ide (or Iodide) and TMB4 (1, 1'-trimethylene-bis(4-formylpyridinium bromide) dioxime) when injected intraperitoneally in the mouse at 50% of LD_{50}, raise the intraperitoneal lethal dose of anticholinesterase, Phospholine (echothiophate) Iodide, by factors of 50 and 280 respectively. When the same oximes are given orally 30 to 60 minutes before Phospholine the intraperitoneal LD_{50} is increased by factors of 23 and 450. Graphs relating log dose of oxime and log dosage ratio of Phospholine are linear over a considerable range. The therapeutic indices for arbitrary increase of 10 times in LD_{50} of Phospholine are 23 and 9 for Protopam and 114 and 120 for TMB4 by oral and intraperitoneal administration respectively. The possible nature of the antagonism is discussed.

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Comparative Inhibition of Pyridoxal Kinase and Glutamic Acid Decarboxylase by Carbonyl Reagents.* (25905)

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γ-Aminobutyric acid (γABA) has been implicated as a transmission regulator in the central nervous system(1), though experimental evidence for such a role is ambiguous. Formation of this compound in brain from L-glutamic acid is catalyzed by a decarboxylase which requires pyridoxal phosphate as coenzyme(2). Carbonyl reagents are known to inhibit most pyridoxal phosphate enzymes through formation of a coenzymatically inactive derivative with the cofactor(3). Many of these reagents are now recognized as stimulants of the central nervous system ("psychic energizers") which in sufficient excess, produce epileptiform seizures that may be alleviated by administration of Vit. B_6(4). Localization of glutamic decarboxylase in brain(2), together with an observed reduction in levels of γABA following treatment by carbonyl reagents(5) have been interpreted to mean that both analeptic and convulsive activities of active carbonyl reagents may result from interference with production of this inhibitor of neuronal transmission by the decarboxylase (6). However, pyridoxal kinase catalyzes formation of pyridoxal phosphate from Vit. B_6 and has been shown to be extremely sensitive to inhibition by carbonyl reagents(7). The possibility therefore exists that the decrease in concentration of γABA results from lowered pyridoxal phosphate levels as a consequence of inhibition of pyridoxal kinase rather than from inhibition of the glutamic decarboxylase *per se*. This report demonstrates that pyridoxal kinase is much more sensitive than glutamic decarboxylase to inhibition by carbonyl reagents both *in vitro* and *in vivo*. These findings emphasize the possibility that inhibition of the kinase, with consequent decrease in pyridoxal phosphate production,

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may be one mechanism by which certain carbonyl reagents limit production of γABA and produce *in vivo* other symptoms related to those of Vit. B_6 deficiency.

Methods. Adult rats received intraperitoneal injections of 1 ml of water or of aqueous solutions of test compound. In some instances urine was collected, chromatographed on paper, and chromatograms examined for pyridoxal catabolites by spraying with 2,6-dichloroquinonechlorimide(8). At specified time intervals, animals were decapitated and the desired organs removed, rinsed, blotted, weighed, and homogenized with 4 volumes of 0.05-0.1 M potassium phosphate, pH 6.5-7.0, or with 9 volumes of 0.25 M sucrose. Homogenates were centrifuged at 18,500 x g for 30 to 60 minutes to obtain supernatant solutions which were assayed both for pyridoxal kinase and for glutamic decarboxylase. Pyridoxal kinase in the phosphate buffer extract from beef brain was purified further by collecting that fraction of supernatant solution insoluble between 40 and 60% saturated $(NH_4)_2SO_4$ and dialyzing(7). Glutamic decarboxylase from beef brain appeared to be more stable in acetone powder extracts than in fresh tissue homogenates. Acetone powders were prepared in the usual way(9) and extracts were obtained by grinding the preparations with 0.1 M phosphate buffer, pH 6.4, containing 0.01% 2-mercaptoethanol and 5 mg of Tween 80 per ml. Protein was determined by the method of Lowry *et al.*(10). Incubation mixtures for pyridoxal kinase contained 5×10^{-4} M pyridoxal and ATP, 10^{-5} M Zn^{++}, 0.1 M potassium phosphate, pH 6.0-6.5, and enzyme with or without inhibitor. After incubating 1 hr at 37°C, the reaction was stopped by heating 3 min at 100°. When pyridoxine was used as substrate in place of pyridoxal, the product, pyridoxine phosphate, was converted to pyridoxal phosphate by a second incubation for 1 hr at 37° and pH 8 with an oxidase preparation from rabbit liver(11). Pyridoxal phosphate formation was followed by a modification of the apotryptophanase method of Wada *et al.*(12). Glutamic decarboxylase activities were measured by the fluorimetric method of Lowe *et al.*(13). A lower concentration of pyridoxal phosphate (0.02 mM in place of 0.5 mM) was used in incubation mixtures. This concentration was lowest that permitted optimal decarboxylase activity, and was used to insure maximum sensitivity of decarboxylase to inhibitors. The intensity of fluorescence of the product formed by reaction of γABA with ninhydrin was determined with the Aminco-Bowman spectrophotofluorimeter, set at activation wave length of 376 mμ and a fluorescence wave length of 452 mμ.

Results. Inhibitory concentrations of several carbonyl reagents for partially purified preparations of pyridoxal kinase and of glutamic decarboxylase from beef brain are compared in Fig. 1. The kinase is from 100 to 1000 times more sensitive than the decarboxylase to these inhibitory compounds.

When hydrazine was injected into rats, and activities of the 2 enzymes subsequently were compared, both enzymes were markedly depressed (Table I). Again the effect upon the kinase appears more severe. Under these same conditions, no significant inhibition of either kinase or decarboxylase was observed one hour after administration of isonicotinyl hydrazide (isoniazid), hydroxylamine, or pyridoxal oxime in amounts sufficient to give an initial "body concentration" in excess of 10^{-3} M. All of these compounds are potent inhibitors of the kinase *in vitro*(7), eliciting 50% inhibitions at concentrations between 5×10^{-7} and 5×10^{-6} M. The dihydrazone (azine) of pyridoxal, like hydrazine, is a potent inhibitor of pyridoxal kinase both *in vivo* and *in vitro* but does not inhibit glutamic decarboxylase under either condition. The time course of inhibition in liver and brain following injection of the azine is shown in Fig. 2.

Animals which received either hydrazine or hydroxylamine developed convulsions and died with larger doses. Hydroxylamine produced severe methemoglobinemia, and onset of convulsions was rapid. Neither pyridoxal azine nor pyridoxal oxime produced convulsions in amounts equimolar with convulsive doses of the free carbonyl reagents. Pyridoxal oxime was rapidly catabolized to 4-pyridoxic acid and its lactone, and these were

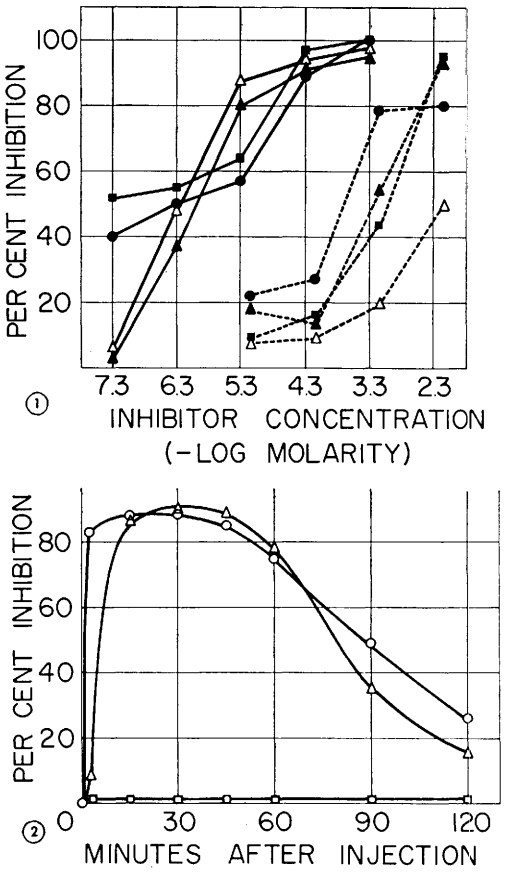

FIG. 1. Comparative effects of carbonyl reagents on activity of partially purified preparations of pyridoxal kinase and of glutamic decarboxylase from beef brain. Conditions are described under *Methods*. Solid lines show kinase activities, broken lines show decarboxylase activities. ●, Hydroxylamine; ▲, α-methylphenethylhydrazine; ■, semicarbazide; △, hydrazine.

FIG. 2. Effect of intraper. injections of pyridoxal azine on activities of pyridoxal kinase and of glutamic decarboxylase in livers and brains of rats as a function of time. Pyridoxal azine (15 mg) was administered as its hydrochloride by intraper. injections to 200-250 g female rats. Incubation conditions as described under *Methods*. Kinase activities in liver (○) and brain (△); decarboxylase activity in brain (□).

excreted in urine. Recovery of rats from an apparent weakness following injection of pyridoxal azine coincided with recovery of kinase activity.

Inhibition of pyridoxal kinase by pyridoxal azine *in vivo* could, in principle, result from its hydrolysis to pyridoxal and hydrazine with subsequent inhibition by the latter of the kinase during its assay *in vitro*. Such inhibition requires condensation of hydrazine with pyridoxal added as substrate, the resulting hydrazone or azine being the actual inhibitor (7). This possibility was eliminated by assaying the kinase with pyridoxine, which cannot condense with hydrazine, as substrate. The results (Table II) show that kinase levels are depressed about equally with either substrate; consequently inhibition by the injected pyridoxal azine cannot result from its preliminary hydrolysis to hydrazine.

Discussion. These findings demonstrate that inhibition of both pyridoxal kinase and glutamic decarboxylase can be produced *in vivo* by a single dose of some, but not all, of the carbonyl reagents that produce these effects *in vitro*. The mechanism of the 2 inhibitions is quite different. Carbonyl reagents inhibit pyridoxal kinase *in vitro*, and presumably also *in vivo* by combining with pyridoxal to form extremely inhibitory substrate analogues(7). That hydrazine inhibits *in vivo* thus infers that there are sufficient amounts of this agent and of free pyridoxal present in tissues to form the active inhibitor. Agents such as hydroxylamine and isoniazid, which inhibit the kinase *in vitro* but not *in vivo*, may be inactive under latter conditions because (a) they are not accumulated in tissues to the same extent as hydrazine, or (b) they require higher concentrations of pyridoxal or longer times to form the inhibitor, or (c) they are destroyed before they reach the site of inhibition. The latter explanation presumably holds for pyridoxal oxime, which rapidly gives rise to pyridoxic acid after injection. This immediate increase in excretion of pyridoxic acid was not observed following injection of pyridoxal azine, which inhibits *in vivo*.

Although these condensation products of carbonyl reagents with pyridoxal are potent inhibitors of the pyridoxal kinase, tests *in vitro* have shown them to be essentially without effect on glutamic decarboxylase. Carbonyl reagents inhibit this enzyme by condensing with pyridoxal phosphate to remove this functionally active coenzyme as a comparatively inactive coenzyme analogue. We prepared such analogues chemically and find them essentially without inhibitory activity

TABLE I. Effect of Hydrazine Injection on Levels of Pyridoxal Kinase and Glutamic Decarboxylase in Rat Tissues.

		Kinase activities*				Decarboxylase activities*	
		Liver		Brain		Brain	
Hydrazine inj., mg	Min. before sacrifice	Units/mg protein	Total units	Units/mg protein	Total units	Units/mg protein	Total units
0	0	11.6 ±3.5(σ)‡	7,140 ±1,840	23.4 ±5.2	834 ±196	216 ±37	7,380 ±1,480
5	60	5.8	3,700	17.2	693	297	12,000
10	120	2.5	1,470	10.4	335	94	3,030
100	7†	.0	0	2.1	70	95	3,160

* One unit of activity is that amount of protein (in phosphate buffer extract) that forms one mμmole of pyridoxal phosphate or of γABA in 1 hr at 37° under assay conditions. Total activity is calculated on basis of total wet wt of organ.

† Time of death due to injected hydrazine.

‡ σ Stand. dev. with 10 control animals. Single animals were used for each level of hydrazine.

for either glutamic decarboxylase or for pyridoxal kinase. The fact that injected pyridoxal azine inhibits pyridoxal kinase, but not glutamic decarboxylase *in vivo,* is consistent with each of these observations.

Summary. 1. Carbonyl reagents such as hydrazine, hydroxylamine and isoniazid inhibit purified preparations of pyridoxal kinase and of glutamic decarboxylase from beef brain, but the kinase is 100 to 1000 times more sensitive. 2. Administration of hydrazine to rats decreases activity of these 2 enzymes in liver and brain; kinase levels are affected more severely. 3. In contrast to their effects *in vitro,* isoniazid, hydroxylamine, or pyridoxal oxime produced no significant inhibition of kinase or decarboxylase activity *in vivo.* Pyridoxal oxime appears rapidly catabolized *in vivo* since its injection results in extensive excretion of 4-pyridoxic acid and its lactone. Unlike free hydroxylamine, injection of pyridoxal oxime does not produce convulsions or extensive methemoglobin formation. 4. Similar administration of pyridoxal azine resulted in rapid and extensive decrease of pyridoxal kinase levels in liver, kidney, and brain. No effects on glutamic acid decarboxylase in brain were observed. 5. These observations demonstrate that individual carbonyl reagents inhibit quite different enzymes *in vivo,* and indicate that appropriate derivatives of such agents may show enhanced selectivity in their inhibitory actions.

TABLE II. Inhibition of Pyridoxal Kinase in Tissues from Rats Injected with Pyridoxal Azine.

	Inhibition of kinase, %* (30 min. after 10 mg dose)	
Organ	Pyridoxal as substrate	Pyridoxine as substrate
Liver	58	50
Kidney	63	62
Brain	6	3

* Avg values for 6 male rats (200-250 g each).

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