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STABILIZATION OF COENZYME BINDING BY CONJUGATED STEROIDS AND CARBOXYLIC ACIDS

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VARIOUS conjugated steroids inhibit the kynurenine transaminase of rat kidney by interfering with its association with pyridoxal phosphate[1,2]. They also protect the apoenzyme from proteolytic inactivation[2], apparently as a result of the association that causes the inhibition. In the present study, estradiol disulfate (EDS), diethylstilbestrol disulfate (SDS), dehydroepiandrosterone sulfate, and several carboxylic acids were found to retard the dissociation of pyridoxal phosphate (PLP) from the kynurenine holotransaminase of fresh kidney homogenates. Similar retardation was observed with partially-purified serine transhydroxymethylase of rabbit liver and the tyrosine-α-ketoglutarate transaminase of rat liver homogenates. An irreversible loss of tyrosine transaminase activity, which occurs during incubation of the homogenates at 37°, was retarded by SDS, α-naphthoate, benzoate, p-aminobenzoate, and salicylate, all of which have been shown[3] to induce this enzyme. This inactivation was also retarded in homogenates prepared from rats injected with SDS or hydrocortisone.

Methods and Results

Kynurenine transaminase, serine transhydroxymethylase, and tyrosine transaminase were assayed as described previously[3-5] unless otherwise indicated.

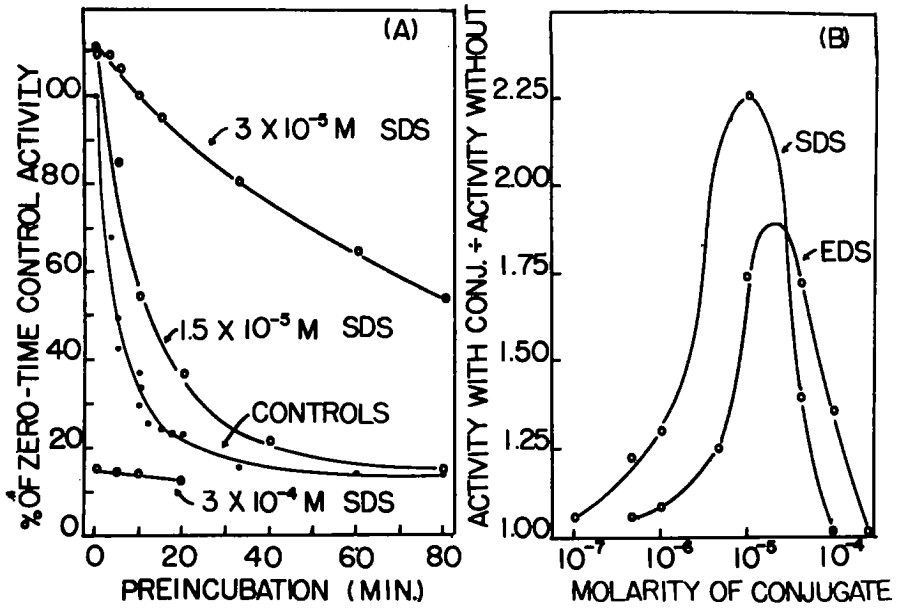

FIG. 1

(A) Retardation of the dissociation of PLP from kynurenine transaminase by various concentrations of SDS. Rat kidney was homogenized (1 gm per 3 ml 0.01 Tris buffer, pH 7.4) and 0.25 ml aliquots were combined immediately with 0.25 ml of 0.24 M sodium phosphate buffer, pH 6.3. These tubes were incubated at 37° with or without added SDS. After the indicated time intervals, 0.25 ml of 0.024 M α-ketoglutarate was added to individual tubes to prevent further dissociation. Thirty minutes after beginning the preliminary incubations, 0.25 ml of 0.0148 M L-kynurenine was added to each tube and the activities were assayed as described previously[4]. For preliminary incubation periods extending beyond 30 minutes, L-kynurenine sulfate was added immediately following the addition of α-ketoglutarate.

(B) Retardation of the dissociation of pyridoxal phosphate from kynurenine transaminase at various concentrations of SDS and EDS. Conditions were the same as described in (A) except that preliminary incubation was limited in all cases to 10 minutes in 0.12 M sodium phosphate buffer, pH 6.5. The curves represent the differences in activity obtained with varying concentrations of EDS and SDS in comparison with controls.

Freshly-homogenized rat kidney exhibits kynurenine

transaminase activity when assayed without added PLP[4]. This activity is lost rapidly when the homogenate is pre-incubated in phosphate buffer, pH 6.3, but is regained on the addition of PLP. We thought it probable that this apparent dissociation of PLP would be accelerated by the addition of EDS or SDS, since these compounds were assumed[2] to compete for the site that binds the coenzyme. Instead, SDS (1.5×10^{-5} M and 3×10^{-5} M) decreased the rate of inactivation (Fig. 1A). With 3×10^{-4} M SDS, on the other hand, enzyme activity was strongly depressed even before preincubation. Retardation of dissociation was observed with concentrations of EDS and SDS in the range of 10^{-7} to 10^{-4} M (Fig. 1B); higher levels caused inhibition as predicted from the results in Fig. 1A.

Dehydroepiandrosterone sulfate and several carboxylic acids, some of which appear to be competitive inhibitors of kynurenine transaminase[4], also stabilized PLP binding. Dehydroepiandrosterone sulfate had little effect at levels below 10^{-4} M. Glutarate and succinate exhibited maximal stabilization at 10^{-2} M levels. Benzoate (10^{-2} M) also had a strong stabilizing action.

Retardation of PLP dissociation was also observed with serine transhydroxymethylase. The enzyme preparation, assayed[5] as approximately 50% pure, was incubated at 37° in 0.1 M phosphate buffer, pH 7.3, for 20 to 30 minutes. It loses activity under these conditions and can be reactivated by added PLP. The loss of activity was retarded at SDS levels in the range of 10^{-5} to 10^{-4} M.

Similar behavior was observed with tyrosine transaminase. Freshly-homogenized rat liver (1 gm liver per 7 ml water) lost activity slowly during storage at 0°; the activity was restored by adding PLP (1 µmole per 5 ml incubation mixture). At 37°,

similar unbuffered homogenates (1 gm liver per 12 ml water) lost about 50% of their activity during one hour. This loss was largely prevented by added PLP (2×10^{-5} M) but could not be reversed by adding it afterwards. The rate of enzyme inactivation was strongly decreased by 8×10^{-3} M benzoate, 5×10^{-3} M α-naphthoate, 7.5×10^{-3} M p-aminobenzoate, 7.5×10^{-3} M salicylate, and 10^{-3} M α-ketoglutarate. SDS was effective in the range of 10^{-3} to 2×10^{-5} M.

The aromatic carboxylic acids and SDS share with the glucocorticoids the ability to induce tyrosine transaminase in adrenalectomized rats[3]. We therefore examined the possibility that hydrocortisone injection might lead to a similar stabilization. Hydrocortisone (3.3 mg per 100 gm body weight) was injected intraperitoneally into 12 male Sprague-Dawley rats weighing 160 to 185 gms. Half of the rats were killed immediately after the injection and the rate of irreversible inactivation of the enzyme during a 30 minute preincubation of the unbuffered liver homogenates at 37° was determined. The rest of the animals were killed 3 hours after the injection and the activities were examined in the same way. The rate of inactivation in the latter group was about 30% less than that found with the controls that were tested immediately after injection. In similar experiments, SDS injections (10 mg per 100 gm body weight) retarded the inactivation by about 50%.

Additional experiments were done to test whether hydrocortisone injections cause retardation of PLP dissociation from the tyrosine transaminase. When the homogenates were incubated 30 minutes at 0°, the rate of dissociation was found to be about 40% less for rats killed one hour after hydrocortisone injection than for the controls that were killed immediately.

Discussion

EDS and SDS are assumed to be analogs of PLP since they compete with it for kynurenine apotransaminase[2,6] and share with it the ability to protect the enzyme against proteolytic inactivation[2]. Their ability to retard PLP dissociation may therefore be related in some way to their ability to bind the active site.

The stabilization of PLP binding by α-ketoglutarate[4] was attributed to its ability, acting as an amino group acceptor, to maintain the coenzyme in its aldehydic form. In view of the present observation that non-substrate dicarboxylic and aromatic acids also stabilize PLP binding, other explanations must be considered. A plausible unifying explanation for these actions of the substrate, substrate analogs, and coenzyme analogs is that they stabilize an active form of the enzyme which has a higher affinity for the coenzyme and a greater resistance to proteolytic action.

The possibility that these and the previously observed actions[1,2] may have a significant metabolic role is suggested by analogy with studies of tryptophan pyrrolase. Tryptophan, the substrate, stabilizes the binding of hemin, the cofactor, to the apoenzyme[7]. This action apparently stabilizes the enzyme *in vivo*, leading to its accumulation in tryptophan-injected rats[7,8]. A similar stabilizing effect of PLP binding is suggested by the decline in the apoenzyme levels of several PLP-dependent enzymes during vitamin-B_6 deficiency[9-11] and by the rapid elevation of hepatic tyrosine transaminase levels following the injection of pyridoxine[12]. It was suggested in the latter case[12] that PLP, formed from the injected pyridoxine, associates with the apotransaminase and, in some way, increases its synthesis or

decreases its breakdown. Our present study demonstrates that added PLP can protect the enzyme against irreversible inactivation during incubation in vitro. This ability is shared with benzoate, p-aminobenzoate, α-naphthoate, salicylate, and SDS, all of which elevated tyrosine transaminase levels in vivo[3]. These observations strongly suggest that these agents cause the accumulation of hepatic tyrosine transaminase by retarding its destruction.

The observation that injected hydrocortisone caused retardation of PLP dissociation from hepatic tyrosine transaminase and that it also retarded the irreversible inactivation suggests that the glucocorticoid, its metabolic derivatives, or other compounds that accumulate in the liver in response to the injection may have actions in vivo similar to those reported for SDS and the organic acids. The retardation of dissociation may explain the increase in the degree of saturation of hepatic tyrosine transaminase with PLP that was reported[13] to occur within one hour after the injection of cortisone.

PLP is bound to various enzymes[14-16] and to serum albumin[17] by Schiff base formation with ε-amino groups; it is reasonable to assume that the ester phosphate group is also associated with cationic groups of the enzymes. The anionic character of the conjugates and carboxylic acids and their ability to interfere with PLP binding suggests that they also bind amino groups. Recently Whitehouse and Skidmore[18] have pointed out that most of the non-steroid anti-inflammatory agents are aromatic acids which interfere with the binding of PLP to serum albumin. They cited this and other evidence in support of their view that many of the actions of these agents on enzyme systems are related to their ability to bind ε-amino groups of proteins. Our earlier observation that various aromatic acids, including salicylate,

resemble the conjugates and glucocorticoids in their ability to induce tyrosine transaminase[3] and the present indications that they share with them the ability to stabilize the enzyme suggest similarities in their modes of action. Whether these actions are related in any way to the anti-inflammatory effects of various steroids and aromatic acids remains to be determined.

Summary

Estradiol disulfate and diethylstilbestrol disulfate, which interfere with the binding of pyridoxal phosphate to kynurenine apotransaminase and protect the apoenzyme from proteolytic inactivation, retarded the dissociation of pyridoxal phosphate from the holotransaminase in freshly homogenized rat kidney. At higher concentrations, dehydroepiandrosterone sulfate and several carboxylic acids also stabilized pyridoxal phosphate binding. Similar stabilization was observed with partially purified serine transhydroxymethylase of rabbit liver and with the tyrosine-α-ketoglutarate transaminase of freshly homogenized rat liver.

Tyrosine transaminase activity in fresh liver homogenates (1 gm liver per 12 mls of water) lost 50% of its activity during incubation for 1 hr at 37°. This activity could not be restored by adding pyridoxal phosphate although the loss could be largely prevented by the presence of 10^{-5} M PLP and by 10^{-3} M α-ketoglutarate. Benzoate, p-aminobenzoate, α-naphthoate, salicylate, and diethylstilbestrol disulfate, all of which were shown previously to induce hepatic tyrosine transaminase in vivo, retarded the inactivation. Hydrocortisone or diethylstilbestrol disulfate injections also resulted in decreased rates of inactivation in liver homogenates prepared 3 hours after injection as compared with those prepared immediately after injection.

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