# SOME NOTES ON THE COENZYME-ACTIVITY OF PHOSPHOPYRIDOXAL DERIVATIVES FOR THE BRAIN GLUTAMIC DECARBOXYLASE

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According to Snell (1) and Braunstein (1) the aldehyde group of pyridoxal phosphate (PAL-P) is indispensable to its role as coenzyme for various $B_6$-dependent enzymes. Against this, Gonnard (2) et al. have found that acyl hydrazones of PAL-P (isonicotinic, nicotinic, picolic, p-amino-salicylic and thienylic acid hydrazones of PAL-P) act as coenzymes of glutamic-aspartic transaminase, dopadecarboxylase and kynureinase like pyridoxal phosphate, and have stated that acyl hydrazones act as such in these reactions without liberation of PAL-P because they could not detect the liberated acyl hydrazines in the reaction mixtures.

The present authors have prepared various derivatives of PAL-P and tested their coenzyme activity or anticoenzyme activity. When phosphate of PAL-P is replaced with sulfate (3), the coenzyme activity is lost and a fairly strong competitive-inhibitory action appears. When the phosphate and the formyl group of PAL-P are replaced with sulfate and hydroxymethyl or amino-methyl respectively, not only the coenzymic but also the inhibitory action disappears.

It has been already shown that the recombination of PAL-P with the apoenzymes is inhibited by 4-deoxypyridoxine-5-phosphate (1,4). We have found that hydrazone, semicarbazone and isonicotinyl hydrazone (INPP) of PAL-P can act as coenzymes of brain glutamic decarboxylase, while oxime

and unexpectedly, phenylhydrazone of PAL-P are inactive as coenzymes. The former is a fairly strong inhibitor. Therefore we have attempted to determine whether the above coenzyme-active compounds are effective as such or through the liberation of PAL-P.

For this experiment, the enzyme used was prepared as follows: 7 g of mouse brain is homogenized with 70 ml of water and centrifuged at 20,000 X g for 30 minutes. 2 ml of 0.5 M phosphate buffer (pH 7.2) and 1 ml of 1 M calcium acetate are added to the supernatant solution and the resultant precipitate of calcium phosphate gel is centrifuged off. Ammonium sulfate is added to 15% saturation followed by centrifugation at 20,000 X g for 20 minutes. To the supernatant solution ammonium sulfate is again added to 35% saturation. The resultant precipitate is dissolved in 12 ml of 0.05 M phosphate buffer (pH 6.2) and dialyzed against 1 liter of the same buffer changed three times in the cold. By the above procedure 85-90% of the PAL-P was removed from the enzyme protein. The enzyme activity is estimated with Warburg's manometer. In the main chamber are placed 1 ml of the enzyme preparation, a solution of each coenzyme dissolved in 0.2 ml of phosphate buffer and 2 ml of phosphate buffer (0.05 M, pH 6.2), and in one side arm 15mg of monosodium glutamate in 0.2 ml of water, and also, if necessary, in another side arm 0.2 ml of 3 N $H_2SO_4$ (for the estimation of $CO_2$-retention). After temperature equilibrium for 10 minutes under 100% $N_2$ at 35°C, the solution of monosodium glutamate is tipped into the main chamber and the $CO_2$ evolution is recorded every ten minutes for 60 minutes.

In the case of PAL-P maximal enzyme activity is produced with $3.3 \times 10^{-5}$M, but above $10^{-4}$M inhibition of the activity occurs. In the case of INPP the enzyme activity observed by the addition of $3.3 \times 10^{-5}$M corresponds to 65% of the activity of the same concentration of PAL-P. With $3.3 \times 10^{-4}$M of INPP the same maximal activity as with PAL-P is attained. Km of PAL-P for the apoenzyme is $5.5 \times 10^{-7}$, while that of INPP is $1.1 \times 10^{-5}$.

After the enzyme reaction with the addition of $10^{-3}$M INPP was finished, the reaction mixture was deproteinized with 20% trichloroacetic acid and

centrifuged. The supernatant solution was concentrated under diminished pressure at low temperature to a syrup and applied in a band (8 centimeters in length) to a filter paper No. 50 (Toyo Roshi Company) and developed with a solvent system of n-butanol, acetic acid and water (4 : 1 : 5) or 69% butyric acid (V/V) containing 0.85% NaOH (5). Compounds were detected by spraying with Gibbs' reagent (6). If the above coenzyme activity of INPP were due to the liberation of PAL-P from INPP, the amount of the liberated PAL-P might be expected to be enough for the detection by the above paper chromatographic procedure. But no spot other than INPP can be detectable up to 60 minutes. In case of the addition to the reaction system of INPP purified by paper chromatography, no lag of $CO_2$-evolution is seen whether INPP is preincubated with the apoenzyme for ten minutes or not. These facts seem to show that INPP itself acts as the coenzyme of glutamic decarboxylase.

Using DL-penicillamine, which has been considered to inhibit PAL-P action by forming a thiazolidine derivative, we determined the effect of this inhibitor on enzyme activity with PAL-P and INPP respectively. For this purpose, 2 X $10^{-5}$M of each coenzyme and $10^{-3}$M or 2 X $10^{-3}$M penicillamine were put into the main chamber of Warburg flasks and then the apoenzyme solution added. After tipping glutamate from the side arm into the main chamber the $CO_2$ evolution was measured. Though the enzyme activity due to 2 X $10^{-5}$M PAL-P is entirely inhibited by $10^{-3}$M or 2 X $10^{-3}$ penicillamine, the activation due to 2 X $10^{-5}$M INPP is inhibited by the addition of $10^{-3}$M penicillamine no more than 20% or 30% respectively (Fig. 1)

This fact seems to show that INPP as such can act as coenzyme of brain glutamic decarboxylase. The slight inhibition by penicillamine found in the experiment with INPP may be due to the effect of DL-penicillamine upon the apoenzyme. This assumption is based on the following analogy found in the inhibition experiment with isonicotinyl hydrazide (INAH): the brain glutamic decarboxylase activation by $10^{-5}$M INPP is inhibited as much as 50% by the addition of 5 X $10^{-4}$M INAH indicating that the latter decreases the enzyme activity by affecting the apoenzyme portion.

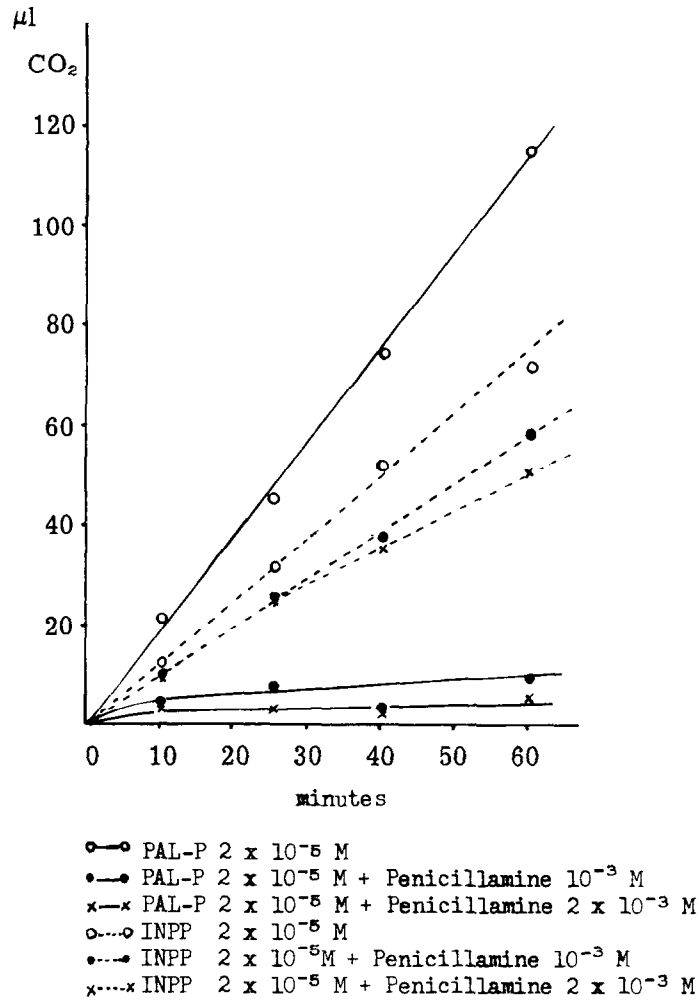


Figure 1

Even when the coenzymes are preincubated with the apoenzyme, the inhibition of INPP-activity by DL-penicillamine added afterwards is far less than that of PAL-P: in the experiments using $10^{-5}$M of each coenzyme, the amount of which is sufficient for the saturation of the apoenzyme in case of PAL-P, the inhibition of the holoenzyme activity by 5 X $10^{-4}$M penicillamine after the 10 minutes' preincubation of the coenzyme with apoenzyme, is 85% in case of PAL-P and 40% in case of INPP when compared in the reaction time from 30 minutes to 70 minutes (Fig. 2).

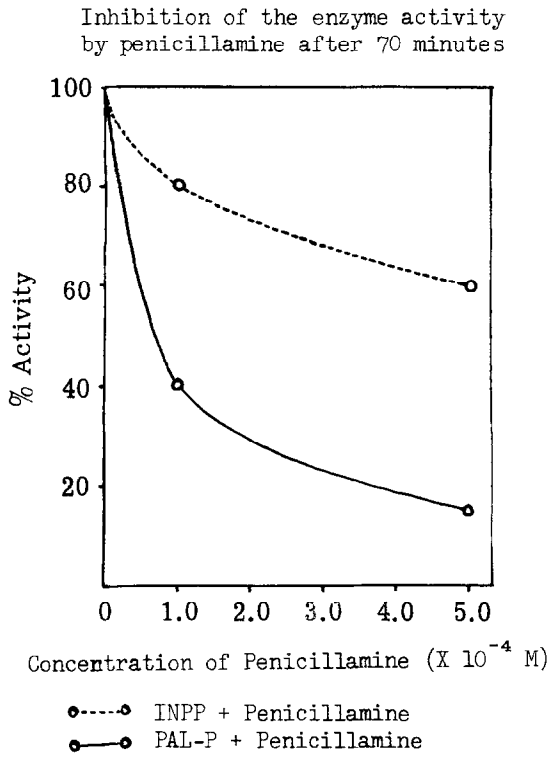


Figure 2

The possibility that in the above experiment INPP acts by liberating PAL-P which rapidly recombines with INAH to form INPP, is excluded by the fact that 2 X $10^{-5}$M PAL-P, when added to a mixture of 2 X $10^{-5}$M INAH and 5 X $10^{-4}$M penicillamine, loses its coenzyme activity completely indicating that INAH cannot compete with penicillamine in combining with PAL-P under these experimental conditions.

Phosphopyridoxal hydrazone and semicarbazone, which were mentioned above, show nearly the same effect as INPP on the inhibition due to DL-penicillamine.

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