AM-05384 and HE-04139) from the U. S. Public Health Service.

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[1] Abbreviations used: DPN^+, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN^+, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; AMP, adenosine monophosphate; ACTH, adrenocorticotropic hormone.

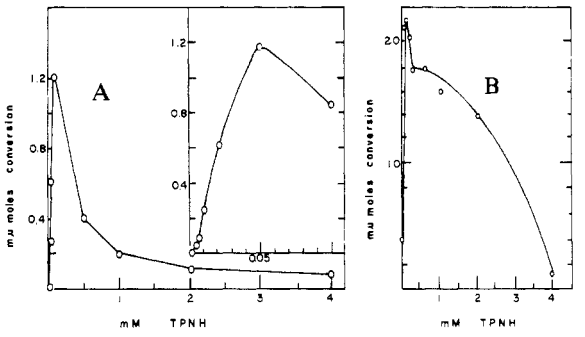

FIGURE 1: TPNH as cofactor. The reaction mixture (2 ml) contained 4 mμmoles of cholesterol and enzyme preparation equivalent to 1 g of adrenal. It was buffered at pH 7.0 with 0.01 M phosphate. In Figure 1A, chemically or enzymatically reduced TPNH was added. In Figure 1B, TPNH and excess of G-6-P dehydrogenase and G-6-P (5 μmoles) were added. The effectiveness of low concentrations of TPNH is shown in expanded scale in the inset of Figure 1A.

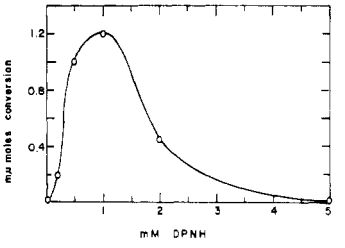

FIGURE 2: DPNH as cofactor. The conditions were the same as in Figure 1 with the substitution of DPNH for TPNH.

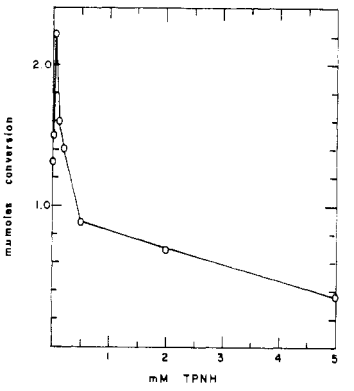

FIGURE 3: Addition of TPNH at different concentrations to reaction mixture already containing 1 mM DPNH. Different concentrations of TPNH were added to a reaction mixture containing 1 mM DPNH and other standard components.

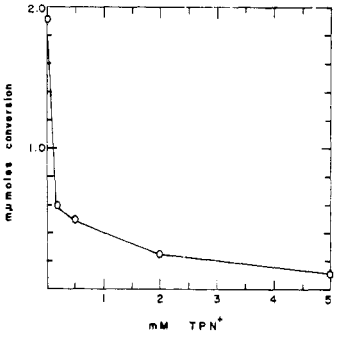

FIGURE 4: Inhibition by TPN^+ when DPNH was used as cofactor. Different concentrations of TPN^+ were added to a reaction mixture containing 1 mM DPNH and other standard components.

while the highest conversion values, obtained under the most favorable conditions, would still be substantially less than quantitative. This incubation period resulted in amplification of the low conversion values relative to the high conversion values, a point which should be borne in mind if one wishes to compare these values on an absolute quantitative basis. However, this does not affect the conclusions to be drawn in this paper, which deals primarily with the determination of the optimal concentrations of DPNH and TPNH and the stimulatory effects of inorganic phosphate and arsenate.

Results

The conversion of cholesterol to pregnenolone at different concentrations of TPNH is shown in Figure 1. If TPNH, either chemically or enzymatically reduced, was added without a regenerating system, there was an extremely sharp concentration optimum (curve A). If a regenerating system, G-6-P and G-6-P dehydrogenase, was also added, there was still an optimal concentration of TPNH, but the inhibition by excess TPNH was less marked than in the absence of the regenerating system. In several experiments, this phenomenon of inhibition by excess TPNH was readily demonstrated although the optimal concentration of TPNH varied from 0.05 to 0.1 mM in different experiments.

The cleavage of cholesterol side chain with DPNH as cofactor is shown in Figure 2. It can be seen that at 0.05–0.1 mM, which is the optimal concentration with TPNH, there was very little conversion with DPNH as cofactor. However, at 1 mM DPNH, very good conversion was obtained. In different experiments, the optimal concentration of DPNH varied between 1 and 2 mm. Using optimal concentration of DPNH, addition of low concentrations of TPNH stimulated the reaction, whereas addition of TPN^+ or excess TPNH led to inhibition (Figures 3 and 4). The reaction with TPNH as cofactor, on the contrary, was not inhibited by DPN^+ or DPNH. However, the addition of DPN^+ did affect the nature of the steroid produced. The yield in pregnenolone, which was the principal product of cleavage with TPNH alone, decreased upon the addition of

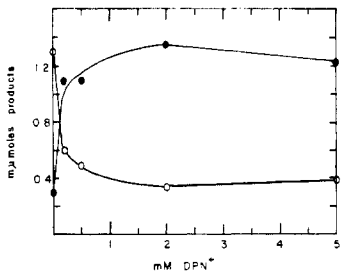

FIGURE 5: Effect of DPN^+ when TPNH was used as cofactor. Different concentrations of DPN^+ were added to reaction mixtures containing 0.05 mM TPNH and excess G-6-P and G-6-P dehydrogenase. Solid and open circles represent the amounts of progesterone and pregnenolone, respectively.

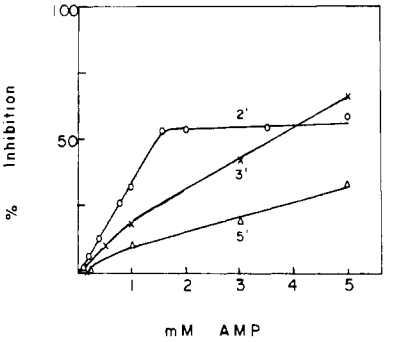

FIGURE 7: Inhibition by AMP when TPNH was used as cofactor. 2'-, 3'- or 5'-AMP was added to reaction mixtures containing 0.05 mM TPNH and other standard components.

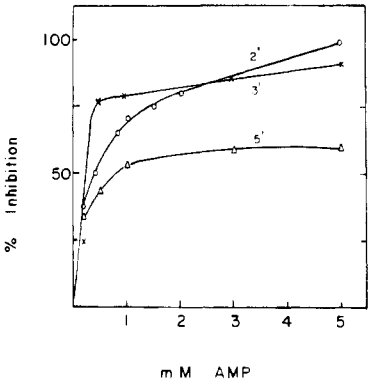

FIGURE 6: Inhibition by AMP when DPNH was used as cofactor. 2'-, 3'-, or 5'-AMP was added to reaction mixtures containing 1 mM DPNH and other standard components.

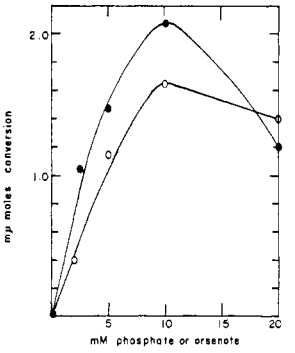

FIGURE 8: Requirement for inorganic phosphate or arsenate with DPNH as cofactor. Inorganic phosphate (solid circles) or arsenate (open circles) is absolutely required when DPNH (1 mM) was used as cofactor. Enzymes were dialyzed *vs.* 0.005 M Tris buffer, pH 7.4, for 18 hr.

DPN^+ and in its place progesterone was formed (Figure 5).

The reaction with DPNH as cofactor is inhibited strongly by 2'- and 3'-AMP and less strongly by 5'-AMP. With TPNH as cofactor, the reaction is much less sensitive to these compounds (Figures 6 and 7). 3',5'-AMP has little or no effect.

With either DPNH or TPNH as cofactor, the conversion of cholesterol to pregnenolone was greatly stimulated by inorganic phosphate (Figures 8 and 9). The optimal concentration for inorganic phosphate was *ca.* 10 mM. Higher concentrations of inorganic phosphate were inhibitory. In other experiments where the concentrations of inorganic phosphate used were higher than shown in Figures 8 and 9, it was found that 0.1 M inorganic phosphate led to essentially complete inhibition.

Discussion

Stone and Hechter (1954) demonstrated that ACTH increased the formation of corticosteroids by stimulating the conversion of cholesterol to progesterone. Several theories have been proposed concerning the mechanism of action of ACTH. In a review article, Hechter (1955) proposed that the mechanism of stimulation may reside in an increased transport of cholesterol either across cell membrane or from one subcellular compartment to another. A second hypothesis on the mode of action of ACTH, proposed by Haynes and Berthet (1957), is that ACTH increases TPNH supply which is normally the rate-limiting factor. Koritz (1962a), on the other hand, has shown that the effect of ACTH and of 3',5'-AMP cannot be entirely explained by increase in TPNH supply and has proposed that the rate-limiting step may be the conversion of pregnenolone to progesterone and that DPN^+ may be the limiting factor. Still a fourth hypothesis, proposed by Ferguson (1963) and based on the inhibition of the ACTH stimulation by puromycin, is that protein synthesis may be required for the stimulation by ACTH of adrenocorticoid output.

Although these hypotheses are all based on experi-

mental observations, none of them can explain all the known effects of ACTH, TPNH, 3′,5′-AMP, Ca^{2+}, freezing and thawing, puromycin, and hypophysectomy on the production of the corticosteroids. These effects are briefly summarized in the following paragraphs.

With intact cells, corticosteroid production is stimulated by ACTH, 3′,5′-AMP (Haynes *et al.*, 1959; Koritz, 1962b; Birmingham *et al.*, 1960; Hilton *et al.*, 1961), Ca^{2+} (Birmingham *et al.*, 1960), TPNH, and a combination of TPN^+ and G-6-P (Koritz and Peron, 1958; Koritz, 1962b). The effect of ACTH requires also glucose and Ca^{2+} (Schönbaum *et al.*, 1956; Birmingham *et al.*, 1960) and is inhibited by puromycin (Ferguson, 1963). The effect of 3′,5′-AMP is stimulated by Ca^{2+} and not inhibited by puromycin (Ferguson, 1963). TPN^+ or G-6-P is further stimulated by 3′,5′-AMP or ACTH (Koritz, 1962b). The stimulation produced by 3′,5′-AMP and ACTH is not additive (Koritz, 1962b). In ACTH treated cells, 3′,5′-AMP accumulates (Haynes, 1958), total reduced pyridine nucleotide (DPNH + TPNH) is reduced (Chance *et al.*, 1962), and protein synthesis (as measured by labeled amino acid incorporation) may or may not be enhanced (Koritz *et al.*, 1957; Scriba and Reddy, 1963).

With cell-free systems, the stimulation by ACTH is observed in unfortified extracts, but not observed in extracts already fortified and stimulated by the addition of G-6-P (Haynes and Berthet, 1957). Different TPNH regenerating systems differ widely in their ability to stimulate corticoid production. G-6-P, TPN^+, and G-6-P dehydrogenase, and to a lesser extent 6-P-gluconate, TPN^+, and 6-P-gluconate dehydrogenase are severalfold more effective than TPNH, whereas isocitrate, TPN^+, and isocitric dehydrogenase is less effective than TPNH (Koritz, 1962b; McKerns, 1962). The addition of Ca^{2+} or freezing and thawing provides additional stimulation in the presence of added G-6-P. Combination of adding Ca^{2+} and freezing and thawing does not further increase the activity beyond that caused by either of these stimulants alone, nor is the activity affected by 3′,5′-AMP or by inorganic phosphate (Koritz and Peron, 1959). Presence of DPNH, but not DPN^+, greatly inhibits corticoid production in rat adrenal homogenate (Koritz, 1962a) but not in cow adrenal homogenate (Koritz, 1962b). Puromycin has no effect in cell-free system (Ferguson, 1963).

Also pertinent to the control of corticoid production, although not directly related, one might mention the swelling of mitochondria by ACTH treatment and the correspondence of Ca^{2+} and inorganic phosphate uptake by mitochondria under certain experimental conditions (Melhuish and Greenbaum, 1961; Vasington and Murphy, 1962; Lehninger *et al.*, 1963; Rossi and Lehninger, 1963; Brierley *et al.*, 1962). The latter has not been demonstrated in adrenal cortex mitochondria but has been demonstrated in both kidney and liver mitochondria.

From the above summary of reports from the many laboratories, it is clear that the control of corticosteroid output is very complex and not well understood. In the present studies, we have sought to obtain some information on the properties of the soluble enzyme system. The results show that DPNH and TPNH can both serve efficiently as cofactor, although with very different optimal concentration. Based on the literature value of TPNH and DPNH content of adrenal mitochondria of 3×10^{-5} M each (Nichols *et al.*, 1956; Nakamura *et al.*, 1963), one may conclude that *in vivo*, TPNH is the actual coenzyme unless extremely high concentrations of DPNH exist locally due to submitochondrial compartmentalization. For optimal activity, inorganic phosphate must be present at the proper concentration. This effect of inorganic phosphate is, however, not of any physiological significance as the activity of intact mitochondria is not affected by the presence or absence of inorganic phosphate.

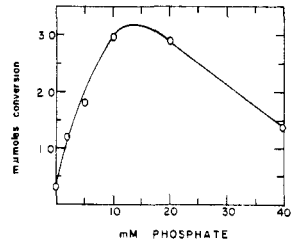

FIGURE 9: Effect of inorganic phosphate with TPNH as cofactor. TPNH (0.05 mM) with the regenerating system was used. Significant but low activity was observed in the absence of added phosphate. However, the possibility has not been excluded that this is due to the presence of inorganic phosphate in the TPNH solution. Enzymes were treated the same as in Figure 8.

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Formation of Isocaproaldehyde in the Enzymatic Cleavage of Cholesterol Side Chain by Adrenal Extract*

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ABSTRACT: The enzymatic conversion of cholesterol to pregnenolone by adrenal extracts produces isocaproaldehyde and does not normally involve 20α-hydroxy-22-ketocholesterol as an intermediate.

It is well established that the conversion of cholesterol to pregnenolone involves as intermediates 20α-hydroxycholesterol and 20α-22ξ-dihydroxycholesterol (Solomon *et al.*, 1956; Shimizu *et al.*, 1960; Constantopoulos and Tchen, 1961b; Shimizu *et al.*, 1961; Shimizu *et al.*, 1962; and Constantopoulos *et al.*, 1962). The conversion of the latter compound to pregnenolone requires TPNH[1] and we have postulated in preliminary communications that it proceeds *via* an oxygenase type reaction to yield pregnenolone and isocaproaldehyde (Constantopoulos *et al.*, 1962). On the other hand, attempts to recover isocaproaldehyde from reaction mixtures in the absence of carrier aldehyde have been unsuccessful (Lantos *et al.*, 1964) and the involvement of 20-hydroxy-22-ketocholesterol, which has been shown to be convertible to pregnenolone at a slower rate than 20α-22ξ-dihydroxycholesterol (Shimizu *et al.*, 1962), has not been rigorously excluded. We wish to report here the details of the experimental results on the formation of isocaproaldehyde and additional results obtained

* From the Department of Chemistry, Wayne State University, Detroit, Michigan. *Received December 17, 1966.* Supported by grants (HE 04139 and AM 05384) from the U. S. Public Health Service and taken in part from theses submitted by G. Constantopoulos and P. S. Satoh to Wayne State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary report has appeared previously (Constantopoulos and Tchen, 1961b). All reprint requests should be addressed to the last author.

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[1] Abbreviations used: TPNH, reduced triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide.