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THE EFFECTS OF THE ADMINISTRATION OF SODIUM BENZOATE AND DIETHYLSTILBESTROL DISULFATE ON THE HEPATIC LEVELS OF SEVERAL GLUCOCORTICOID-SENSITIVE ENZYMES IN ADRENALECTOMIZED RATS*

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SUMMARY

1. In a previous study the administration of benzoate, α-naphthoate, diethylstilbestrol disulfate and various aromatic carboxylic acids to adrenalectomized rats was shown to increase hepatic tyrosine transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) levels several fold. The action of benzoate was strongly inhibited by the concurrent administration of puromycin or actinomycin D. In the present study, the administration of actinomycin D strongly inhibited the increase in tyrosine transaminase activity caused by the administration of diethylstilbestrol disulfate or α-naphthoate to adrenalectomized rats. In this respect the effect of these aromatic acids resembles that of the glucocorticoids.

2. To further compare the effects of cortisol and the aromatic acids *in vivo*, several other systems known to be sensitive to the glucocorticoids were examined. Cortisol administration, under the indicated conditions, significantly increased the hepatic levels of glycogen, tryptophan oxygenase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12), D-amino acid oxidase (D-amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3), and L-threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16). Under equivalent conditions doses of diethylstilbestrol disulfate and benzoate that increased hepatic tyrosine transaminase levels had no effect on hepatic glycogen and tryptophan oxygenase levels. Benzoate had no effect on hepatic D-amino acid oxidase levels, but increased L-threonine dehydratase levels.

INTRODUCTION

The administration of glucocorticoids[1,2], pyridoxine[3], L-tryptophan[4], and various indole derivatives[4] to adrenalectomized rats results in increased hepatic

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levels of tyrosine transaminase (L-tyrosine:2-oxoglutarate amino transferase, EC 2.6.1.5). In an earlier report[5] we demonstrated that various aromatic acids and diethylstilbestrol disulfate caused a similar effect. Like the glucocorticoid-mediated induction of tyrosine transaminase[2], the effect of benzoate was prevented by the simultaneous injection of puromycin or actinomycin D (ref. 5). The present study, designed to further compare the actions of these non-steroid inducers* with those of cortisol, deals with the effects *in vivo* of benzoate and diethylstilbestrol disulfate on the levels of other hepatic enzymes and on hepatic glycogen deposition.

MATERIALS AND METHODS

Chemicals

α-Ketoglutarate, pyridoxal 5-phosphate, L-tyrosine, DL-threonine, and D-alanine were purchased from Nutritional Biochemical Corp.; actinomycin D was a gift from Merck, Sharp and Dohme. Sodium pyruvate, FAD, cortisol, and diethylstilbestrol were obtained from the Sigma Chemical Co., L-kynurenine sulfate from the California Corporation for Biochemical Research, and anthrone from Eastman Organic Chemicals, Inc. All solutions used for intraperitoneal treatment were neutralized to pH 7.4 and administered in volumes proportional to 1 ml of solution per 150 g body weight. For studies using the intramuscular route of administration, volumes of 0.20–0.40 ml were administered.

Animals

In most of the experiments described below, male albino rats, weighing 125–130 g were obtained from the Holtzman Rat Co., Madison, Wisc., or from Rawley Farms, Plymouth, Mich. They were adrenalectomized and maintained as described earlier[5]. The test for the completeness of adrenalectomy was also described[5].

METHODS

Tyrosine transaminase induction

For these studies the conditions and method of assay were essentially the same as described previously[5]. However, homogenates were made in 14 vol. of cold distilled water. Activity is given as the absorbance change produced in 10 min, as measured by the assay of 2.00 ml of deproteinized reaction mixture for *p*-hydroxyphenylpyruvate by a modification[5] of the Briggs method.

Glycogen deposition and induction of tryptophan oxygenase (L-tryptophan: oxygen oxidoreductase, EC 1.13.1.12)

Each adrenalectomized rat was given 6 g of Rockland Mouse/Rat food twice daily, at 9 a.m. and 6 p.m., for 7 days. All uneaten food was removed 90 min after each feeding. On the day preceding sacrifice, food was removed at 7 p.m. After

* 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 in this report to all administered compounds that cause increased activity as inducers, and to the increase in activity as induction, unless kinetic factors other than apoenzyme concentration are known to be responsible for the increase.

15–17 h, the animals were injected intraperitoneally with saline, cortisol, benzoate, or diethylstilbestrol disulfate. At 0–6 h after injection, they were anesthetized with 1.00 ml of 4% hexobarbital, given intraperitoneally, and their livers were rapidly removed. About 3 g of liver were immediately frozen in isopentane–dry ice and used for the determination of glycogen. The remaining tissue was chilled immediately homogenized in 10 times its weight of ice-cold 0.14 M KCl containing 2.5 mmoles of KOH per l (ref. 6), and used for the determination of tryptophan oxygenase activity by a method described below.

The method of glycogen assay was a modification of the methods of VAN DER VRIES[7] and of CORI[8]. The frozen liver was weighed rapidly. Two ml of hot 30% KOH were added for each gram of frozen liver and the mixture was digested for 40 min on a boiling-water bath. The hot liver digests were centrifuged at 1000 rev./min for 10 min and the pellet was discarded. 3-ml aliquots of the supernatant fluid were combined with 0.20 ml of saturated Na_2SO_4 followed by 4 ml of 95% ethanol. The mixture was brought to a boil, and then cooled on ice for 10 min. Following centrifugation at 1000 rev./min for 10 min, the supernatant fluid was removed and the tube was thoroughly drained. The pellet was redissolved in 3 ml of water and precipitated as before. The white precipitate of glycogen was redissolved and assayed by a modified anthrone method[9], using a glucose standard. Glycogen levels are expressed as mg of glucose equivalent per g of liver.

For the assay of tryptophan oxygenase activity, 2.80 or 3.30 ml of reaction mixture containing 200 μmoles of sodium phosphate (pH 7.0) and 100 μmoles of L-tryptophan (pH 7.0)[6,10] was equilibrated for 5 min at 37°. The reaction was started by the addition of 1.00 or 0.50 ml of fresh whole homogenate. In 1 h the reaction was terminated by the addition of 2.00 ml of 15% (w/v) metaphosphoric acid. After filtration, 2.00-ml aliquots of filtrate were combined with 1.00 ml of 1 M NaOH and the absorbance at 365 mμ was determined. Enzyme activity is expressed as μmoles of L-kynurenine formed per g of liver per h.

The induction of D-*amino acid oxidase (*D-*amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3)*

Immediately after adrenalectomy injections were started. The rats were divided into three groups which were injected intramuscularly twice daily with sodium benzoate or cortisol in 0.25 ml of diluent (saline or corn oil) or with an equivalent amount of diluent. On the morning of the fourth day of treatment, the animals were sacrificed by anesthesia with 1.0 ml of 4% hexobarbital administered intraperitoneally. Their livers were removed rapidly, chilled, and homogenized in 4 vol. of 1.15% KCl (pH 8.3). The resulting homogenates were centrifuged briefly at 4000 $\times$ *g*, at 5°, to remove cell debris.

The supernatant fluid was diluted 1:1 with the homogenizing medium and 0.20- and 0.40-ml aliquots were added to 3.00 ml or 2.8 ml of a reaction mixture[11] which contained 100 μmoles of potassium pyrophosphate buffer (pH 8.3) and 0.30 μmole of FAD. After 5 min at 37° the reaction was initiated by the addition of 0.50 ml of 5% D-alanine. At 0 and 40 min, 0.30 ml of 100% (w/v) trichloroacetic acid was added to stop the reaction. After filtration, 1.0-ml aliquots of filtrate were assayed for pyruvate by a modification of the method of FRIEDEMANN AND HAUGEN[12] in which absorbance at 450 mμ is compared to that produced by a known amount

of pyruvate. Protein was determined by the LOWRY method[13]. Enzyme activity is expressed as μmoles of pyruvate produced per h per mg of protein.

The induction of L-threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16)

Beginning immediately after adrenalectomy, rats were injected intramuscularly each day for 5 days with 0.25 ml of 0.9% saline or the same amount of saline containing sodium benzoate or cortisol. On the sixth day, they were sacrificed by decapitation 3.5–5 h after a final injection. Their livers were removed and homogenized in 4 vol. of ice-cold 0.20 M potassium phosphate buffer (pH 8.0) containing 0.01 M mercaptoethanol[14]. The homogenates were centrifuged briefly at 4000 $\times$ g to remove cell debris, and the supernatant solutions were then diluted 1:1 with the homogenization medium. The diluted homogenate, 0.40- and 0.80-ml aliquots, was added to 3.30 ml or 2.9 ml of reaction mixture containing 800 μmoles of potassium phosphate buffer (pH 8.1) and 1.6 μmole of pyridoxal phosphate[14]. After incubation for 5 min at 37°, the reaction was started by the addition of 500 μmoles of DL-threonine in 1.00 ml of water (pH 8.0). At 0 and 10 min, the reaction was stopped by the addition of 0.40 ml of 100% (w/v) trichloroacetic acid. The reaction mixture was filtered and assayed for keto acid by the method described previously for D-amino acid oxidase. Enzyme activity is expressed as μmoles of keto acid formed per 10 min per mg of liver protein.

RESULTS

The effect of actinomycin D administration on the induction of hepatic tyrosine transaminase by α-napthoate and diethylstilbestrol disulfate

The administration of puromycin or actinomycin D markedly inhibits the benzoate-mediated induction of hepatic tyrosine transaminase in adrenalectomized rats[5]. It was assumed, therefore, that the effect of benzoate on this enzyme required intact protein- and RNA-synthesizing systems. In order to determine whether these requirements apply to the inducing actions of other aromatic acids, actinomycin D was administered to adrenalectomized rats along with diethylstilbestrol disulfate or α-napthoate. Similar inhibitory effects on the inductions mediated by these compounds were observed (Table I).

The effect of benzoate, diethylstilbestrol disulfate and cortisol administration on several other parameters of glucocorticoid action

Sodium benzoate, α-naphthoate and diethylstilbestrol disulfate resemble cortisol in its ability to elevate hepatic tyrosine transaminase levels in adrenalectomized rats, and in the sensitivity of the response to the administration of actinomycin D (ref. 5) (Table I). For further comparison with the action of injected cortisol, we examined the effect of benzoate and diethylstilbestrol disulfate on several other biological systems that were known to be affected by glucocorticoid administration.

The effect on hepatic tryptophan oxygenase and glycogen levels

Increases in both tryptophan oxygenase levels[15], and in glycogen deposition[16,17]

TABLE I

INHIBITION OF THE α-NAPHTHOATE- AND DIETHYLSTILBESTROL DISULFATE-MEDIATED INDUCTION OF TYROSINE TRANSAMINASE BY THE SIMULTANEOUS ADMINISTRATION OF ACTINOMYCIN D

In *Expt. I*, adrenalectomized rats were injected intraperitoneally with 1.0 ml of 0.9% NaCl, an equal volume of saline containing 30 mg of α-naphthoate, or an equal volume containing 30 mg of α-naphthoate and 0.20 mg of actinomycin D (per 150 g body wt.). In *Expt. II*, α-naphthoate was replaced with 12 mg of diethylstilbestrol disulfate. *Expt. III* was the same as *Expt. II* except that 0.30 mg of actinomycin D was used. In each case, the animals were sacrificed by decapitation 3 h after injection. Activity data are given as the average absorbance ± the standard deviation (see METHODS). The numbers in parentheses indicate the number of animals used. The percent inhibition is defined as $100 \times [1-(B-1)/(A-1)]$.

Treatment	*Activity*	*Fold induction***	*Inhibition (%)*
Expt. I			
Saline	0.086 ± 0.007 (3)†		
α-Naphthoate	0.282 ± 0.040 (3)*	(A) 3.30	
α-Naphthoate + actinomycin D	0.160 ± 0.032 (3)*,†	(B) 1.86	63
Expt. II			
Saline	0.084 ± 0.005 (2)†		
Diethylstilbestrol disulfate	0.212 ± 0.019 (2)*	(A) 2.52	
Diethylstilbestrol disulfate + actinomycin D	0.118 ± 0.002 (3)*,†	(B) 1.40	73
Expt. III			
Saline	0.084 ± 0.007 (4)†		
Diethylstilbestrol disulfate	0.197 ± 0.014 (4)*	(A) 2.35	
Diethylstilbestrol disulfate + actinomycin D	0.092 ± 0.006 (4)†	(B) 1.09	93

* Statistically significant variation from the saline control.

** Fold induction is defined as experimental activity/control activity.

† Statistically significant variation from the diethylstilbestrol disulfate-treated or α-naphthoate-treated group.

have been reported to occur within 3–5 h after the administration of glucocorticoids to adrenalectomized rats. Sodium benzoate was tested concurrently with cortisol for its ability to induce changes in these two systems (Table II). Cortisol treatment (5 mg/150 g, administered intraperitoneally) induced increases in hepatic tryptophan oxygenase and glycogen levels that were obvious within 3 h and reached 8.7- and 74-fold maxima within 6 h. Sodium benzoate administration did not cause any significant changes in either of these two parameters, in the time interval studied, at dosages which induced tyrosine transaminase. Diethylstilbestrol disulfate (Table III) was also ineffective at concentrations which caused 3-fold increases in tyrosine transaminase levels.

The effect on hepatic D-*amino acid oxidase levels*

Several reports[18,19] indicate that treatment of adrenalectomized rats with cortisone for several days is followed by increases of hepatic D-amino acid oxidase levels. Our preliminary experiments showed no changes in the hepatic levels of this enzyme after a single dose of benzoate or cortisol. Daily intramuscular injection of cortisol (10 mg/150 g) in corn oil or saline, for several days, caused 1.93- and 2.3-fold inductions of hepatic D-amino acid oxidase activity (Table IV). Similar treatment with sodium benzoate (40 mg/150 g) did not significantly change hepatic D-amino acid oxidase levels as compared to saline-injected controls.

TABLE II

THE EFFECT OF INTRAPERITONEAL CORTISOL OR SODIUM BENZOATE ADMINISTRATION ON HEPATIC GLYCOGEN CONTENT AND HEPATIC TRYPTOPHAN OXYGENASE ACTIVITY IN THE ADRENALECTOMIZED RAT

One ml of physiological saline per 150 g body wt., or equivalent volumes of saline containing 16 mg of sodium benzoate or 5 mg of cortisol were administered intraperitoneally at zero time. At the indicated time the animals were sacrificed and their livers were assayed for tryptophan oxygenase and glycogen as described under METHODS. The data are given as the average activity ± the standard deviation.

Expt. No.	*Treatment*	*Time of sacrifice (h)*	*Glycogen*			*Tryptophan oxygenase*		
			Number of animals	*(mg/g) liver*	*Fold induction*	*Number of animals*	*Activity*	*Fold induction*
1	Saline	0	3	0.155 ± 0.017		3	0.79 ± 0.095	
2	Saline	1	3	0.140 ± 0.007		3	0.79 ± 0.041	
	Benzoate		3	0.126 ± 0.070	0.90	4	0.83 ± 0.093	1.1
	Cortisol		3	0.371 ± 0.060*	2.7	4	1.15 ± 0.25	1.5
3	Saline	3	3	0.103 ± 0.016		4	0.72 ± 0.14	
	Benzoate		6	0.121 ± 0.035	1.2	7	0.83 ± 0.20	1.2
	Cortisol		3	2.88 ± 0.250	24	4	2.92 ± 0.67*	4.1
4	Saline	3.5	3	0.124 ± 0.011				
	Benzoate		3	0.101 ± 0.035	0.81			
	Cortisol		3	4.53 ± 1.25*	37			
5	Saline	4.5	5	0.118 ± 0.031		3	0.58 ± 0.15	
	Benzoate		5	0.116 ± 0.027	1.0	5	0.80 ± 0.12	1.4
	Cortisol		4	8.40 ± 1.40*	72	4	4.51 ± 0.68*	8.0
6	Saline	6.0	3	0.125 ± 0.008		3	0.79 ± 0.008	
	Benzoate		4	0.127 ± 0.002	1.0	3	0.84 ± 0.075	1.1
	Cortisol		4	9.27 ± 0.780*	74	3	6.90 ± 0.57*	8.7

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

TABLE III

THE EFFECT OF DIETHYLSTILBESTROL DISULFATE ON GLYCOGEN AND TRYPTOPHAN OXYGENASE LEVELS IN THE LIVERS OF ADRENALECTOMIZED RATS

Physiological saline (1.0 ml/150 g body wt.) or equivalent volumes of physiological saline containing 15 mg of diethylstilbestrol disulfate per 150 g body weight were injected intraperitoneally at zero time. The animals were sacrificed at the indicated time and their livers were assayed for tryptophan pyrrolase and glycogen levels, as described under METHODS. The data are expressed as the average value ± the standard deviation. The numbers in parentheses are the numbers of animals used.

Treatment	*Time (h)*	*Tryptophan oxygenase*		*Glycogen*	
		Activity	*Fold induction*	*(mg/g) liver*	*Fold induction*
Saline (2)	3.5	0.74 ± 0.065		0.097 ± 0.002	
Diethylstilbestrol sulfate (4)	3.5	0.70 ± 0.071	0.95	0.097 ± 0.010	1.0
Saline (2)	5.0	0.83 ± 0.15		0.096 ± 0.003	
Diethylstilbestrol sulfate (4)	5.0	0.74 ± 0.096	0.89	0.095 ± 0.010	1.0

thanol. La Fig. 1 montre la phase initiale d'activation lors de l'hydrolyse trypsique de la LEE en présence de méthanol.

Dans ces conditions, pour déterminer une vitesse initiale qui ait un sens, il est absolument nécessaire d'effectuer la mesure au tout début de la réaction (pour moins de 2% de réaction, soit un degré d'avancement $\xi < 0.02$) (réf. 2).

La Fig. 2 montre l'allure globale d'une cinétique d'hydrolyse avec transesterification. La cinétique n'est plus du tout d'ordre I, comme l'indique la représentation log $(a - z)/a$ en fonction de t (Fig. 3).

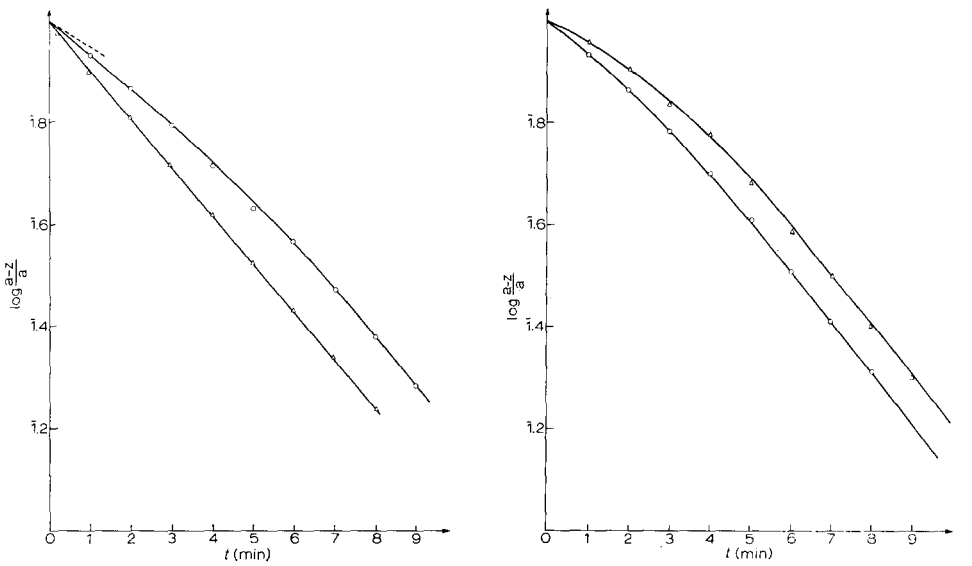

Fig. 3. Cinétique de la transesterification LEE → LME. Transformée logarithmique: log $(a - z)/a$ en fonction de t (voir texte). ○, LEE; △, LME. Conditions strictement identiques voir la figure précédente.

Fig. 4. Cinétique de transesterification en présence de butanol et de pentanol. Transformée logarithmique log $(a - z)/a$ en fonction de t (voir texte). △, pentanol, 0.37 M; ○, butanol, 0.437 M. LEE, 0.8 mM (pH 4.80); $CaCl_2$, 25 mM; temp. 25°; trypsine, 11.3 μM.

Par contre, la cinétique d'hydrolyse de la LME dans les mêmes conditions est rigoureusement d'ordre I (Fig. 3).

Conformément à la discussion de l'Eqn. 9, les pentes des deux courbes sont identiques à la fin de la réaction. Ceci indique que pour un degré d'avancement suffisant de la réaction la transformation LEE → LME est pratiquement complète dans nos conditions ($k_4 N \sim 4\, k'_3$).

Il est donc possible de calculer à partir de telles cinétiques, la constante de vitesse correspondant à l'hydrolyse de l'entité formée au cours de la transesterification (LME ici).

Ainsi, à partir d'un seul ester, il est possible d'estimer les paramètres cinétiques d'autres esters du même acide sans avoir besoin de les synthétiser, à condition bien entendu, de disposer de l'alcool correspondant.

II. Transesterification avec d'autres alcools

Nous avons également obtenu des cinétiques d'activation avec d'autres alcools aliphatiques normaux primaires, le butanol et le pentanol ainsi qu'avec l'alcool benzylique (Figs. 4 et 5).

Nos résultats indiquent clairement que les esters formés par la lysine avec ces alcools sont hydrolysés plus rapidement par la trypsine que la LEE, dans des conditions où $K_m \gg S$.

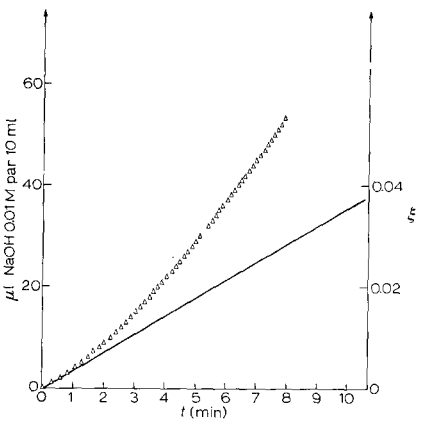

Fig. 5. Hydrolyse trypsique de la LEE en présence d'alcool benzylique. La transesterification LEE → LBzE se traduit par une activation très importante (>100%). LEE, 1 mM (pH 4.80); $CaCl_2$, 25 mM; temp. 25°; alcool benzylique, 0.194 M; trypsine, 1.22 μM.

Ceci est assez surprenant si on se réfère aux vitesses d'hydrolyse alcaline de divers esters acétiques aliphatiques pour lesquels aucun effet de ce type n'est observé[9,10].

Ce phénomène semble donc caractéristique de l'hydrolyse enzymatique; il peut probablement être interprété en termes d'interactions non covalentes enzyme-substrat.

Nos résultats sont en accord avec les travaux de GLAZER qui a pu mettre en évidence de telles transesterifications en isolant l'intermédiaire formé par électrophorèse ou chromatographie. Cet auteur a d'ailleurs montré que de telles transesterifications[3] s'observent également avec la chymotrypsine (EC 3.4.4.5), la subtilisine[4] (EC 3.4.4.16) et la papaïne[5] (EC 3.4.4.10).

APPENDICE

La résolution du système décrit par le Schéma I à l'état stationnaire conduit aux expressions suivantes pour

$$-\frac{dS}{dt}, \frac{dS^*}{dt} \text{ et } \frac{dP_2}{dt} :$$

$$-\frac{dS}{dt} = \frac{\dfrac{k_2\,(k^*_{-2}\,P^*_1 + k_3')\,E_t}{k_2 + k^*_2\,\dfrac{K_S}{K^*_S}\,\dfrac{S^*}{S} + k^*_{-2}\,P^*_1 + k_3'}}{1 + \dfrac{K_S}{S}\,\dfrac{k^*_{-2}\,P^*_1 + k_3'}{k_2 + k^*_2\,\dfrac{K_S}{K^*_S}\,\dfrac{S^*}{S} + k^*_{-2}\,P^*_1 + k_3'}\left(1 + \dfrac{S^*}{K^*_S}\right)}$$

$$\frac{dS^*}{dt} = \frac{\dfrac{k_2\,k^*_{-2}\,P^*_1 - k^*_2\,k_3'\,\dfrac{K_S}{K^*_S}\,\dfrac{S^*}{S}}{k_2 + k^*_2\,\dfrac{K_S}{K^*_S}\,\dfrac{S^*}{S} + k^*_{-2}\,P^*_1 + k_3'}\,E_t}{1 + \dfrac{K_S}{S}\,\dfrac{k^*_{-2}\,P^*_1 + k_3'}{k_2 + k^*_2\,\dfrac{K_S}{K^*_S}\,\dfrac{S^*}{S} + k^*_{-2}\,P^*_1 + k_3'}\left(1 + \dfrac{S^*}{K^*_S}\right)}$$

$$\frac{dP_2}{dt} = \frac{\dfrac{k_3'\left(k_2 + k^*_2\,\dfrac{K_S}{K^*_S}\,\dfrac{S^*}{S}\right)}{k_2 + k^*_2\,\dfrac{K_S}{K^*_S}\,\dfrac{S^*}{S} + k^*_{-2}\,P^*_1 + k_3'}\,E_t}{1 + \dfrac{K_S}{S}\,\dfrac{k^*_{-2}\,P^*_1 + k_3'}{k_2 + k^*_2\,\dfrac{K_S}{K^*_S}\,\dfrac{S^*}{S} + k^*_{-2}\,P^*_1 + k_3'}\left(1 + \dfrac{S^*}{K^*_S}\right)}$$

RÉSUMÉ

La compétition nucléophile entre l'eau et ses analogues dans les réactions d'hydrolyse enzymatique peut se traduire par l'apparition de nouveaux produits hydrolysables par l'enzyme. Les produits ainsi formés peuvent perturber le cours de la cinétique d'hydrolyse du substrat initial.

A partir d'un schéma général mettant en jeu un intermédiaire covalent comme l'acylenzyme, on obtient des équations simples lorsque les concentrations du substrat initial et des espèces formées par compétition nucléophile sont petites devant leurs constantes de Michaelis apparentes respectives.

Ces équations ont été vérifiées dans le cas particulier de l'hydrolyse trypsique de l'ester éthylique de la lysine en présence de divers alcools.

L'analyse de ces cinétiques montre que le paramètre k_2/K_S d'hydrolyse trypsique des esters benzylique, butylique et pentylique de la lysine est supérieur à celui de son ester éthylique.