BBA 65653

STUDIES OF THE *IN VITRO* AND *IN VIVO* EFFECTS OF CONJUGATED STEROIDS AND CARBOXYLIC ACIDS ON HEPATIC TYROSINE TRANSAMINASE IN THE RAT*

S. SINGER** AND M. MASON

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Mich. (U.S.A.)

(Received May 8th, 1967)

SUMMARY

1. Tyrosine transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) activity was lost rapidly in fresh rat-liver homogenates (pH 6.9), that were incubated at 38°. The inactivation was paralleled by the loss of the coenzyme but was not reversed by the subsequent addition of pyridoxal 5-phosphate.

2. The coenzyme, the keto acid substrates, and their anionic analogs retarded the inactivation and dissociation. Various anionic steroids and diethylstilbestrol disulfate ($5 \cdot 10^{-4}$–$5 \cdot 10^{-5}$ M) also retarded the inactivation and dissociation; free steroids were ineffective at saturation levels. Aromatic carboxylic acids were effective at 10^{-2}–10^{-3} M, 5-hydroxytryptophan at 10^{-3} M, and L-glutamate, bicarbonate, and P_i at 10^{-2} M. Several other amino acids and NaCl were ineffective at 10^{-2} M. Many of the *in vitro* stabilizing agents caused elevated levels of hepatic tyrosine transaminase when injected into adrenalectomized rats. In general, the most potent stabilizers were also the most effective agents in causing the elevated enzyme levels *in vivo*.

3. Estradiol disulfate and diethylstilbestrol disulfate also retarded the inactivation and dissociation that occurred when the homogenates were incubated at 25° in 1.1 or 2.2 M urea or when partially-purified tyrosine transaminase was incubated with trypsin (EC 3.4.4.4) or chymotrypsin (EC 3.4.4.5). The rate of inactivation in homogenates was not significantly changed by the presence of 0.001 M EDTA or mercaptoethanol nor by incubation with alkaline phosphatase (EC 3.1.3.1).

4. A small but significantly greater degree of association of the tyrosine transaminase with its coenzyme was found in rat-liver homogenates prepared 1 h after cortisol administration than in the injected controls that were sacrificed immediately. There was also a significantly slower rate of coenzyme dissociation in the 1-h animals. Similar doses of cortisone were ineffective in the latter case.

* This work was included in a thesis submitted by S. S. in partial fulfillment of the requirements for the Ph.D. degree.

** Present address: Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Eastchester Road and Morris Park Avenue, Bronx, N.Y., U.S.A.

INTRODUCTION

Various conjugated steroids[1,2] and carboxylic acids[3] inhibit the kynurenine transaminase (EC 2.6.1.7) of rat kidney. This action of the conjugated steroids was attributable to their interference with the association of pyridoxal 5-phosphate with the apoenzyme[2]; the carboxylic acids, on the other hand, inhibited by competition with the substrates[3]. The conjugated steroids retarded the inactivation of the apoenzyme that occurred during incubation at 37° and also prevented the rapid inactivation catalyzed by chymotrypsin (EC 3.4.4.5) (ref 2). More recently[4], they were also found to retard the dissociation of pyridoxal 5-phosphate from the holoenzyme in freshly-prepared homogenates.

Conjugated steroids and carboxylic acids also influence the activity and stability of other pyridoxal 5-phosphate enzymes. JENKINS, YPHANTIS AND SIZER[5] reported that various dicarboxylic acids inhibit aspartate aminotransferase (EC 2.6.1.1) and protect it from heat inactivation. Other authors have reported that preparations of this enzyme from placenta[6] and from heart muscle[7,8] are strongly inhibited by conjugated steroids. Preliminary tests of other pyridoxal 5-phosphate enzymes indicate that they are also inhibited by conjugated steroids and that diethylstilbestrol disulfate also retards the dissociation of pyridoxal 5-phosphate from rabbit-liver serine transhydroxymethylase (EC 2.1.2.1) and from rat-liver tyrosine transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) (ref. 4).

In the earlier work with kynurenine transaminase[2], it was suggested that the interaction of the conjugated steroids with pyridoxal 5-phosphate enzymes might also occur *in vivo* and thereby alter metabolic activity. More recently[9] we have shown that the administration of diethylstilbestrol disulfate and various aromatic acids to adrenalectomized rats results in the induction* of hepatic tyrosine transaminase. The response to diethylstilbestrol disulfate and benzoate appears to be somewhat specific in that two other inducible enzymes and glycogen synthesis were not affected[10]. In the present study, we have examined the effects *in vitro* and *in vivo*, of a variety of anionic steroids and of a number of non-steroid compounds on tyrosine transaminase in an attempt to clarify the relation between the ability of compounds to stabilize the enzyme and their ability to induce it.

MATERIALS AND METHODS

Materials

The free steroids, diethylstilbestrol, disodium cortisol 21-succinate, indole 3-acetic acid, pyridoxal 5-phosphate, intestinal alkaline phosphatase (EC 3.1.3.1), and crystalline preparations of trypsin (EC 3.4.4.4) and chymotrypsin were purchased from the Sigma Chemical Co. Dipotassium estradiol diphosphate, 'ultra-pure' urea, and androst-5-ene-3β-ol-17β-oic acid were obtained from Mann Research Laboratories, and deoxypyridoxal phosphate from Calbiochem. Benzoic acid and related compounds were from lots previously described[9]. The steroid sulfates were prepared by the

* The increased enzyme activity caused by non-steroid compounds has not been shown by rigorous criteria to be due to increased apoenzyme concentrations. For convenience, however, we shall refer to all compounds that cause increase as inducers, unless kinetic factors other than apoenzyme concentration are known to be responsible for the increase.

method of MORREN[11,2]. The two steroid mercaptoles, cortisol-21-(3',3'-dimethylglutarate) (Table I), and 11β-hydroxyestradiol were gifts from the Upjohn Co.

Male albino rats were purchased from Rawley Farms, Plymouth, Michigan and maintained as described earlier[9], before and after adrenalectomy, except as indicated.

Methods

In studies of the inactivation of tyrosine transaminase in liver homogenates, intact unfasted rats, weighing 150–190 g, were killed and their livers removed, immediately chilled, and homogenized in 12 times (except as indicated) their weight of ice-cold distilled water. The homogenates were centrifuged briefly at 4000 $\times$ g. Aliquots of the supernatant fluid (9.00 ml) were combined with 1.00-ml aliquots of water and with 1.00-ml aliquots of an aqueous solution (pH 6.9) of the compound to be tested. The mixtures were maintained at the desired temperature and samples (0.80 ml, except as indicated) were assayed at zero time and after the indicated periods of incubation.

Each sample was added to 4.60 ml of reaction mixture (pH 7.6), containing 0.90 μmole pyridoxal 5-phosphate (or water), 30 μmoles α-ketoglutarate, 25 μmoles sodium diethylthiocarbamate, 25 μmoles L-tyrosine, and 100 μmoles sodium phosphate. After incubation at 37° for 10 or 15 min, the reaction was stopped by the addition of 0.40 ml of 100% (w/v) trichloroacetic acid. The reaction mixture was filtered and 2.00-ml aliquots of the filtrate were assayed for p-hydroxyphenylpyruvate as described previously[9]. Activities are expressed, except as indicated, as the change in absorbance obtained, per 0.8 ml of diluted homogenate, after a 15-min incubation period.

In testing the action of proteolytic enzymes on tyrosine transaminase, a 7–8-fold purified transaminase preparation (the heated and dialyzed fraction of JACOBY AND LADU[12]) was incubated with the proteinases and assayed for tyrosine transaminase activity. The preincubation mixture consisted of 0.20 ml of the transaminase preparation (30 mg protein per ml), 0.10 ml of water, and 0.20 ml of 0.30 M sodium phosphate (pH 7.6). The proteolytic reaction was initiated by adding 0.1 ml of a freshly-prepared solution of trypsin or chymotrypsin (250 μg/ml of water) and immersing the mixture in a 38° water bath. After 30 min, the mixture was diluted with 2.00 ml of ice-cold water and 1.00-ml aliquots were assayed for tyrosine transaminase activity as described above. The activity of these aliquots was compared with those obtained by substituting 0.10 ml of water for the proteinase solutions.

For the induction studies, male rats, weighing 140–150 g initially, were adrenalectomized bilaterally and maintained for 3–5 days as described earlier[9]. On the evening before use, food was removed from their cages and, 13–16 h later, saline or the compound to be tested was administered intraperitoneally. The rats were killed by decapitation, 3.5 or 4.5 h later, and the livers were homogenized in 15 times their weight of ice-cold distilled water. The homogenate was centrifuged briefly at 4000 $\times$ g and the supernatant, 0.50–1.00 ml, was assayed immediately for tyrosine transaminase activity as described above.

RESULTS

Inactivation of tyrosine transaminase in rat-liver homogenates

Freshly-prepared rat-liver homogenates lost activity rapidly when they were

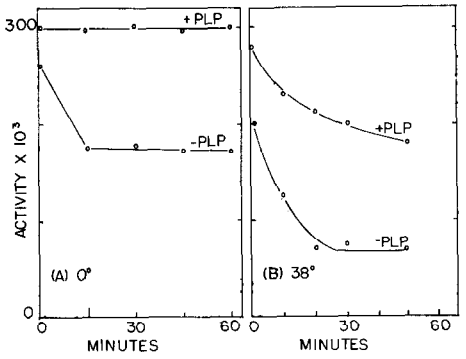

Fig. 1. The inactivation of tyrosine transaminase in liver homogenates at 0° and 38°. In Expt. A, homogenates containing 8 ml of cold distilled water per g of fresh liver were prepared and immediately placed in an ice-bath. After 0, 15, 30, 45, and 60 min, 0.50-ml aliquots were assayed for tyrosine transaminase activity (see METHODS) in the presence and absence of 0.17 μmole pyridoxal 5-phosphate (PLP). Activity is based on a 10-min period of incubation. In Expt. B fresh rat liver was homogenized, preincubated at 38° and assayed (in the presence and absence of PLP) as described in METHODS.

incubated at 0° and then assayed in the absence of pyridoxal 5-phosphate (Fig. 1A). When pyridoxal 5-phosphate was added to the assay mixtures the initial activity was regained. This indicated that the inactivation was a result of pyridoxal 5-phosphate dissociation. When homogenates were held at 38°, on the other hand, the enzyme was rapidly inactivated but could not be restored to the initial level of activity by the addition of pyridoxal 5-phosphate (Fig. 1B). The inactivation at 38° paralleled the dissociation of cofactor, suggesting that it is the apoenzyme that is destroyed. There was no significant change in the pH of the homogenates during the first 45 min of preincubation at 38°, so the effect is not complicated by pH changes in the weakly-buffered system.

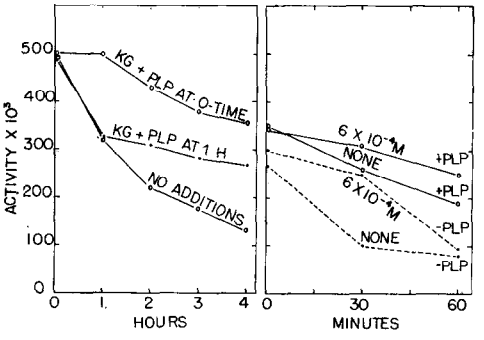

Fig. 2. The effect of pyridoxal 5-phosphate (PLP) and α-ketoglutarate (KG) on the inactivation of tyrosine transaminase at 38°. The homogenates were prepared, preincubated at 38° and assayed (in the presence of PLP) essentially as described in METHODS. In the preincubation, either water or 10 μmoles KG *plus* 0.30 μmole PLP in a volume of 0.1 ml were added at zero time or after 1 h.

Fig. 3. The effect of α-ketoglutarate on the inactivation and dissociation of tyrosine transaminase at 38°. The homogenate was prepared, preincubated at 38° and assayed (in the presence and absence of pyridoxal 5-phosphate (PLP)) as described in METHODS. α-Ketoglutarate concentrations in the preincubation media are shown on the curves.

The effect of substrates, cofactor, and cofactor analogs on the inactivation of tyrosine transaminase at 38°

The addition of α-ketoglutarate and pyridoxal 5-phosphate at zero time prevented the inactivation for 1 h, after which time the activity decreased slowly (Fig. 2). When they were added after 1 h, further decline in the enzyme activity was largely prevented. In other experiments (not shown) the addition of α-ketoglutarate and pyridoxal 5-phosphate every hour from zero time prevented enzyme inactivation for 4 h. These data suggest that the addition of substrate and cofactor results in stabilization and that the stabilization declines as the stabilizers are destroyed.

α-Ketoglutarate alone retarded both the inactivation and the dissociation of holoenzyme at a concentration of $6 \cdot 10^{-4}$ M (Fig. 3). In other experiments (not shown), phenylpyruvate and *p*-hydroxyphenylpyruvate were strongly effective at $5 \cdot 10^{-4}$ M, whereas oxaloacetate was only weakly effective at that level. Glutarate was ineffective at 10^{-3} M.

Pyridoxal 5-phosphate alone stabilized the enzyme to varying degrees at concentrations from $3 \cdot 10^{-4}$ M to 10^{-6} M, with the greatest effect occurring at 10^{-5} M (Fig. 4). At $3 \cdot 10^{-4}$ M, an actual increase in the rate of inactivation was observed. At concentrations of 10^{-5} M or higher, there appeared to be more protection 1 h after pyridoxal 5-phosphate addition than at 30 min after its addition. This suggests that the coenzyme may act both to protect and, at the higher concentrations, to inhibit the enzyme and that pyridoxal 5-phosphate destruction during the course of the experiment releases the inhibition, permitting a more complete expression of the protection. LITWACK, SEARS-GESSEL AND WINICOU[13] have shown that high concentrations of pyridoxal 5-phosphate inhibit the 200-fold purified enzyme. In other experiments (not shown) both the dissociation and inactivation occurred in the presence of $3 \cdot 10^{-5}$ M pyridoxal 5-phosphate, although at a much reduced rate.

No significant retardation of the inactivation was observed with pyridoxal or pyridoxine at concentrations as high as 10^{-3} M. However deoxypyridoxine 5-phos-

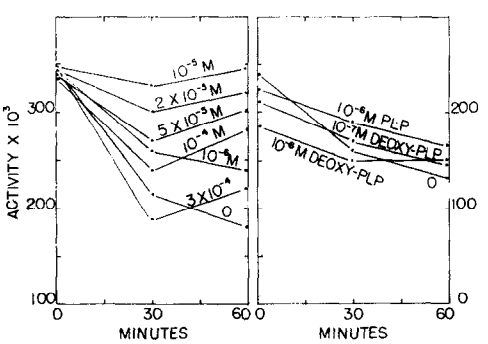

Fig. 4. The effect of pyridoxal 5-phosphate (PLP) on the inactivation of tyrosine transaminase at 38°. Homogenates were prepared, preincubated at 38° and assayed (in the presence of PLP) as described in METHODS. PLP concentrations in the preincubation media are shown on the curves.

Fig. 5. The effect of deoxypyridoxine 5-phosphate (deoxy-PLP) on the inactivation of tyrosine transaminase at 38°. Homogenates were prepared, preincubated, and assayed (in the presence of pyridoxal 5-phosphate) as described in METHODS. Deoxy-PLP concentrations are indicated in the figure.

phate, which inhibited the enzyme activity at 10^{-6} M (Fig. 5), prevented further enzyme inactivation. At 10^{-7} M deoxypyridoxine 5-phosphate also inhibited the initial activity and retarded the inactivation process.

The effect of diethylstilbestrol disulfate on the inactivation of tyrosine transaminase

Diethylstilbestrol disulfate has been shown to retard the dissociation of pyridoxal 5-phosphate from the kynurenine transaminase of rat kidney[4] and to stabilize the apoenzyme against inactivation[2]. In the present study, aliquots of fresh liver homogenate were preincubated in the presence and absence of pyridoxal 5-phosphate in buffered mixtures containing tyrosine and varying amounts of diethylstilbestrol disulfate. At a level of $8 \cdot 10^{-5}$ M, diethylstilbestrol disulfate greatly decreased the rate of pyridoxal 5-phosphate dissociation (Fig. 6A). Diethylstilbestrol disulfate also retarded pyridoxal 5-phosphate dissociation when added to aqueous homogenates that did not contain other components of the reaction mixture (Fig. 6B); it also

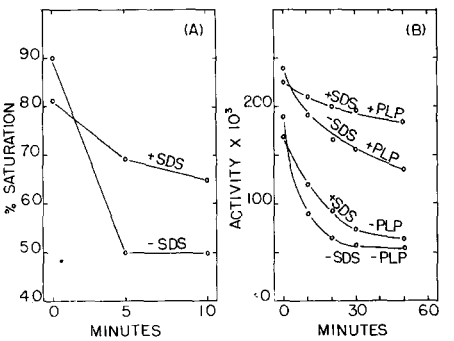

Fig. 6. The effects of diethylstilbestrol disulfate (SDS) on the inactivation and dissociation of tyrosine transaminase at 38°. In Expt. A, 0.50-ml aliquots of fresh homogenate containing 7 ml of water per g of liver were incubated at 38° in 5.1 ml of preincubation mixture containing 25 μmoles of L-tyrosine, 0.90 μmole of pyridoxal 5-phosphate (PLP) (or water), 100 μmoles of sodium phosphate (pH 7.6) and $8 \cdot 10^{-5}$ M SDS. After various periods of incubation, the reaction was started by the addition of 25 μmoles of α-ketoglutarate and 15 μmoles of disodium diethyldithiocarbamate in 0.50 ml of solution (pH 7.6). In Expt. B the homogenate was prepared, preincubated at 38° and assayed (in PLP) as described in METHODS. The concentration of SDS was 4×10^{-4} M in the preincubation medium.

retarded the enzyme inactivation. As with α-ketoglutarate (Fig. 3), the effect on inactivation substantially outlasted the effect on dissociation. In separate experiments (not presented), diethylstilbestrol disulfate was shown to be effective at various concentrations between $2 \cdot 10^{-5}$ M and 10^{-3} M. The stabilizing effect observed at 10^{-3} M, which was virtually complete during 1 h of incubation, was accompanied by a marked inhibition of the initial (zero time) activity. A similar association of stabilization and inhibition was noted earlier for kynurenine transaminase[2,4].

The effect of various other compounds on the inactivation of tyrosine transaminase

A large number of compounds were tested at various concentrations in experiments similar to those described for diethylstilbestrol disulfate in Fig. 6B. Representative data are listed in Table I. A rough comparison of the relative effectiveness

TABLE I

THE EFFECT OF VARIOUS COMPOUNDS ON THE RATE OF INACTIVATION OF TYROSINE TRANSAMINASE IN RAT-LIVER HOMOGENATES AT 38°

Abbreviations: dehydroepiandrosterone sulfate, androst-5-ene-17-one-3β-yl sulfate; steroid mercaptole I, androst-5-ene-3 β-ol-17-one, bis(carboxymethyl)mercaptole; steroid mercaptole II, 5 β-pregnane-17 α,21-diol-3,11,20-trione, 3-bis(carboxymethyl)mercaptole; cortisol hemiester I, pregn-4-ene-11 β,17 α-diol-3,20-dione-21-yl (3',3'-dimethylglutarate); cortisol hemiester II, pregn-4-ene-11 β,17 α-diol-3,20-dione-21-yl succinate.

Compound tested	*Concn. (mM)***	*Initial activity* $\times 10^{3}$†	*Relative inactivation rate*† *(30 min)*
H_2O*	—	340	100
p-Aminobenzoate	10.00	328	58
Diethylstilbestrol disulfate	0.20	320	48
Estradiol disulfate	0.20	338	39
Diethylstilbestrol disulfate	0.05	350	79
Estradiol disulfate	0.05	340	73
H_2O	—	185	100
Dehydroepiandrosterone sulfate	0.50	178	58
Testosterone sulfate	0.50	172	50
Hyodeoxycholate	0.50	178	79
H_2O	—	175	100
Steroid mercaptole I	0.50	170	42
Steroid mercaptole II	0.50	178	50
Cortisol hemiester I	0.50	175	89
Estradiol disulfate	0.50	163	0
H_2O	—	330	100
Androst-5-ene-3β-ol-17β-oic acid	0.50	320	62
H_2O	—	158	100
Cortisol hemiester II	5.00	155	94
Cortisol	0.10	156	97
H_2O	—	320	100
Diethylstilbestrol diphosphate	0.60	320	100
H_2O	—	262	100
Estradiol diphosphate	0.40	262	85
NaCl	10.00	272	80
H_2O	—	178	100
Estradiol-17β***	0.60	180	96
11β-hydroxyestradiol***	0.60	180	92
Estriol	0.60	178	90
H_2O	—	370	100
Diethylstilbestrol***	0.60	352	98
Diethylstilbestrol disulfate	0.25	370	30
H_2O	—	290	100
α-Naphthoate	1.00	290	44
H_2O	—	360	100
β-Naphthoate	1.70	360	51
α-Naphthalene acetate	1.70	360	56
o-Diphenate	10.00	370	62

TABLE I (continued)

Compound tested	*Concn. (mM)***	*Initial activity* $\times 10^{3}$†	*Relative inactivation rate*† *(30 min)*
H_2O	—	350	100
DL-5-Hydroxytryptophan	1.00	320	70
DL-Tryptophan	1.00	375	110
H_2O	—	430	100
p-Hydroxybenzoate	1.00	420	68
Indole 3-acetate	1.00	440	48
Anthranilate	10.00	420	34
H_2O	—	200	100
EDTA	1.00	200	100
H_2O	1.00	430	100
Mercaptoethanol	1.00	424	96
Ethanol	1.00	428	89
H_2O	—	360	100
Salicylate	7.50	370	63
H_2O	—	320	100
Benzoate	8.00	320	50
p-Aminobenzoate	7.5	330	67
H_2O	—	438	100
DL-Alanine	10.00	420	97
DL-Methionine	10.00	420	86
H_2O	—	270	100
Glycine	10.00	275	100
α-Aminoisobutyrate	10.00	275	97
L-Histidine	10.00	265	80
L-Glutamate	10.00	264	66
H_2O	—	350	100
Sodium phosphate	10.00	338	40
H_2O	—	300	100
Sodium bicarbonate	10.00	305	66

* Frozen-thawed homogenate was used instead of fresh homogenate.

** Represents the millimolarity of the compounds in the preincubation mixture.

*** The water-insoluble compounds were added to the vessels as ethanolic solutions, evaporated to dryness and resuspended in water.

† Relative inactivation rate is determined as the amount of activity lost during preincubation at 38° as compared to the amount lost in the aqueous controls. Initial activity is the activity observed before preincubation.

of the various compounds as stabilizers can be made by listing the concentrations of the compounds required to retard enzyme inactivation in the treated homogenates by 30–70% of the rate observed with the controls. This concentration was $2 \cdot 10^{-4}$ M for estradiol disulfate and diethylstilbestrol disulfate, $5 \cdot 10^{-4}$ M for dehydroepiandrosterone sulfate, testosterone sulfate, androst-5-ene-3 β-ol-17 β-oic acid, and the two steroid mercaptoles, 10^{-3}–2×10^{-3} M for α-naphthoate, β-naphthoate, α-naphthalene

acetate, indole 3-acetate, 5-hydroxytryptophan, and *p*-hydroxybenzoate, $7.5 \cdot 10^{-3}$–10^{-2} M for salicylate, *p*-aminobenzoate, benzoate, anthranilate, *o*-diphenate, glutamate, phosphate and bicarbonate.

Several free steroids (estradiol-17β, 11β-hydroxyestradiol, estriol, and cortisol) and diethylstilbestrol were ineffective at saturating levels. Estradiol diphosphate and diethylstilbestrol diphosphate were ineffective at $5 \cdot 10^{-4}$ M, possibly due to rapid hydrolysis. Two steroid hemi-esters of dicarboxylic acids (cortisol-21-(3',3'-dimethylglutarate) and cortisol-21-hemisuccinate) were ineffective at $5 \cdot 10^{-4}$ M and $5 \cdot 10^{-3}$ M levels, respectively. Several amino acids (glycine, alanine, α-aminoisobutyric acid, methionine, and histidine), had little or no retarding actions at 10^{-2} M levels.

Preliminary studies of the nature of the inactivation process

Earlier experiments by MASON AND GULLEKSON[2] showed that diethylstilbestrol disulfate stabilized kynurenine apotransaminase against inactivation by chymotrypsin. To test the possibility that the conjugates also protect tyrosine transaminase against proteinase action, we examined the effect of trypsin and chymotrypsin on the partially-purified enzyme in the presence and absence of $5 \cdot 10^{-4}$ M diethylstilbestrol disulfate and estradiol disulfate. Both of these compounds retarded the

TABLE II

THE EFFECT OF DIETHYLSTILBESTROL DISULFATE AND ESTRADIOL DISULFATE ON THE PROTEOLYTIC DESTRUCTION OF PARTIALLY-PURIFIED TYROSINE TRANSAMINASE BY TRYPSIN AND CHYMOTRYPSIN

Diethylstilbestrol disulfate or estradiol disulfate were present in the incubation mixtures at final concentrations of $5 \cdot 10^{-4}$ M; otherwise the experiments were carried out as described under METHODS.

Expt. No.		*Activity retained after 30 min*		*Inactivation (%)*
		Without chymotrypsin	*With chymotrypsin*	
1	Enzyme	0.280	0.050	82
	Enzyme + diethylstilbestrol disulfate	0.320	0.160	50
2	Enzyme	0.315	0.155	50
	Enzyme + estradiol disulfate	0.340	0.238	30
		Without trypsin	*With trypsin*	
1	Enzyme	0.240	0.042	83
	Enzyme + diethylstilbestrol disulfate	0.260	0.195	23
2	Enzyme	0.315	0.215	32
	Enzyme+ estradiol disulfate	0.340	0.302	11

proteolytic inactivation (Table II). In experiments similar to that reported by MASON AND GULLEKSON[2], $5 \cdot 10^{-4}$ M diethylstilbestrol disulfate did not interfere significantly with the digestion of casein by trypsin. We therefore assumed that diethylstilbestrol disulfate inhibited proteolysis by binding the transaminase rather than the proteinase.

If, as suggested earlier[4], the effective compounds impart stability to the enzyme by stabilizing its tertiary or quaternary structure, they would be expected to render

the enzyme more resistant to urea denaturation. As shown in Fig. 7A, 1.1 M or 2.2 M urea strongly accelerated the enzyme inactivation that normally occurred slowly at 25°. Similar effects on a 200-fold purified enzyme were previously shown by LITWACK, SEARS-GESSEL AND WINICOU[13]. Diethylstilbestrol disulfate ($5 \cdot 10^{-4}$ M) retarded this inactivation (Fig. 7B). The inactivation was halted but not reversed when diethylstilbestrol disulfate was added 30 min after the addition of urea. These effects of diethylstilbestrol disulfate are similar to those observed when it is incubated with the homogenates at 38° in the absence of urea. Our data are consistent therefore with the above suggestion that the protection of the enzyme in the homogenates is a result of the stabilization of its tertiary or quaternary structure.

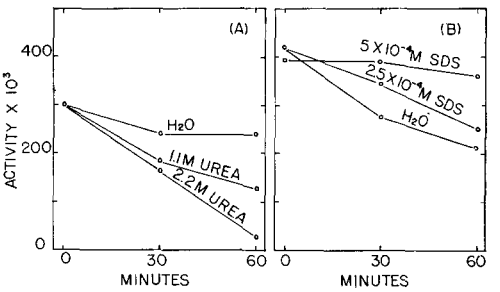

Fig. 7. The effect of urea and diethylstilbestrol disulfate (SDS) on the inactivation of tyrosine transaminase at 25°. In Expt. A, 8 ml of homogenate (11 ml of water per g of liver) were added to 2.00 ml of water or 2.00 ml of water containing enough urea to give the indicated urea concentrations. The treated homogenates were incubated at 25° and then assayed as described in METHODS. In Expt. B, 8 ml of fresh homogenate was added to 1 ml of water containing 0, 2.5, or 5 μmoles of SDS. These mixtures were allowed to stand for 4 min at 0° and 1.00 ml of 11 M urea was added to each. Aliquots (0.80 ml) were removed at zero time and after 30 and 60 min in a 25° bath and assayed for tyrosine transaminase in the presence of 0.90 μmole of pyridoxal 5-phosphate (see METHODS).

Added pyridoxal 5-phosphate retarded the inactivation of tyrosine transaminase in the freshly-prepared homogenates (Fig. 4) and, as reported by LITWACK, SEARS-GESSEL AND WINICOU[13], in the 200-fold purified preparation. However, the rate of pyridoxal 5-phosphate dissociation in the homogenates is much more rapid than the rate of inactivation (Fig. 1B), so other stabilizing factors probably play a dominant role in determining the rate of inactivation. The destruction of pyridoxal 5-phosphate in the homogenates by phosphatase action, for example, would be expected to favor dissociation but would not significantly accelerate the inactivation if other factors are rate-limiting. Consistent with this expectation, the addition of alkaline phosphatase to the homogenates in amounts sufficient to increase the rate of pyridoxal 5-phosphate hydrolysis several-fold did not result in a significantly greater rate of enzyme inactivation. In this connection, it should also be noted that 10^{-3} M EDTA, an effective inhibitor of phosphatase action, did not alter the rate of inactivation (Table I).

Other factors that might influence the stability of the enzyme include the concentrations of hydrogen ions and of sulfhydryl compounds and the incubation temperature. Dissociation occurred readily at 0° without appreciable inactivation (Fig. 1A). As expected, the rate of inactivation was greater at 38° than at 23° and

was still greater at 46°. Diethylstilbestrol disulfate protected against inactivation at all three temperatures. Dissociation without inactivation also occurred when the pH of the homogenates was changed to 6.0 by the addition of dilute HCl. At pH 8.4, the dissociation and inactivation were similar to that observed at pH 6.9. The presence of 10^{-3} M mercaptoethanol in homogenates incubated at pH 6.9 at 38° did not significantly alter the rate of inactivation (Table I), so the oxidation of enzymic sulfhydryl groups does not appear to be important in the inactivation process.

The correlation between the protection in vitro and induction in vivo of tyrosine transaminase

Several of the compounds that protected against inactivation (Table I) were shown earlier to induce tyrosine transaminase *in vivo*. The list includes diethylstilbestrol disulfate, α-naphthoate, salicylate, β-naphthoate, α-naphthalene acetate,

TABLE III

THE EFFECTS *in vivo* OF SOME STABILIZERS *in vitro* OF TYROSINE TRANSAMINASE

The numbers in parentheses represent the number of animals injected with the compound. Two or three saline-injected controls were used in each experiment. Animals that were injected at zero time and 2.5 h were killed at 4.5 h, the others at 3.5 h. Procedure as described in METHODS.

Compound tested	*Dosage per 150 g body wt. (mmoles)*		*Exptl. enzyme level/control enzyme level*
	Zero time	*2.5 h*	
Indole 3-acetate	0.2	—	2.65* (6)
Anthranilate	0.2	—	1.50* (4)
Anthranilate	0.4	0.2	3.85* (3)
p-Hydroxybenzoate	0.4	0.2	4.10* (3)
o-Diphenate	0.35	0.17	1.90* (4)
Androst-5-ene-3 β-ol-17 β-oic acid	0.2	0.08	3.60* (3)
Sodium phosphate	0.4	0.2	1.30 (3)
Glycine	0.27	0.27	0.82 (3)
Estradiol disulfate	0.03	—	1.20 (3)
Estradiol-17 β	0.03	—	1.50* (3)
Diethylstilbestrol	0.030	—	1.60* (3)
Steroid mercaptole I	0.030	—	3.00* (3)
Steroid mercaptole II	0.036	—	2.00* (4)

* Statistically significant variation from controls ($P = 0.05$).

benzoate, *p*-aminobenzoate[9], and 5-hydroxytryptophan[14]. Pyridoxal 5-phosphate may also be included if we assume that it is the active inducing agent formed from injected pyridoxine[15].

The suggested correlation between ability to induce and ability to retard inactivation prompted further *in vivo* tests of compounds which protected *in vitro* but which either had not been tested as inducers or had been tested and found to be ineffective in adrenalectomized rats. Indole 3-acetate, which had been reported ineffectual[14] was found to induce in our experiments (Table III). Anthranilate, *p*-hydroxybenzoate, and *o*-diphenate, which were found to be weak inducers in our earlier studies[9], were more effective at higher dosage levels and with longer time

intervals (Table III). The two steroid mercaptoles and androst-5-ene-3β-ol-17β-oic acid, which had not been tested before, were found to induce the enzyme at relatively low dosage levels (Table III). Glycine, methionine, and alanine, did not protect significantly (Table I). Methionine and alanine have been reported not to induce in intact rats[14,16]. Glycine was also tested in our *in vivo* experiments and found to be ineffectual as an inducer (Table III). Both estradiol-17 β and diethylstilbestrol were weak inducers. Surprisingly, estradiol disulfate, which was at least as effective as diethylstilbestrol disulfate as a stabilizer, did not cause a significant effect when administered in amounts equivalent to the inducing levels of diethylstilbestrol disulfate (Table III). Large doses of inorganic phosphate, which protected *in vitro* (Table I), did not induce (Table III).

The effect of cortisol administration on the association of the tyrosine transaminase in liver homogenates with pyridoxal 5-phosphate

GREENGARD[17] reported that rats injected with cortisone or pyridoxine showed, after 1 h a large increase in the degree of saturation of tyrosine transaminase in liver homogenates with pyridoxal 5-phosphate. This effect of cortisone appeared to be a manifestation *in vivo* of the type of stabilization that we had observed *in vitro*

TABLE IV

THE EFFECT OF CORTISOL ADMINISTRATION *in vivo* ON THE PERCENT SATURATION OF HEPATIC TYROSINE TRANSAMINASE IN HOMOGENATES FROM INTACT RATS

The per cent saturation is defined as B/A × 100. Intact rats were injected intraperitoneally with 5 mg of cortisol per 150 g rat. At the indicated times, they were killed and their livers were removed and chilled. Aliquots (0.50 or 1.00 ml) of whole homogenate (7.00 ml of 0.14 M KCl containing 0.02 M sodium phosphate (pH 7.6) per g of liver) were added to reaction mixture containing pyridoxal 5-phosphate (A) or without pyridoxal 5-phosphate (B) immediately after preparation. The enzyme activity obtained with both aliquots is expressed as the average absorbance change produced in 10 min ± the standard deviation.

Treatment	*Number of animals*	*Enzyme activity with added cofactor (A)*	*Enzyme activity without added cofactor (B)*	*Saturation (%)*
Killed 2 min		0.112 ± 0.027	0.074 ± 0.020	65.5 ± 4.2
After injection	10	0.202 ± 0.042	0.136 ± 0.035	68.7 ± 6.3
Killed 1 h		0.135 ± 0.030	0.103 ± 0.024	77.1 ± 5.6*
After injection	10	0.236 ± 0.042	0.185 ± 0.033	77.3 ± 6.2*

* Statistically significant variation ($P = 0.05$).

with diethylstilbestrol disulfate (Fig. 6). We found similar but much smaller increases using 5-mg doses of cortisol under presumably equivalent conditions, which included the use of 1:7 homogenates and 0.02 M phosphate buffer (Table IV). Under these conditions there was a small but statistically significant increase in the per cent saturation in rats that were killed 1 h after injection as compared with those killed immediately after injection.

When the homogenates from these two groups of animals were prepared in distilled water, the initial per cent saturation levels were 80–85%. During 1 h in

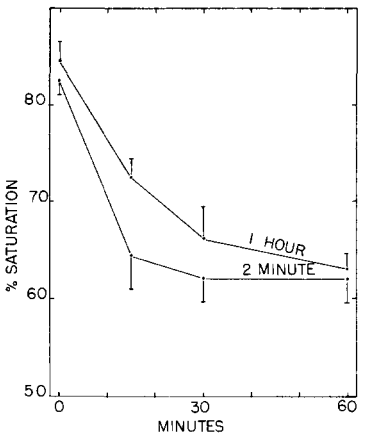

Fig. 8. The effect of cortisol injection on the association of tyrosine transaminase with cofactor in liver homogenates at 0°. Intact male rats were injected with 5 mg of cortisol and killed after 2 min or 1 h. Their livers were removed rapidly, chilled, and homogenized in 8 vol. of water. Aliquots (0.50 ml) were immediately assayed for tyrosine transaminase activity (see METHODS) in the presence and absence of 0.90 μmole of pyridoxal 5-phosphate. The rest of the homogenate was held in an ice-bath and aliquots were assayed after the indicated periods of time. The average initial activity levels were 0.350 for animals killed after 2 min and 0.375 for animals killed after 1 h. The vertical bars represent the standard deviation from the mean values for 4 2-min, and 5 1-h animals.

an ice bath, the values for the two groups declined to the same equilibrium level (Fig. 8). The rate of decline was slower, however, in the homogenates from the 1-h animals. In equivalent experiments, 5- and 10-mg doses of cortisone had no significant effect on the rate of dissociation.

As reported briefly before[4], we also attempted to determine whether homogenates from cortisol- or diethylstilbestrol disulfate-injected rats exhibited a retardation of the inactivation of tyrosine transaminase as compared to controls sacrificed immediately after injection. The data, which is not presented, indicated that homogenates from the treated animals killed 3 h after injection retained a higher percentage of the initial activity during various periods of incubation at 38°. More complete studies with the diethylstilbestrol disulfate-treated animals showed that this apparent retardation paralleled the extent of induction during a 12-h post-injection period. It was not certain, however, that this basis of comparison is valid over the wide range of initial activities occurring during induction. A more definitive test of the accumulation of stabilizing compounds in the liver of the injected rats is under investigation and is described below.

DISCUSSION

The accumulation of an enzyme *in vivo* must be due either to increased synthesis relative to destruction or decreased destruction relative to synthesis. Tyrosine transaminase is very labile *in vivo*, exhibiting a half-life of 2–3 h (ref. 18) as compared to several days for total hepatic protein[19]. It appears to have a corresponding lability *in vitro*, since the enzyme in fresh liver homogenates was rapidly inactivated by incubation at 38° (Fig. 1), and is exceptionally unstable in purified form[13]. The

observation that a number of compounds that stabilize against inactivation also induce the enzyme suggests that the *in vitro* and *in vivo* effects are related.

The effectiveness of the stabilizing agents *in vitro* (Table I) seems to be related to the presence of anionic groups and to the size of the hydrocarbon moiety. Neutral steroids and diethylstilbestrol were ineffective at saturating levels whereas most of the anionic steroids were highly effective. Monocyclic aromatic carboxylic acids were effective at concentrations near 10^{-2} M, bicyclic aromatic acids at about 10^{-3}–$2 \cdot 10^{-3}$ M, and tetracyclic acids at $5 \cdot 10^{-4}$–10^{-5} M. A somewhat similar relation seems to hold also for the induction process; tetracyclic acids were, in general, effective inducers at much lower doses than mono- or bicyclic ones[9] (Table III). These correlations suggest that the steroid and non-steroid inducers may differ in their potency but not necessarily in their mode of action.

Although rat liver rapidly converts free steroids to anionic derivatives, one must consider the possibility that injected steroid conjugates may undergo hydrolysis prior to their action. It is interesting, in this connection, that the action of diethylstilbestrol on the enzyme level *in vivo* was much weaker than that found earlier for diethylstilbestrol disulfate[9]. That anionic steroids may be active without hydrolysis is also suggested by the observation that a steroid carboxylic acid (androst-5-ene-3 β-ol-17 β-oic acid), which cannot be hydrolyzed to yield a neutral steroid, was also a fairly potent inducing agent (Table III).

It may be significant that the concentrations of the compounds required for stabilization *in vitro* is of a similar order of magnitude to the levels that one might find in the livers of the injected rats. For example, 1/10 to 1/100 of the 15 mg inducing dose of diethylstilbestrol disulfate[9], uniformly distributed in 6 ml of cellular water in the liver (livers of the 150-g rats weighed 5.5–6.5 g), would give a concentration of approx. $5 \cdot 10^{-4}$–$5 \cdot 10^{-5}$ M. Levels of diethylstilbestrol disulfate in that range were highly protective *in vitro* (Tables I, II; Figs. 6, 7) so it is logical to expect that its presence might influence the properties and fate of the enzyme *in vivo*.

Although a precise correlation of structure and action *in vitro* with effectiveness in induction cannot be expected in view of the many factors that control the fate of injected compounds, it would seem possible to predict from the effectiveness of a compound as an stabilizer *in vitro* whether it is likely to be an effective inducer. This was, in fact, the reason that indole 3-acetate was tested (and found to induce (Table III)) even though it was reported earlier to be ineffective[14]. Similarly, several other compounds were tested as inducers (Table III) on the basis of their action *in vitro* (Table I) and found to behave as predicted.

Among the most effective stabilizers of tyrosine transaminase *in vitro* are its keto acid substrates (Figs. 2, 3, and text), the coenzyme (Figs. 2, 4) and various anionic compounds that may be regarded as coenzyme or substrate analogs (Table I). The suggested specificity might serve as a guide for the selection of stabilizers that are much more effective than any tested thus far. One substrate analog that might be tested, for example, is the steroid α-keto acid, 3,20-diketo-4-pregnen-21-oic acid, which is formed from deoxycorticosterone by guinea-pig liver[20]. If such compounds were found to be highly effective their potency *in vivo* could then be tested.

Of the amino acids studied, those giving some protection (5-hydroxytryptophan, glutamate and histidine) are among the ones that have been found to be substrates or inhibitors of the enzyme[12,21]. Tryptophan, which is also a substrate[12], was inactive

at 10^{-3} M however; higher concentrations were not tested. The other amino acids tested (glycine, alanine, methionine, and α-aminoisobutyrate) were ineffective at 10^{-2} M. Phosphate and bicarbonate buffers were effective at that level, presumably as a result of the association of the buffer anions with the enzyme.

The small effects of injected cortisol (Table IV, Fig. 8) on the degree of association of pyridoxal 5-phosphate with the hepatic tyrosine transaminase in the homogenates and on the rate of dissociation suggest that the steroid, its metabolites, or non-steroid compounds that may accumulate in the liver as a result of the glucocorticoid injection, may exert stabilizing actions similar to those observed with the anionic compounds that were added *in vitro*. This type of experiment is not well suited, however, for further examination of the possible role of enzyme stabilization in the glucocorticoid-mediated induction. A more fruitful approach would be to test extracts of the livers of the glucocorticoid-injected rats for their ability to stabilize a partially-purified tyrosine transaminase preparation. Such extracts of the soluble fraction of rat liver have been reported by FIALA AND LITWAK[22] to contain a large proportion of the hepatic steroid in the form of anionic derivatives.

The study of the tryptophan-mediated induction of hepatic tryptophan oxygenase (EC 1.13.1.12) (refs. 23, 24) providess an example of mammalian enzyme induction in which the elevation of enzyme levels appears to be a result of the inhibition of enzyme degradation. L-Tryptophan and those analogs that induced the oxygenase protected it against inactivation *in vivo* as well as *in vitro*[24–26]. Further study is required to determine whether stabilization *in vitro* is related to the induction of tyrosine transaminase in a similar way.

REFERENCES

1 M. MASON AND E. GULLEKSON, *J. Am. Chem. Soc.*, 81 (1959) 1517.
2 M. MASON AND E. GULLEKSON, *J. Biol. Chem.*, 235 (1960) 1312.
3 M. MASON, *J. Biol. Chem.*, 234 (1959) 2770.
4 S. SINGER, J. FORD, L. SCHIRCH AND M. MASON, *Life Sci.*, 5 (1966) 837.
5 W. T. JENKINS, D. A. YPHANTIS AND I. W. SIZER, *J. Biol. Chem.*, 234 (1959) 51.
6 M. O. PULKKININ AND K. WILLMAN, *Steroids*, 8 (1966) 51.
7 V. SCARDI, S. MAGNO AND E. SCARANO, *Boll. Soc. Ital. Biol. Sper.*, 36 (1960) 1719.
8 V. SCARDI, M. IACCARINO AND E. SCARANO, *Biochem. J.*, 83 (1962) 413.
9 S. SINGER AND M. MASON, *Biochim. Biophys. Acta*, 110 (1965) 370.
10 S. SINGER AND M. MASON, *Biochim. Biophys. Acta*, 146 (1967) 443.
11 H. MORREN, *J. Pharm. Belg.*, 1 (1942) 129.
12 G. A. JACOBY AND B. N. LADU, *J. Biol. Chem.*, 239 (1964) 419.
13 G. LITWAK, M. L. SEARS-GESSEL AND I. WINICOU, *Biochim. Biophys. Acta*, 118 (1966) 351.
14 F. ROSEN AND R. J. MILHOLLAND, *J. Biol. Chem.*, 238 (1960) 3720.
15 O. GREENGARD AND M. GORDON, *J. Biol. Chem.*, 238 (1963) 3708.
16 F. T. KENNEY AND R. M. FLORA, *J. Biol. Chem.*, 236 (1961) 2699.
17 O. GREENGARD, *Advan. Enzyme Regulation*, 2 (1963) 277.
18 E. C. C. LIN AND W. E. KNOX, *J. Biol. Chem.*, 233 (1958) 1186.
19 D. SHEMIN AND D. RITTENBERG, *J. Biol. Chem.*, 153 (1944) 401.
20 J. SCHNEIDER, *Proc. 1st Intern. Congr. Hormonal Steroids, Milan, 1964*, Vol. 1, Academic Press, New York, 1964, p. 127.
21 Z. N. CANELLAKIS AND P. P. COHEN, *J. Biol. Chem.*, 222 (1956) 63.
22 E. S. FIALA AND G. LITWAK, *Biochim. Biophys. Acta*, 124 (1966) 260.
23 R. T. SCHIMKE, E. W. SWEENY AND C. M. BERLIN, *J. Biol. Chem.*, 240 (1965) 322.
24 R. T. SCHIMKE, W. E. SWEENY AND C. M. BERLIN, *J. Biol. Chem.*, 240 (1965) 4609.
25 J. W. DUBNOFF AND M. DIMICK, *Biochim. Biophys. Acta*, 31 (1959) 541.
26 M. CIVEN AND W. E. KNOX, *J. Biol. Chem.*, 235 (1960) 1716.