Inhibition of Steroid Mediated Pyridine Nucleotide Transhydrogenase and 17 β Hydroxysteroid Dehydrogenase by 2'Adenylic Acid.* (24986)

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Kaplan *et al.*,(1) have shown that 2'-adenylic acid (2'AMP) increases the rate of bacterial transhydrogenase. Inhibition by 2'AMP of placental estradiol-17β mediated pyridine nucleotide transhydrogenase and 17-β hydroxysteroid dehydrogenase has been demonstrated(2). At pH 6.8, 17-β hydroxysteroid dehydrogenase was markedly inhibited with respect to oxidation of estradiol by DPN and less affected with respect to reduction of estrone by TPNH while reactions of estradiol with TPN and of estrone with DPNH were unaffected. Evidence will be presented regarding the specificity and mechanism of this inhibition.

Materials and methods. Transhydrogenase preparations. 20% placental homogenate was prepared from normal term human placenta in 0.25 M sucrose in Potter homogenizer by rotation of teflon pestle for 1 minute. After straining thru nylon gauze the supernatant fraction was centrifuged 45 minutes at 50,000 x g and 2°C. The supernatant was then dialyzed 12 hours against 0.005 M phosphate buffer pH 7.0. This preparation is hereafter referred to as dialyzed fraction. It contained isocitric dehydrogenase-TPN, 17β-hydroxysteroid dehydrogenase, and traces of TPN. For study of the effect of 2'AMP on acetyl pyridine DPN transhydrogenation and dehydrogenation the dialyzed fraction was freed of nucleotides by addition of equal volume of saturated ammonium sulfate. The precipitate was redissolved in ¼ volume of distilled water and dialyzed 2 hours in cold room against 0.005 M phosphate buffer. Both dialyzed and ammonium sulfate purified preparations were stored at –20°C. DPN and TPN were obtained from Sigma. Acetyl pyridine DPN was obtained from Pabst. Deoxyadenylic acid, Deoxyguanilic acid, Uridine 5'monophosphate, Guanosine 5'Triphosphate, Adenosine 3'monophosphate were obtained from California Corp. for Biochemical Research. Cytidine 2'3'cyclic phosphate and Uridine 2'3'cyclic phosphate were obtained from Schwarz Labs. When Ba salts were obtained, these were converted to sodium salts. 2'AMP was obtained from Schwarz Labs. and purified by chromatography on Dowex-1 in the formate form. The product was recrystallized from water. 2'5'diphosphoadenosine and nicotinamide mononucleotide were prepared by hydrolysis of TPN by venom diesterase according to Wang, Shuster and Kaplan(3). 2'Inosinic monophosphate (2'IMP) was prepared by modification of that used by Shuster and Kaplan(4) and purified by chromatography on Dowex-1 in the formate form. Enzymatic assay for transhydrogenation between TPNH and DPN by measurement of α-ketoglutarate produced from isocitrate has been described (5). 17β-hydroxysteroid dehydrogenase was measured at same concentration of estradiol-17β (3×10^{-6} M) as that employed in measurement of transhydrogenase. This was accomplished by measurement of formation of reduced pyridine nucleotide in an Aminco-Bowman spectrophotofluorimeter. Sensitivity of this instrument permitted reaction rates to be followed when only catalytic quantities of steroid were present. The incubation system consisted of 0.006 M phosphate buffer pH 6.8, Versene 0.001 M, estradiol-17β 3×10^{-6} M, DPN 3×10^{-4} M, $MgCl_2$ 0.006 M and 0.1 ml dialyzed enzyme. The reaction was started by addition of enzyme to the incubation mixture at room temperature and the linear reaction rate determined. The exciting wave length was 340 mμ and the fluorescent wave length 450 mμ. Assays for dehydrogenase and transhydrogenase could be duplicated within 5%.

Results. Inhibition of transhydrogenase by 2'AMP. Table I indicates that 2'AMP inhib-

* This work supported by grant from U. S. Public Health Service.

TABLE I. Inhibition of 17-β Hydroxysteroid Dehydrogenase and Estradiol Mediated Transhydrogenase by Nucleotides.

Substance	Conc., M	% inhibition	
		De-hydrogenase	Trans-hydrogenase
2'AMP	1×10^{-3}	100	95
	1×10^{-4}	99	54
	1×10^{-5}	65	0
	1×10^{-6}	6	
2'IMP	1×10^{-3}	88	97
	1×10^{-4}	82	
	2×10^{-5}	38	36
5'AMP	1×10^{-3}	50	
	1×10^{-4}	25	0
3'AMP	1×10^{-3}	33	20
	1×10^{-4}	0	16
5'UMP	1×10^{-3}	0	24
	1×10^{-4}	0	16
NMN	1×10^{-3}	40	17
	5×10^{-5}	0	0
2'5'ADP	1×10^{-3}	65	0
	5×10^{-5}	58	0

5'Deoxyguanylic, 5'deoxyadenylic acid, cytidine 2'3'cyclic monophosphate, uridine 2'3'cyclic monophosphate, 5'IMP and 5'guanosine triphosphate showed no inhibition of either enzyme at 10^{-3} M.

its dehydrogenase and transhydrogenase activity to a significant degree. The inhibition of dehydrogenase was usually somewhat more than that of transhydrogenase activity. 2'IMP also showed significant inhibition of both activities. 2'5'ADP showed significant inhibition of dehydrogenase activity but no measurable inhibition of transhydrogenation. The 5'AMP and 3'AMP were far less effective inhibitors than 2'AMP. A variety of other nucleotides showed little inhibition of either system.

Effect of 2'AMP on dehydrogenation and transhydrogenation involving acetyl pyridine DPN. Rate of transhydrogenation between DPNH and acetyl pyridine DPN (APDPN) was measured spectrophotometrically at 400 mμ. The incubation mixture consisted of 0.1 M Tris buffer pH 7.4, DPNH 0.0002 M, APDPN 0.001 M, estradiol-17β 6 x 10^{-6} M, and 0.10 ml ammonium sulfate purified placental enzyme which contained approximately 0.1 mg protein. In accordance with observations of Talalay(6) transhydrogenation between DPNH and APDPN was readily measured at 400 mμ because of negligible adsorption of the former substance. Transhydrogenation was inhibited completely at 10^{-3} M 2'AMP and 65% inhibited at 10^{-4} M. No inhibition was observed at 10^{-5} M. Oxidation of estradiol-17β by APDPN was studied at 6 x 10^{-6} M steroid, similar to that employed in the study of transhydrogenation to APDPN. The reaction was measured fluorimetrically with excitation wave length 400 mμ and fluorescence wave length 475 mμ. The incubation mixture was similar to that used for study of DPN except for the indicated steroid concentration. 2'AMP caused 60, 45 and 25% inhibition at concentrations of 1 x 10^{-4}, 1 x 10^{-5}, and 5 x 10^{-6} M respectively.

Attempts to reverse 2'inhibition. Variation of estradiol, Mg^{++}, or phosphate concentrations over a 10-fold range, or doubling DPN concentration had no effect on 2'AMP inhibition of dehydrogenation of estradiol by DPN or transhydrogenation between TPNH and DPN. Addition of nicotinamide, cysteine, or pyrophosphate did not alter inhibition in either the dehydrogenation or transhydrogenation systems.

Discussion. These observations indicate that the estrogen sensitive enzyme system in human placenta is inhibited by low levels of 2'AMP. Inhibition appears to depend on the 2' structure since 2'IMP is also an effective inhibitor while 5'IMP inhibits neither reaction. This inhibition appears fairly specific since a variety of other nucleotides are considerably less effective. Inhibition of transhydrogenation is consistently less than that for dehydrogenation. Studies by Talalay *et al.*, (6) indicate that transhydrogenation between TPNH and DPN is very dependent on TPN concentration. The lesser inhibition of transhydrogenation may be due to competition of TPN and the 2'AMP for the same site on the enzyme. 2'AMP is known to inhibit TPN enzyme systems competitively at somewhat higher concentrations than used in these studies(7). However, 2'AMP is an effective inhibitor when transhydrogenation involves DPNH and APDPN in absence of TPN. TPN completely inhibits transhydrogenase at 10^{-5} M(6), 2'AMP is a less effective inhibitor.

Appropriate hydrolysis of TPN yields nicotinamide mononucleotide, a poor inhibitor of either dehydrogenase or transhydrogenase, and 2'5' diphosphoadenosine, a substance with

considerable activity to inhibit dehydrogenase but no demonstrable effect on transhydrogenation. This latter lack of inhibition is probably due to easy displacement from the active site by TPN. These data are consistent with the identity of 17β hydroxysteroid dehydrogenase and steroid mediated pyridine nucleotide transhydrogenase proposed by Talalay and Williams-Ashman(8). If these 2 enzyme activities do not reside in the same molecule the similarity in their inhibition by 2'AMP is remarkable.

Evaluation of the role of steroid mediated transhydrogenation in slices of estrogen sensitive target organs is now in progress with the aid of 2'AMP as inhibitor of this process.

Summary. 2'AMP inhibits placental 17β hydroxysteroid dehydrogenase and steroid mediated pyridine nucleotide transhydrogenase. This inhibition is due to the 2' structure and occurs in transhydrogenation which does not involve TPN.

1. Kaplan, N. O., Colowick, S. P., Neufeld, E. F., Ciotti, M. M., *J. Biol. Chem.,* 1953, v205, 17.

2. Hollander, V. P., Hollander, N., Brown, J. D., *ibid.,* 1959, v234, 1678.

3. Wang, T. P., Shuster, L., Kaplan, N. O., *ibid.,* 1954, v206, 299.

4. Shuster, L., Kaplan, N. O., *ibid.,* 1953, v201, 535.

5. Hollander, V. P., Nolan, H., Hollander, N., *ibid.,* 1958, v233, 580.

6. Talalay, P., Hurlock, B., Williams-Ashman, H. G., *Proc. Nat. Acad. Sci.,* 1959, v44, 862.

7. Neufeld, E., Kaplan, N. O., Colowick, S., *Biochimica et Bioph. Acta,* 1955, v17, 525.

8. Talalay, P., Williams-Ashman, H. G., *Proc. Nat. Acad. Sci.,* 1958, v44, 15.

Received April 29, 1959. P.S.E.B.M., 1959, v101.

Transient Secretion of Phosphate in the Mammalian Kidney* (24987)

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Even though phosphate secretion into the renal tubule has been invoked to account for a wide variety of data(1-6), such a process has never been demonstrated reproducibly in the dog. Secretion of phosphate, demonstrated reproducibly by clearance technic, has been reported in cats and chickens. Taugner(4) reported net secretion by the cat under conditions of loading with various organic phosphates esters *i.e.,* fructose 1,6-di-phosphate, phosphoglyceric acid, and glycerophosphate. Davidson reported net secretion in the chicken after intravenous administration of parathormone(6). Such findings suggest that secretion of phosphate might occur in all mammals under certain conditions. A comprehensive working hypothesis would require that phosphate is excreted *via* glomerular filtration, tubular reabsorption, and tubular secretion. If rate of reabsorption exceeded that of secretion under steady-state conditions of phosphate loading, net secretion could not be demonstrated by conventional clearance technic. On the other hand, a secretory mechanism might respond more rapidly to transient increase of concentration of serum phosphate than would a reabsorptive mechanism. If such an event could occur, the transient excretion of phosphate would be greater than that of creatinine or of any "glomerular substance." Such considerations prompted an exploration of transient renal response to close-arterial instantaneous injection of phosphate into the renal artery. The technic used to study transient responses of dog kidney was developed by Chinard(7).

Methods. Nembutalized dogs were given priming and maintenance infusions of 1 molar mannitol containing creatinine, KH_2PO_4, and NaH_2PO_4 in amounts sufficient to maintain high steady-state levels of urine flow, and to

* Supported by research grant, N.I.H., P.H.S., by Office of Surgeon General, Dept. of Army, and by Kentucky Med. Research Comm.

† Work done during tenure of Fellowship of Am. Heart Assn.

‡ Work done during tenure of Established Investigatorship of Am. Heart Assn.