The Activation of the Δ^5-3-Keto Steroid Isomerase in Rat Adrenal Small Particles by Diphosphopyridine Nucleotides*

Nancy L. Oleinick† and Seymour B. Koritz

ABSTRACT: The Δ^5-3-keto steroid isomerase of rat adrenal small particles is stimulated to a high degree by concentrations of diphosphopyridine nucleotide (DPN^+) or reduced DPN^+ (DPNH) as small as 5×10^{-7} M. A large number of natural nucleotides was tested and only triphosphopyridine nucleotide (TPN^+) and reduced TPN^+ (TPNH) showed any activity at concentrations several orders of magnitude greater than that for DPN^+ and DPNH. Both diphosphopyridine nucleotides appear to activate the enzyme and not to function as electron donors or acceptors. They do not prevent a dilution inactivation; however, they do protect the enzyme against thermal and acid denaturation. This protection at acid pH values may be partially responsible for an observed shift in pH optimum to lower values as the DPN^+ or DPNH concentration is raised. The interaction between DPN^+ and DPNH and the enzyme seems to take place at some locus other than the active site, since the activation, but not the unstimulated, basal level of activity could be abolished by *p*-chloromercuriphenylsulfonic acid (PCMS) and by other nucleotides structurally related to DPN^+ and DPNH. The inhibition by PCMS suggests the involvement of sulfhydryl group(s) in the activation process. In this respect, the pyridine nucleotides will partially protect the enzyme against PCMS inhibition. Parallel lines are obtained upon subjecting the kinetic data for androst-5-ene-3,17-dione (Δ^5-AND) concentration at various pyridine nucleotide concentrations to Lineweaver–Burk plots. Possible mechanisms of the activation are discussed.

The conversion of pregnenolone to progesterone and of dehydroepiandrosterone to androstenedione requires the participation of two enzymes: a diphosphopyridine nucleotide (DPN^+)[1] linked 3β-hydroxy steroid dehydrogenase (Koritz, 1964; Kowal *et al.*, 1964) and a Δ^5-3-keto steroid isomerase. The latter enzyme catalyzes the isomerization of the double bond from position 5 to position 4. It has been shown to be present in mammalian tissues (Talalay and Wang, 1955; Kruskemper *et al.*, 1964) and in extracts of *Pseudomonas testosteroni* grown in the presence of testosterone (Kawahara *et al.*, 1962). The bacterial isomerase has been crystallized and extensively studied by Wang *et al.* (1963). No effect on its activity by various cofactors has been found (Wang *et al.*, 1963).

The isomerase present in beef adrenal cortex is associated primarily with the particulate fractions (Kruskemper *et al.*, 1964). It has been assumed that, similar to the enzyme from *Pseudomonas*, there was no effect of cofactors on the activity of mammalian isomerases. The data in the present communication indicate that the Δ^5-3-keto steroid isomerase in rat adrenal small particles (microsomal fraction) is stimulated to a high degree by small amounts of reduced or oxidized diphosphopyridine nucleotides and that this stimulation does not involve these substances as cofactors but rather as activators of the enzyme. In addition, other characteristics of this system are described. A preliminary report of some of this work has appeared (Oleinick and Koritz, 1965).

Experimental Section

Preparation of Rat Adrenal Small Particles (Pellet 3). Rat adrenals were excised from decapitated rats, homogenized in 0.154 M KCl, and fractionated as previously described (Koritz, 1964). The pellet 3 from 1000 mg wet weight of adrenal glands was homogenized to 10 ml in 0.154 M KCl. Amount of enzyme preparation is expressed as milliliters of this pellet 3 suspension. When dilutions were required, they were made in 0.154 M KCl just prior to use. The protein content of each preparation was determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as a standard. The pellet 3 suspension usually contained about 50 μg of protein/0.1 ml.

* From the Biochemistry Department, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania. *Received September 8, 1965.* This work was supported by Grant A-3676 END from the National Institutes of Health, U. S. Public Health Service.

† This report is from the Ph.D. dissertation of Nancy L. Oleinick, predoctorate fellow at the University of Pittsburgh.

[1] The abbreviations used are the following: DPN^+, diphosphopyridine nucleotide; DPNH, reduced DPN^+; Δ^5-AND, androst-5-ene-3,17-dione; Δ^4-AND, androst-4-ene-3,17-dione; 3′,5′-AMP, adenosine 3′,5′-cyclic phosphate; 2′,3′-CMP, cytidine 2′,3′-cyclic phosphate; 2′,3′-GMP, guanosine 2′,3′-cyclic phosphate; 2′,3′-UMP, uridine 2′,3′-cyclic phosphate; PCMS, *p*-chloromercuriphenylsulfonic acid; APA, adenosine 5′-phosphoramidate; ADPR, adenosine 5′-diphosphoribose; BSA, bovine serum albumin; NMN, nicotinamide mononucleotide; FMN, flavine mononucleotide; FAD, flavin-adenine dinucleotide. The analogs of DPN^+ are designated 3-acetylpyridine-DPN^+ and 3-pyridinealdehyde-DPN^+. All other abbreviations are in accord with IUPAC rules.

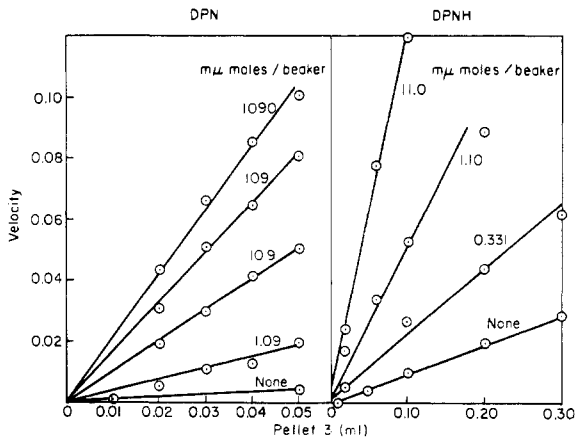

FIGURE 1: Stimulation of isomerase activity by DPN^+ and DPNH. The standard incubation mixture included 60 μmoles of potassium phosphate buffer, pH 7.0, the indicated amounts of DPN^+ or DPNH, and various concentrations of pellet 3. These were incubated for 10 min at 37°.

Preparation of Androst-5-ene-3,17-dione. Androst-5-ene-3,17-dione (Δ^5-AND) was synthesized from dehydroepiandrosterone (androst-5-ene-3β-ol-17-one) by the method of Nes *et al.* (1963) as modified by G. C. Karaboyas (personal communication). Spectro Grade acetone was used without prior refluxing with CrO_3^- reagent. The CrO_3^- reagent was added dropwise over a 10-min period to the steroid solution kept at 10–15° and under nitrogen. The product was purified on an alumina column as described by Nes *et al.* (1963). The purity of the fractions from the column was determined in the following manner. The absorbancy at 240 mμ of ethanolic solutions of known concentration of material from each fraction was measured before and after isomerization with strong base. The difference in absorbancy is a measure of Δ^5-3-keto steroid present in the sample. The total absorbancy after isomerization, a measure of the combined Δ^5- and Δ^4-AND present in the fraction, corresponded to a molar extinction coefficient of 16,300, which had been found for authentic samples of Δ^4-AND. Fractions which were 90% or better Δ^5-AND were pooled. The remaining 10% or less could be accounted for as isomerized Δ^4-AND. Usually, only the first three 3-ml fractions were saved. The product was stored at −20°.

Standard Incubation Procedure. All incubation mixtures were prepared in 20-ml beakers. Standard additions included 90 μmoles of potassium phosphate buffer, pH 7.0, 0.5 mg of BSA (bovine serum albumin), various amounts of DPN^+ or DPNH, 0.175 μmole of Δ^5-AND in 0.05 ml of methanol, 0.01–0.30 ml of pellet 3, and 0.154 M KCl to a final volume of 2.0 ml. Enzyme, substrate, and any other labile substances were added to the beakers on ice. Δ^5-AND was always added last, after the enzyme, to lessen somewhat the nonenzymatic isomerization. Incubation was for 10 min in a Dubnoff metabolic shaking incubator at 23, 30, or 37°. After the 10-min reaction time, the beakers were placed on ice, and a 1.5-ml aliquot was transferred to a glass-stoppered extraction tube containing 1.5 ml of dichloromethane. The tubes were shaken vigorously for 1 min and centrifuged to separate the layers. The top aqueous layer was removed by aspiration, and the lower dichloromethane layer was read at 240 mμ in a Beckman DU spectrophotometer. No more than 0.175 μmole (50 μg) of Δ^5-AND was ever employed, because just beyond that point, the limit of solubility of the steroid in 2.0 ml of H_2O was approached. Velocity is expressed as micromoles of Δ^4-AND formed per beaker per 10 min. The determinations were reproducible to ±0.0006 μmole of Δ^4-AND formed.

Boiled Enzyme Controls. It was always necessary to correct for nonenzymatic isomerization of the substrate, and this was accomplished by running boiled enzyme controls for each experiment. All values reported are corrected. The spontaneous isomerization varied with temperature. The following are ranges usually encountered: 23°, 0.0075–0.010 μmole; 30°, 0.010–0.015 μmole; 37°, 0.015–0.020 μmole/beaker, when 0.175 μmole of Δ^5-AND was present.

More extensive controls were required for measurement of the K_m for Δ^5-AND. The time course of the spontaneous isomerization takes the form of an initial burst (which includes the presence of some Δ^4-AND already in the preparation) and thereafter a much slower, steady reaction. It was, therefore, apparent that the actual substrate concentration at zero time of incubation would be slightly less than that added, some substrate having been converted to product. A partial correction was made by running two boiled enzyme controls at each concentration of Δ^5-AND. The first was not incubated, but was immediately extracted into dichloromethane as described above. The amount of Δ^4-AND appearing in this control was subtracted from the amount of Δ^5-AND initially added to the experimental beaker to give the actual substrate concentration at zero time. The second control was identical with the first, but was incubated along with the experimental beaker and was used to correct the velocity value (micromoles of Δ^4-AND formed per beaker per 10 min). All values in the kinetic experiments are corrected according to these two controls.

Materials

The following substances were purchased from the Sigma Chemical Co., St. Louis, Mo.: DPN^+, DPNH, TPN^+, TPNH, ATP, ADP, AMP, 3′,5′-AMP, NMN, CTP, GTP, UTP, FMN, FAD. Adenosine 5′-phosphoramidate, 3′,5′-AMP, 2′,3′-CMP, 2′,3′-GMP, 2′,3′-UMP, and glutathione were purchased from Schwarz BioResearch Inc., Mt. Vernon, N. Y.; ADPR, adenosine, 3-acetylpyridine-DPN^+, 3-pyridinealdehyde-DPN^+, GDP, CMP, GMP, UMP, PCMS, and the 2′- and 3′-mixed isomers of CMP, GMP, and UMP from Pabst Laboratories, Milwaukee, Wis.; EDTA from Merck and Co., Inc., Rahway, N. J.; iodoacetamide and

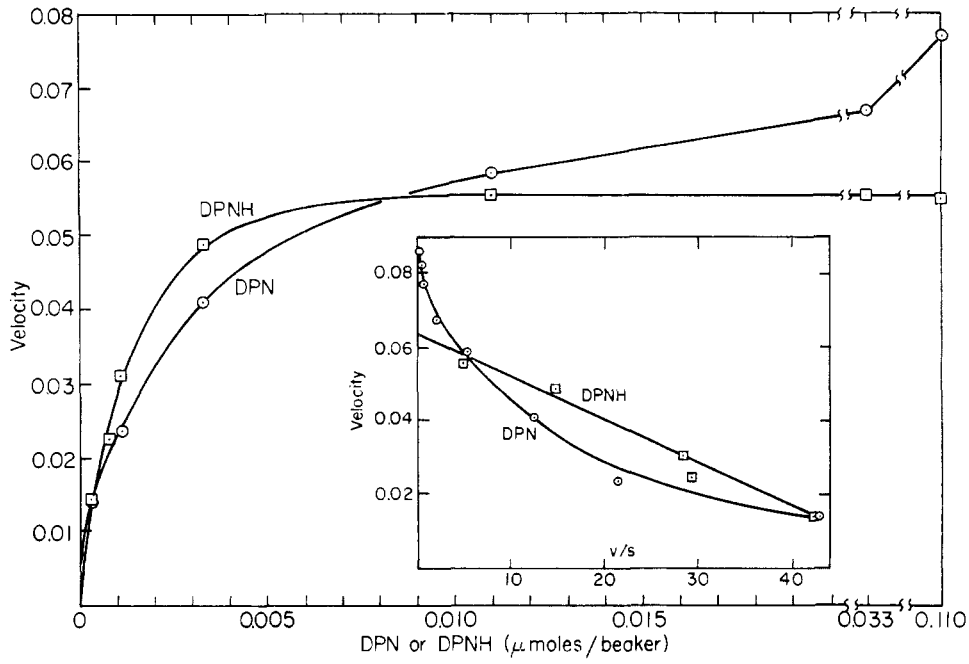

FIGURE 2: Relative effects of DPN^+ and DPNH on isomerase activity. The standard incubation mixture contained DPN^+ or DPNH in the amounts indicated and 0.08 ml of pellet 3. Incubation was at 23° for 10 min. ⊙-⊙ = DPN^+, ⊡-⊡ = DPNH.

L-cysteine hydrochloride from Nutritional Biochemicals Corp., Cleveland, Ohio; 2-mercaptoethanol from Eastman Organic Chemicals, Rochester, N. Y.; and 5-androsten-3β-ol-17-one from Steraloids Inc., Queens, N. Y. Alcohol dehydrogenase was purchased from Worthington Biochemicals Corp., Freehold, N. J., and lactate dehydrogenase from C. F. Boehringer and Soehne, New York, N. Y.

Results

Figure 1 shows the stimulation of isomerase activity by DPN^+ or DPNH. Concentrations of DPN^+ as low as 5.5×10^{-7} M and of DPNH as low as 1.0×10^{-7} M were found to be effective. Hence, it is only catalytic amounts of either pyridine nucleotide which are required. The isomerase activity in the absence of exogenous pyridine nucleotide is shown in the lowest curve of each set in Figure 1. In all cases, with or without the stimulatory agent, initial velocity is linear with concentration of the small particle pellet.

It was possible that interference might be encountered in the form of substrate removal in the presence of DPNH by the 3β-hydroxy steroid dehydrogenase known to be present in rat adrenal small particles (Koritz, 1964). The pellet 3 was therefore assayed for conversion of dehydroepiandrosterone (androst-5-en-3β-ol-17-one) to androstenedione, the final product of the combined dehydrogenase and isomerase activities. The reaction mixture contained 1.10 μmoles of dehydroepiandrosterone, 0.5 mg of BSA, 60 μmoles of phosphate buffer, pH 7.0, or 60 μmoles of Tris buffer, pH 8.6, various

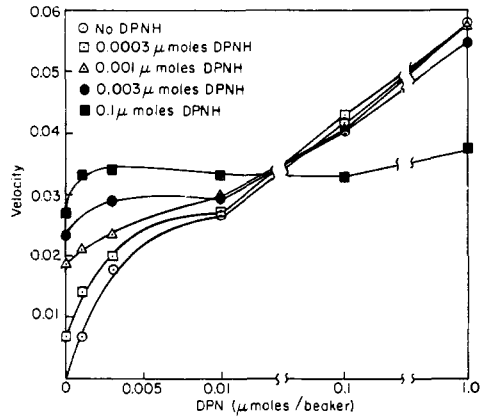

FIGURE 3: The effect of DPNH on the DPN^+ activation of isomerase. The standard incubation mixtures contained the indicated amounts of DPN^+ and/or DPNH, added at the same time, and 0.02 ml of pellet 3. The beakers were incubated for 10 min at 37°.

amounts of pellet 3, and 0.154 M KCl to a final volume of 2.0 ml. At neither pH could any significant dehydrogenase activity be detected below 0.075 ml of the pellet 3 suspension. At pH 7.0, even at 0.30 ml of pellet 3, the largest amount of enzyme protein ever used in the experiments described in this paper, there was less dehydrogenase activity than the unstimulated isomerase activity. At pH 8.6, the increase in dehydrogenase activity with increasing pellet 3 concentration is more pro-

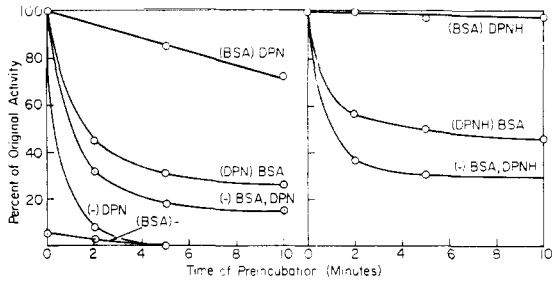

FIGURE 4: The protection against isomerase inactivation by BSA and the activation by pyridine nucleotides. For the preincubation, all beakers contained 60 μmoles of potassium phosphate buffer, pH 7.0, pellet 3 (0.02 ml for the DPN^+ set of beakers and 0.03 ml for the DPNH set), and 0.154 M KCl in an amount such that a total volume of 2.0 ml would be obtained for the final incubation. In addition, various beakers contained 1.1 μmoles of DPN^+, 0.11 μmole of DPNH, or 0.5 mg of BSA as designated by the notations inside the parentheses. After the indicated time of preincubation at 37°, all beakers were placed on ice, and further additions in the amounts given above were made as designated by the notations outside the parentheses. The substrate, 0.175 μmole of Δ^5-AND, was added, and all beakers were incubated for 10 min at 37°. Original activity is the activity of an incubation mixture which had no preincubation, but was assayed in the presence of BSA and DPN^+ or DPNH.

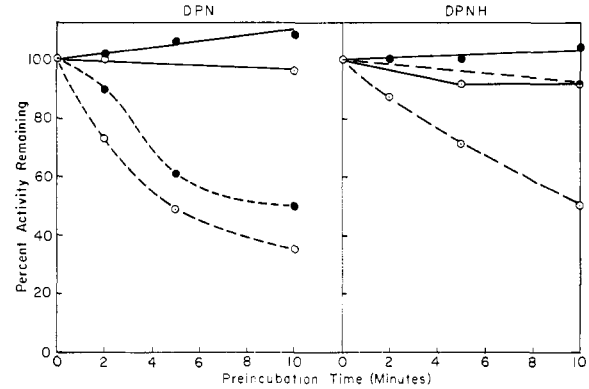

FIGURE 5: Protection by pyridine nucleotides against thermal inactivation. Initially, all beakers contained 90 μmoles of Tris buffer, pH 7.0, 0.5 mg of BSA, 0.02 ml of pellet 3, and sufficient 0.154 M KCl so that a final volume of 2.0 ml would be obtained after all additions have been made. Half of the beakers contained in addition either 1.1 μmoles of DPN^+ or 0.11 μmole of DPNH as indicated in the figure. All beakers were incubated with shaking for the times shown at 37 or 42°, then placed on ice. DPN^+ or DPNH was added to the appropriate beakers so that all beakers now contained either 1.1 μmoles of DPN^+ or 0.11 μmole of DPNH. Δ^5-AND (0.175 μmole) was added, and the usual 10-min incubation was performed at 37°. ● = preincubation with DPN^+ or DPNH, ⊙ = preincubation without DPN^+ or DPNH, ——— = preincubation at 37°, --- = preincubation at 42°.

nounced. Even so, there is rarely any set of conditions in which the dehydrogenase could interfere. When DPN^+ is used as the stimulating agent for isomerase, there would be no steroid substrate present for the dehydrogenase in the direction $DPN^+ \rightarrow$ DPNH. When the isomerase-stimulating agent is DPNH, the levels of DPNH are usually so low as to make the very low dehydrogenase activity negligible.

Consideration was also given to the possibility that only one form of the pyridine nucleotide was active and that the other exerted its effect by first being converted to the active form. This seems unlikely, due to two observations. First, no reversal of this postulated conversion process could be demonstrated. When isomerase activity was measured in the presence of DPN^+, addition of excess pyruvate and lactate dehydrogenase, in amounts sufficient to immediately oxidize any DPNH formed, had no effect on the activity. The reverse experiment was more easily performed with alcohol dehydrogenase. In the presence of ethanol and with DPNH as isomerase activator, sufficient enzyme was added so that 0.1 μmole of DPN^+ could be reduced within 25 sec. Again, no effect on the activity of the isomerase could be found. Second, at low concentrations, DPNH is more effective than DPN^+, while the reverse is true at high concentrations (Figure 2). A comparison of the shapes of the two concentration curves shows that they are inconsistent with the conversion of one (inactive) form into the other (active) form.

Examination of Figure 2 reveals further that activity increases rapidly with increasing DPNH concentration, but reaches a maximum at a rather low concentration. On the other hand, the DPN^+ stimulation increases more slowly as the concentration is raised, reaching no maximum velocity in the range of concentrations employed. This is shown even more clearly in the *v vs. v/s* plot (Figure 2, insert). An apparent K_m for DPNH was calculated to be 1.7×10^{-7} M. No such calculation could be made for DPN^+. Better lines were obtained with *v vs. v/s* plots than with Lineweaver–Burk plots for DPN^+ and DPNH concentration curves, due to the large range of concentrations employed.

It was of interest to consider the effect on isomerase activity of adding the two pyridine nucleotides simultaneously. Figure 3 shows that at the lowest concentrations of both DPN^+ and DPNH, stimulation is nearly additive. However, at higher concentrations, the resultant effect is much less than additive. Note that at a concentration of 0.1 μmole of DPNH/beaker + 0.001 μmole of DPN^+/beaker, saturation of the enzyme is reached. Increasing the DPN^+ concentration 1000-fold does not further enhance the activity.

The isomerase activity in the small particle pellet is very stable for several days in the cold as long as the suspension is kept concentrated. Upon dilution, however, the enzyme is rapidly inactivated. It was found that

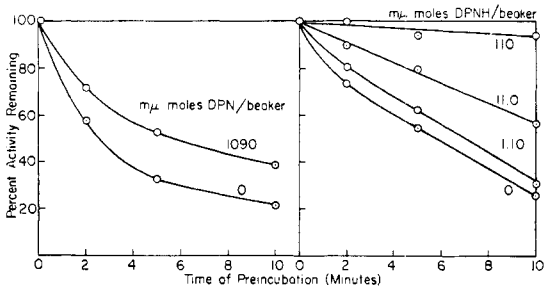

FIGURE 6: Protection by pyridine nucleotides against acid inactivation. For the preincubation, each beaker contained 15 μmoles of acetate buffer, pH 4.73, 0.5 mg of BSA, 0.02 ml of pellet 3, the indicated amounts of DPN^+ or DPNH, and sufficient 0.154 M KCl so that a final volume of 2.0 ml would be obtained when all additions had been made. Beakers were preincubated with shaking for the times shown at 30°, then placed on ice. Potassium phosphate buffer (90 μmoles), pH 6.5, was added immediately, bringing the pH of the mixture to about 6.4. Sufficient DPN^+ or DPNH was then added to the appropriate beakers to equalize the concentrations within a set; *i.e.*, all beakers in the DPN^+ set now contained 1.09 μmoles of DPN^+, while all those in the DPNH set contained 0.11 μmole of DPNH. Finally, 0.175 μmole of Δ^5-AND was added, and the usual 10-min incubation was performed at 37°.

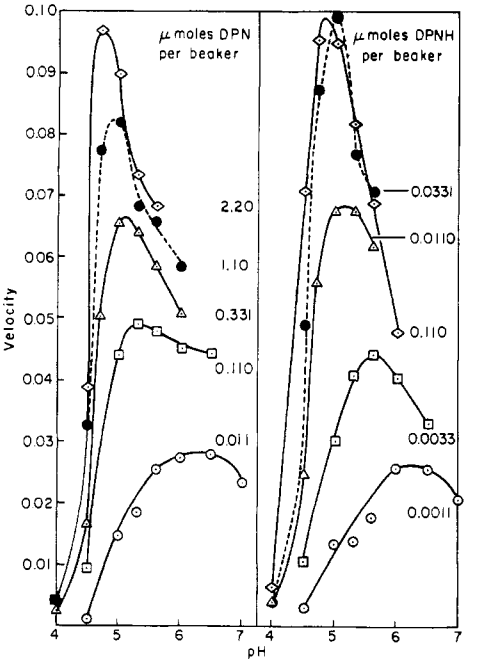

FIGURE 7: Variation of pH optimum with pyridine nucleotide concentration. The incubation mixtures contained 0.5 mg of BSA, 90 μmoles of the appropriate buffer, the indicated concentrations of DPN^+ or DPNH, 0.175 μmole of Δ^5-AND, pellet 3 (0.02 ml for the DPN^+ set of beakers and 0.03 ml for the DPNH set), and 0.154 M KCl to a final volume of 2.0 ml. Acetate buffers were used for pH 4.0–5.6; phosphate for pH 6.0–7.0. In other experiments, where buffer overlaps were used, no significant buffer effect was found.

this inactivation is prevented by bovine serum albumin, hence the inclusion of BSA in the standard assay mixture. The question then arose whether the observed stimulation of isomerase activity by the pyridine nucleotides was in fact a stimulation or merely a prevention against inactivation at the dilution used for assay. The experiment described in Figure 4 shows that a true stimulation was found. The substances given in parentheses in each case were present during a 10-min preincubation; those substances outside parentheses were added for the assay, so that the final incubation mixture contained the sum of the substances. Preincubation with BSA effects a marked protection against inactivation, but unless a pyridine nucleotide is present during the assay, negligible activity is seen. Preincubation with DPN^+ or DPNH exerts some protection over those reaction mixtures containing no added substance, but the protection is far less than that observed with BSA. In each case DPNH curves are higher than the corresponding curve with DPN^+. This is a function both of comparative concentrations and probably of the superior protection by DPNH during the 10-min assay. It is apparent, however, that the pyridine nucleotides are functioning as stimulators and not as protectors against inactivation.

In addition to acting as activating agents, DPN^+ and DPNH function to protect the enzyme against thermal and acid inactivation (Figures 5 and 6). The isomerase activity is stable at pH 7.0 and 37° when protected by BSA in the presence or absence of DPN^+ or DPNH. Figure 5 shows that upon increasing the temperature to 42°, inactivation takes place, and this can be partially prevented by DPN^+ and completely by DPNH. The enzyme is also unstable at acid pH values, but again its inactivation can be partially prevented by the presence of DPN and almost completely by DPNH (Figure 6). As in the case of activation at pH 7.0 and 37°, DPNH appears to be more effective, acting more efficiently at lower concentrations than DPN^+.

The pH dependence of the isomerase is a function of the concentration of added pyridine nucleotide (Figure 7). The apparent pH optimum of the enzyme is not constant, but appears to shift toward more acid values as the concentration of DPN^+ or DPNH is increased. The pH optimum appears to be about 6.5–7.0 for the unstimulated activity and about 4.7–4.9 for the maximally stimulated activity. To eliminate acid denaturation during most experiments, the standard incubation conditions are at pH 7.0. At the acid pH values, the observed activity is probably a combination both of enhanced activity and of additional protection of the enzyme by pyridine nucleotides against inactivation.

It was further noted that the apparent pH optimum for a fixed pyridine nucleotide concentration shifted

TABLE I: Relative Effectiveness of DPN^+, DPNH, TPN^+, and TPNH as Isomerase Activators.[a]

Pyridine Nucleotide Concn (M)	Velocity			
	DPN^+	DPNH	TPN^+	TPNH
3×10^{-7}	0.00761	0.0101		
10^{-6}	0.0128	0.0163		
3×10^{-6}	0.0205	0.0178		
10^{-5}	0.0307	0.0199		0.00332
3×10^{-5}	0.0346	0.0187	0.00466	0.00307
10^{-4}	0.0392	0.0190	0.00528	0.00551
3×10^{-4}			0.00981	0.0122
10^{-3}			0.0184	0.0174
2×10^{-3}			0.0257	0.0206

[a] The standard incubation mixtures contained 0.03 ml of pellet 3 and various amounts of the nucleotides. Incubation was for 10 min at 23°.

toward the acid pH range as the temperature of the incubation was lowered. For example, when the concentration of DPN^+ was 5.5×10^{-6} M with 0.04 ml of pellet 3, the apparent pH optimum was 6.5 at 37°, 6.2 at 30°, 6.0 at 22°, and 5.5 at 15°. These observations are consistent with the pH shift being due at least partially to enzyme stabilization.

The activation of the isomerase is relatively specific for DPN^+ and DPNH. The following group of structurally related compounds have no significant effect as activators or as inhibitors of the DPN^+ or DPNH activation at final concentrations of 10^{-3} M: NMN, ATP, ADP, AMP, adenosine, CTP, GTP, UTP, GDP, 5′-CMP, 5′-GMP, 5′-UMP, the 2′- and 3′-mixed isomers of CMP, GMP, and UMP, 2′,3′-CMP, 2′,3′-GMP, 2′,3′-UMP, and FMN.

Less effective activators are TPN^+, TPNH, 3-acetylpyridine-DPN^+, and 3-pyridinealdehyde-DPN^+. Evaluation of the activity of the two DPN^+ analogs is difficult, because they also catalyze the isomerization nonenzymatically, but it appears that as enzyme activators they are at least one to two orders of magnitude less effective. Furthermore, it is difficult to determine whether or not these substances act as inhibitors of DPN^+ and DPNH, since they are also activators. However, a comparison of the relative reactivities of the di- and triphosphopyridine nucleotides is given in Table I. From a graphical representation of these data, it may be found that to reach a velocity of 0.0160 μmole of Δ^4-AND/beaker per 10 min, it takes 10^{-6} M DPNH or $10^{-5.9}$ M DPN^+, but $10^{-3.2}$ M TPN^+ or $10^{-3.1}$ M TPNH. Therefore, TPN^+ and TPNH are less effective activators by a factor of approximately 10^3. This activity could conceivably be accounted for by a 0.1% contaminant of DPN^+ or DPNH.

A third class of structurally related compounds are those which are inactive as stimulating agents by themselves, but are inhibitors of the activation by DPN^+ and DPNH. These are: 3′,5′-AMP, ADPR, FAD, and adenosine 5′-phosphoramidate. Data concerning the inhibition are summarized in Table II. The inhibition appears to decrease as the concentration of pyridine nucleotide increases. It is also noteworthy that the inhibition is never complete; rather, it is only the DPN^+ or DPNH activation that is inhibited, while the unstimulated, basal level of activity remains unchanged (Table III). To see almost complete inhibition of the pyridine nucleotide activation it is necessary to adjust experimental conditions so that high levels of inhibitor and low levels of DPN^+ and DPNH are present. This is apparently due to the ready reversibility of the inhibition by DPN^+ and DPNH. In addition, preincuba-

TABLE II: Inhibition of DPN^+ and DPNH Stimulation by Nucleotides.[a]

Inhibitor	Concn (M)	% Inhibition			
		DPN^+		DPNH	
		10^{-5} M	10^{-4} M	10^{-6} M	10^{-5} M
3′,5′-AMP	4×10^{-4}	38.9	14.7		
3′,5′-AMP	5×10^{-4}			35.5	11.7
3′,5′-AMP	10^{-3}			48.2	18.5
ADPR	4×10^{-4}	51.5	31.0		
ADPR	5×10^{-4}			44.6	11.2
ADPR	10^{-3}			52.1	16.2
FAD	5×10^{-4}	21.6	17.9	29.8	12.5[b]
		5×10^{-6} M	5×10^{-5} M	5×10^{-7} M	5×10^{-6} M
APA	10^{-3}	23.4	0	37.6	8.2

[a] The standard incubation mixture contained various amounts of nucleotides and 0.10 ml of pellet 3 (except for the conditions of APA or of FAD + DPNH in which cases 0.05 ml of pellet 3 was used). Incubation was for 10 min at 23°.
[b] 3×10^{-6} M DPNH.

TABLE III: Insensitivity of the Basal Level of Isomerase Activity to Inhibition by Certain Nucleotides.[a]

Assay Additions	Velocity				
		Preincubation Additions			
		ADPR	3′,5′-AMP	FAD	APA
	0.0069	0.0066	0.0079	0.0076	0.0071
DPN^+ (2.5×10^{-7} M)	0.0203	0.0077	0.0096	0.0116	0.0103
DPNH (5×10^{-7} M)	0.0119	0.0071	0.0076	0.0084	0.0085

[a] For the preincubation, beakers contained 90 μmoles of potassium phosphate buffer, pH 7.0, 0.5 mg of BSA, 0.1 ml of pellet 3. The nucleotides given under Preincubation Additions had a final concentration of 10^{-3} M, and 0.154 M KCl so that a final volume of 2.0 ml would be obtained after all additions had been made. After the 10-min preincubation at 23°, all beakers received DPN^+ or DPNH as indicated under Assay Additions and 0.175 μmole of Δ^5-AND in 0.05 ml of methanol. Final incubation was at 23° for 10 min. Additional controls in the absence of added steroid substrate with pellet 3 which had not been boiled gave analytical values identical with those obtained when boiled enzyme was used under these conditions.

tion of the enzyme with the inhibiting nucleotides gives a more profound inhibition.

The activity of the isomerase is unaffected by 10^{-3} M EDTA. Zn^{2+} or Fe^{3+} at final concentrations of 10^{-3} M are highly inhibitory, about 90% of the activity having been lost. The enzyme is also inhibited by 10^{-5} M Cu^{2+}, but evaluation of the exact magnitude of this inhibition is difficult because Cu^{2+} alone catalyzes the isomerization of Δ^5-AND nonenzymatically. Fe^{2-}, Ca^{2+}, Mn^{2+}, and Mg^{2+}, each at 10^{-3} M, had negligible effects on the enzyme activity. All metal ions were tested in the presence of Tris buffer, pH 7.0, in place of phosphate.

The thiol compounds, β-mercaptoethanol, glutathione, and L-cysteine, gave only very slight stimulations to the activity at concentrations of 10^{-4} and 10^{-3} M. In most cases, there was no effect at all. On the other hand, the enzyme is markedly inhibited by PCMS at a concentration as low as 10^{-5} M. The inhibition is about 90% at a PCMS concentration of 10^{-4} M. The above thiol compounds will reverse the inhibition.

Preincubation with DPN^+ or DPNH prevents some of the PCMS inhibition, as shown in Table IV. When the PCMS is added at the same time as the pyridine nucleotide, the inhibition is not as great as when the enzyme is preincubated with PCMS. It appears that a residual 10–12% of activity is not inhibited even by increased PCMS concentrations or by preincubation with PCMS. It is possible that this basal level of activity is that present in the unstimulated pellet 3 and that it is only the activation by pyridine nucleotide that is PCMS sensitive. This was tested with larger amounts of pellet 3 protein, so that a greater amount of unstimulated activity would be present (Table V). In each case, the stimulation by the pyridine nucleotide was abolished by preincubation with PCMS, but the unstimulated basal activity was not affected.

The kinetics of the activation of the Δ^5-3-keto steroid isomerase by DPN^+ and DPNH are shown in Figures 8 and 9. The apparent K_m for the substrate, Δ^5-AND, is dependent on the concentrations of DPN^+ or DPNH and on the concentration of pellet 3 protein. The parallel lines obtained upon subjecting the data to double reciprocal plots are indicative of an "uncompetitive" or "anticompetitive" activation.

TABLE IV: PCMS Inhibition of Isomerase Activity and Its Partial Prevention by DPN and DPNH.[a]

	% Inhibition (M)			
	DPNH		DPN^+	
	5×10^{-7}	5×10^{-6}	5×10^{-6}	5×10^{-5}
I. No preincubation				
PCMS 10^{-5} M	64	66	75	74
3×10^{-5} M	77	74	85	88
II. Preincubation with DPN^+ and DPNH				
PCMS 10^{-5} M	44	31	58	52
3×10^{-5} M	60	45	72	69
III. Preincubation with PCMS				
PCMS 10^{-5} M	76	86	87	91
3×10^{-5} M	78	88		92

[a] Standard incubation conditions were employed, except that a 10-min preincubation at 23° of all additions except PCMS and Δ^5-AND was performed in case II, and of all additions but pyridine nucleotide and Δ^5-AND in case III.

Discussion

It now seems clear that the increase in Δ^5-3-keto steroid isomerase activity in the presence of DPN^+ or DPNH is an activation rather than a protective effect against denaturation at the dilutions commonly used for assay (Figure 4). When the enzyme is preincubated

TABLE V: Insensitivity of the Basal Level of Isomerase Activity to Inhibition by PCMS.[a]

Additions	Velocity (μmoles of Δ^4-AND/beaker per 10 min)		
	Pellet 3 (ml)		
	0.1	0.2	0.3
...	0.0089	0.0167	0.0251
... + 3×10^{-5} M PCMS	0.0098	0.0183	0.0254
5×10^{-6} M DPN^+	0.0950	0.139[b]	0.141[b]
5×10^{-6} M DPN^+ + 3×10^{-5} M PCMS	0.0109	0.0192	0.0265
5×10^{-7} M DPN^+	0.0383	0.0702	0.0906
5×10^{-7} M DPN^+ + 3×10^{-5} M PCMS	0.0102	0.0187	0.0264
5×10^{-7} M DPNH	0.0479	0.0781	0.0992
5×10^{-7} M DPNH + 3×10^{-5} M PCMS	0.0106	0.0195	0.0266
5×10^{-8} M DPNH	0.0136	0.0251	0.0358
5×10^{-8} M DPNH + 3×10^{-5} M PCMS	0.0110	0.0198	0.0264

[a] In each case in which PCMS is present, the enzyme and PCMS were preincubated together for 10 min at 23° as in Table IV. Otherwise, the standard incubation was carried out for 10 min at 23°. [b] These values represent a nearly complete transformation of the substrate to the product.

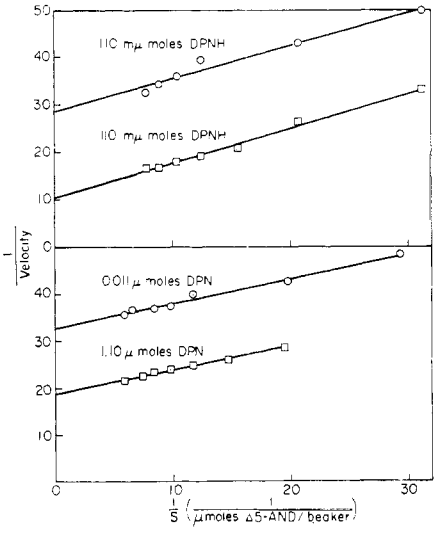

FIGURE 8: Dependence of K_m for Δ^5-AND on pyridine nucleotide concentration. Each reaction mixture contained 90 μmoles of potassium phosphate buffer, pH 7.0, 0.5 mg of BSA, 0.08 ml of pellet 3, the indicated amounts of Δ^5-AND in 0.1 ml methanol, DPN^+ or DPNH in the amounts shown, and 0.154 M KCl to a total volume of 2.0 ml. Incubation was at room temperature, 23°, for 10 min. Boiled enzyme controls were run at zero time and at 10 min.

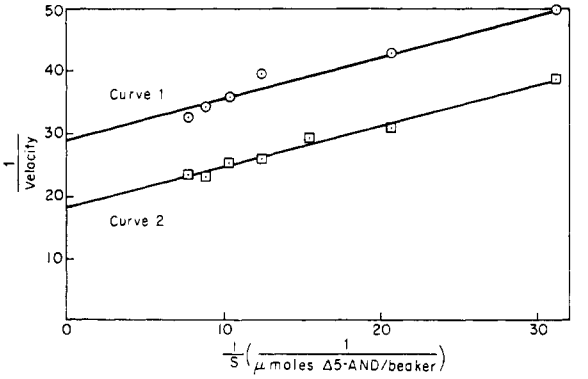

FIGURE 9: Dependence of K_m for Δ^5-AND on pellet 3 concentration. Reaction mixtures were identical with those of Figure 8, except that all beakers contained 1.10 mμmoles of DPNH, and beakers for curve 1 contained 0.08 ml and for curve 2, 0.12 ml of pellet 3.

at pH 7.0 and 37° in the absence of BSA or pyridine nucleotides, inactivation is rapid and is complete at 5 min. Even when DPN^+ is added for assay, less than 10% of the control activity is seen with enzyme which has had as little as 2 min of preincubation without BSA. The presence of DPN^+ or DPNH during the preincubation gives some protection, but it is small compared with that exerted by BSA. However, even when the enzyme is protected by BSA, full activity is measured only if pyridine nucleotide is added for assay. The function of DPN^+ and DPNH as activators and not as protectors against dilution inactivation is, therefore, apparent.

The evidence presented indicates that both DPN^+ and DPNH are activators and not that one of these pyridine nucleotides is active and the other becomes active by conversion into this form. If, for example, DPNH were being converted to DPN^+, and if DPN^+ were the active form, then addition of excess ethanol and alcohol dehydrogenase should inhibit the activation by removal of DPN^+. In like manner, excess pyruvate and lactate dehydrogenase should act by removing any

DPNH formed when DPN^+ is added as activator. In both cases, however, these substances had no effect at all on the DPN^+ or DPNH activation. Furthermore, the comparative shapes of the curves in Figure 2 confirm this conclusion. Since DPNH effects a greater stimulation at low concentrations, while DPN^+ is the better activator at high concentrations, activation of the enzyme by the conversion of one form to the other is doubtful.

The stimulation could be due to DPN^+ and DPNH acting as cofactors, since the biochemical reaction catalyzed by the Δ^5-3-keto steroid isomerase of the small particles of the rat adrenal gland is an internal oxidation–reduction reaction; *i.e.*, C-4 is oxidized while C-6 is reduced. In this process, a hydrogen atom is lost at C-4 and gained at C-6. The function of the pyridine nucleotide could then be to act catalytically in conjunction with the enzyme to alternately accept and donate this hydrogen. A similar mechanism has been proposed to explain the DPN^+ requirement for UDP-galactose-4-epimerase (Maxwell, 1957). However, in this latter case, DPNH, as to be expected, is an inhibitor of the enzyme, whereas it is an activator of the steroid isomerase (Figure 1). In view of the data with the alcohol and lactate dehydrogenases and that of Figure 2 which indicate that both DPN^+ and DPNH are activators, it seems unlikely that both the reduced and oxidized forms of the pyridine nucleotide could function in the same manner as electron donor–acceptors.

Since these substances do not appear to be acting as cofactors, an alternative hypothesis is that DPN^+ and DPNH act in some fashion to modify the enzyme protein, so as to cause increased reactivity of the active site. A modification of structure is suggested by two types of observations. First, the inhibition studies with PCMS and with the structurally related nucleotides are consistent with the involvement of the pyridine nucleotides at some locus on the enzyme other than the active site. Under certain conditions (Tables III and V), all of the stimulated activity could be abolished by these substances, but the unstimulated basal level of activity was not inhibited. It is noteworthy that of all the nucleotides tested for the inhibition, only those with the following partial structure in common with DPN^+ and DPNH possessed the ability to inhibit: (1) the phosphate of the nucleotide is attached to the 5′ position of adenosine; (2) there is no secondary dissociation of any phosphate present; *i.e.*, phosphate must be doubly esterified, the second esterification being to the 3′ position of ribose (as in 3′,5′-AMP), to a second doubly esterified phosphate group (as in ADPR or FAD), or to an amino group to form an ester amide (as in adenosine 5′-phosphoramidate). Esterification to a free phosphate (as in ADP) does not form a structure capable of inhibiting the DPN^+ and DPNH stimulation. The structural characteristics of these inhibitors perhaps give a picture of the requirements for binding of DPN^+ and DPNH to the protein.

The second type of evidence which suggests that the pyridine nucleotide activation is characterized by a modification in the structure of the enzyme is presented in Figures 5 and 6. Preincubation of the enzyme at 42° or at an acid pH causes a marked inactivation, which is at least partially prevented by the presence of pyridine nucleotide in the incubation medium. It appears that the enzyme–pyridine nucleotide complex is more stable to heat and to acid pH values. This protection is probably at least partially responsible for the shift in pH curves when higher amounts of pyridine nucleotide are present (Figure 7). The fully activated enzyme demonstrates an apparent pH optimum between 4.7 and 4.9. However, at suboptimal amounts of DPN^+ or DPNH, rapid inactivation of the unprotected enzyme takes place, and the observed optimum is closer to neutrality. That the shift in pH optimum is a result of protection against enzyme denaturation is also suggested by the fact that a similar shift is obtained upon lowering the temperature of the reaction. The pH curves of Figure 7 show not only a decrease in apparent pH optimum with increased pyridine nucleotide, but also an increase in velocity of the reaction at the optimum. It is obvious that the enzymatic activity is much greater at the acid pH values. This could be the result of a dissociation or association of some essential group on the protein.

The different shapes of the DPN^+ and DPNH curves given in Figure 2 invite some comments. A possible explanation for this involves the assumption that DPNH binds more tightly to the enzyme than does DPN^+, but that the enzyme–DPNH complex resulting is less active than the enzyme–DPN^+ complex. On this basis, DPNH would be more active than DPN^+ at low concentrations, and the reverse would be true at high concentrations.

This hypothesis not only explains these curves, but also accounts for two other observations. First, it was noted in the studies of acid (Figure 5) and thermal (Figure 6) inactivation that DPNH provides much more efficient protection for the enzyme than DPN^+, and, second, when the isomerization is carried out in the presence of various combinations of both DPN^+ and DPNH, the activation is essentially additive at low concentrations of both pyridine nucleotides (Figure 3). However, at nearly saturation levels of DPNH, addition of DPN^+, even in very large amounts, has no effect. The mutual interaction of the two pyridine nucleotides also suggests that these substances act at the same site on the enzyme.

The observations described in Tables IV and V indicate that sulfhydryl group(s) are in some way involved in the activation of the isomerase by the pyridine nucleotides. Thus it is seen that preincubation of the enzyme with the pyridine nucleotide results in a diminished inhibition by PCMS, while preincubation with PCMS gives a greater inhibition than when PCMS and pyridine nucleotide are added together. These data suggest several possibilities: first, these SH group(s) may actually participate in the binding of DPN^+ and DPNH; second, the PCMS-sensitive SH group(s) may be found elsewhere on the protein molecule, but reaction with PCMS may cause a conformational change in the protein, thereby inhibiting interaction of DPN^+ and

DPNH with the enzyme; and, third, interaction of the pyridine nucleotide with the enzyme may expose SH group(s) which are essential for the reaction. With the present system, it is not possible to distinguish among these or other possible explanations. However, the fact that the presence of DPN^+ or DPNH partially prevents the PCMS inhibition and that the PCMS inhibition does not at all affect the basal activity (Table V) can be interpreted to favor the first possibility. In this respect, the interaction between pyridine nucleotide and protein SH groups has been observed (Racker, 1955).

When the velocity was determined as a function of the concentration of substrate, Δ^5-AND, it was found that the apparent K_m varied both with DPN^+ and DPNH concentration (Figure 8) and with the amount of pellet 3 (Figure 9). The Lineweaver–Burk plots of the data assume the form of parallel lines, a type of behavior which has been called "uncompetitive" or "anticompetitive" activation. A kinetic interpretation of double reciprocal plots of this nature has recently been presented by Frieden (1964). According to this treatment, the activity of the enzyme is a function of the relative concentrations of enzyme–substrate and enzyme–substrate–modifier complexes. Hence, it would appear that the concentration of the latter complex should be dependent upon the amounts of enzyme and of modifier present. This might account for the effect of enzyme concentration on the double reciprocal plots noted above.

The literature contains many reports of enzymes which are modified by substances unrelated chemically to either the substrate or the product of the reaction catalyzed by that enzyme. It is unknown whether the essential features of all these modifications are similar, but it appears that many are consistent with the model proposed by Monod *et al.* (1963). Such "allosteric" enzymes are characterized by having at least two sites: an active site, at which binding of the substrate and conversion to product occur, and a second, "allosteric" site, at which certain specific small molecules bind, causing some alteration of the protein and hence of the active site The rat adrenal Δ^5-3-keto steroid isomerase possesses certain features in common with this model: *i.e.*, activation by substances structurally unrelated to the substrate; inhibition of the pyridine nucleotide activation by PCMS without affecting the unstimulated enzyme activity; inhibition of the activation by compounds which are structurally related to DPN^+ and DPNH, but which have no effect by themselves on the enzyme activity; and kinetics consistent with such a modification. However, it would be premature to catalog the enzyme as an allosteric protein until further evidence is obtained.

References

Frieden, C. (1964), *J. Biol. Chem. 239*, 3522.

Kawahara, F. S., Wang, S.-F., and Talalay, P. (1962), *J. Biol. Chem. 237*, 1500.

Koritz, S. B. (1964), *Biochemistry 3*, 1098.

Kowal, J., Forchielli, E., and Dorfman, R. I. (1964), *Steroids 3*, 531.

Kruskemper, H. L., Forchielli, E., and Ringold, H. J. (1964), *Steroids 3*, 295.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Maxwell, E. S. (1957), *J. Biol. Chem. 229*, 139.

Monod, J., Changeaux, J.-P., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.

Nes, W. R., Loeser, E., Kirdani, R., and Marsh, J. (1963), *Tetrahedron 19*, 299.

Oleinick, N. L., and Koritz, S. B. (1965), *Biochim. Biophys. Acta 99*, 394.

Racker, E. (1955), *Physiol. Rev. 35*, 1.

Talalay, P., and Wang, S.-F. (1955), *Biochim. Biophys. Acta 18*, 300.

Wang, S.-F., Kawahara, F. S., and Talalay, P. (1963), *J. Biol. Chem. 238*, 576.