10. Hirsch, J. G., ibid., 1958, v108, 925.

11. Burger, W. C., Stahmann, M. A., Arch. Biochem. Biophys., 1952, v39, 27.

12. Sela, M., Katchalski, E., Advances in Prot. Chem., 1959, v14, 391.

13. Jeffries, C., Holtman, D., Guse, D., J. Bact., 1957, v73, 590.

14. Maver, M., Greco, A., J. Biol. Chem., 1949, v181, 853.

15. Fischer, N., Wagner, L., Naturwissenshaften, 1954, v41, 533.

16. Allfrey, V. G., Littau, V. C., Mirsky, A. E., Proc. Nat. Acad. Sci., 1963, v49, 414.

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Effects of Acid Catabolites on Activity *in vitro* of Phenylalanine Hydroxylase from Rat Liver.* (29776)

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Wang and Waisman(1) demonstrated a significant decrease of phenylalanine hydroxylase in male rats which received high levels of phenylalanine for 3 weeks. Woods and McCormick(2) confirmed these findings, quantitated more exactly the amount of phenylalanine necessary to produce a given lowering in hydroxylase activity, and indicated that the primary decrease in measured activity may not be due to accumulation of catabolites directly inhibitory to the enzyme, since loss in activities upon dialysis of preparations from both experimental and control rats is approximately the same. However, such indirect evidence does not preclude some secondary inhibition of the hydroxylase caused by certain of the phenylalanine catabolites, *e.g.*, phenylpyruvate. The potential influence of accumulated levels of catabolites and similar compounds has not been sufficiently assessed, although Udenfriend and Cooper(3) showed that even pyruvate added in a concentration equimolar to substrate phenylalanine markedly inhibits the hydroxylase, and recently Ross and Haljasmaa(4) found that esculetin and some dihydroxyphenylacetamide derivatives are quite active inhibitors of the enzyme *in vitro*. In addition, the relative ability of the pteridine cofactor of phenylalanine hydroxylase, characterized by Kaufman(5), in effecting the degree of any such inhibition has not been defined, though Gal *et al*(6) showed a differential inhibition of the hydroxylase is relieved by a synthetic analogue of the cofactor.

The present investigation was undertaken to assess the influence of common acids and catabolites of phenylalanine on phenylalanine hydroxylase, to quantitate any inhibition of crude and purified enzyme in the absence and presence of additional cofactor, and to characterize the nature of any such inhibition elicited.

Materials and methods. The organic acids were obtained from Eastman Organic Chemicals. 6,7-Dimethyl-5,6,7,8-tetrahydropteridine, a synthetic cofactor for phenylalanine hydroxylation, was purchased from Calbiochem. Young, male Sprague-Dawley rats were fed *ad libitum* on commercial diet. For measuring activities of crude phenylalanine hydroxylase in supernatant solutions from liver homogenates, preparations were made according to Kenney *et al*(7); partially purified enzyme was prepared by carrying the fractionation through an ammonium sulfate precipitation as described by Kaufman(8). Incubation mixtures were made generally according to Freedland *et al*(9), and contained 10^{-3} M L-phenylalanine, 10^{-4} M reduced diphosphopyridine nucleotide, 2.5×10^{-3} M nicotinamide, 3.3×10^{-4} M pteridine cofactor when added, and enzyme in 0.1 M potassium phosphate buffer, pH 6.7. Tyrosine formation was determined by the colorimetric method of Udenfriend and Cooper (10).

Results. The effects of phenylalanine ca-

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tabolites and common acids with similar structural features on activity of crude and partially purified phenylalanine hydroxylase with and without synthetic cofactor are shown in Table I. In general, sensitivity of the hydroxylase to inhibition increases with purification, but is decreased by additional pteridine cofactor. The enzyme is insensitive to benzoate and acetate at concentrations of these compounds (10^{-3} M) which are in large, non-physiological excess over phenylalanine substrate (10^{-4} M). Acids which bear hydroxyl substituents in the *alpha* position of the alkyl chain (Mandelate, lactate, and phenyllactate) or *para* position in the phenyl ring (*p*-hydroxyphenyllactate, and *p*-hydroxyphenylpyruvate) are relatively inactive with crude enzyme, with the exception of the less typical example of *p*-hydroxybenzoate. However, as shown previously by McCormick *et al*(11), the actual amount of tyrosine formed is difficult to measure when certain *p*-hydroxyphenyl acids are tested in relatively large amounts because of their contribution to the color reaction. Pyruvate proved to be the most potent inhibitor tested, and among the natural acid catabolites of phenylalanine, only homogentisate and phenylpyruvate elicited significant inhibition regardless of purity of enzyme or amount of cofactor added.

TABLE I. Effects of Compounds on Activity *in vitro* of Phenylalanine Hydroxylase from Rat Liver.

Compounds added* (10^{-3} M)	% Inhibition of hydroxylase activity†		
	Crude	Partially purified −Cofactor	Partially purified +Cofactor
Benzoate	0	0	0
p-Hydroxybenzoate	40	44	12
Acetate	0	0	0
Phenylacetate	52	42	0
Mandelate	0	48	60
Homogentisate	40	42	24
Hydrocinnamate	16	46	0
Lactate	0	48	0
Phenyllactate	1	48	0
p-Hydroxyphenyllactate	0(+)	0(+)	0(+)
Pyruvate	87	80	78
Phenylpyruvate	31	56	6
p-Hydroxyphenylpyruvate	0(+)	28(+)	54(+)

* Added as fresh solutions of sodium salts.

† Duplicate experiments were run to obtain values ± 10% of the mean. Plus signs in parentheses indicate these compounds enhance color due to apparent tyrosine in the assay.

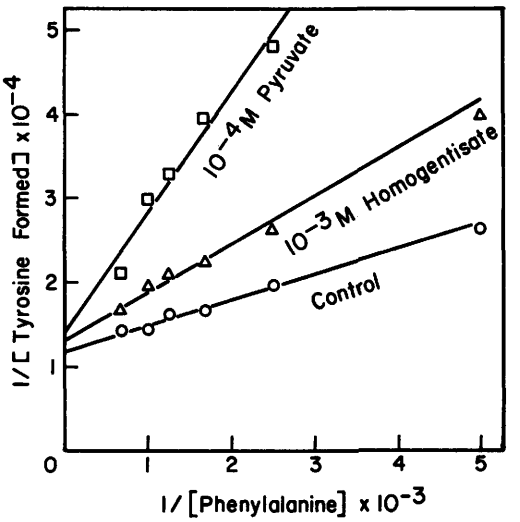

FIG. 1. Inhibition of phenylalanine hydroxylase by pyruvate and homogentisate. Supernatant solution from rat liver without added pteridine cofactor was used.

The inhibition of phenylalanine hydroxylase by pyruvate and homogentisate with varying concentrations of phenylalanine is shown by the double-reciprocal plots in Fig. 1. The compounds exhibit inhibition of a near competitive type wherein sufficient substrate is largely able to overcome the effect, and the same maximum rate of tyrosine production is approached. From these data, the Km value for phenylalanine is calculated as 2.7×10^{-4} M; K_i values for pyruvate and homogentisate are 2.9×10^{-5} and 1.2×10^{-3} M respectively. In relation to phenylalanine, these values may reflect that affinity of the enzyme is greater for pyruvate and less for homogentisate.

Discussion. Although certain phenylalanine catabolites, such as phenylpyruvate and homogentisate, and closely related compounds cause some inhibition of phenylalanine hydroxylase *in vitro,* the amounts necessary to reduce significantly the activity even of partially purified enzyme is greater than the amount of substrate added and hence far greater than the amounts of catabolites which can be formed from the substrate under a normal physiological situation. Up to a point, increased amounts of phenylalanine

tend to relieve such inhibition. It would appear improbable, then, for the decrease of hydroxylase activity seen under conditions of experimental phenylketonuria, where excess substrate is administered, to be mainly accounted for in terms of catabolite inhibitions. The data of Udenfriend and Cooper(3) show a decrease of approximately 30% of hydroxylase activity when substrate in 10-fold excess of the optimum is added. This indicates that substrate choking of the hydroxylase's active site is of the same order of magnitude as the relatively weak inhibition shown herein for most catabolites. The value of Km reported for phenylalanine by these authors is in good agreement with that found in the present investigation. Also in phenylketonuria, whether of genetic or dietary origin, the fault is not with insufficient cofactor, but apparently enzyme *per se*. The greater inhibition observed with partially purified enzyme alone is somewhat overcome in those instances *in vitro* where protective cofactor is present, *i.e.*, crude hydroxylase and partially purified hydroxylase plus added cofactor. These latter are more representative in the state *in vivo*.

Summary. The activity of phenylalanine hydroxylase from rat liver can be inhibited *in vitro* by several organic acids including a few catabolites of phenylalanine, *e.g.*, phenylpyruvate and homogentisate. However, the concentrations of such compounds necessary to produce significant inhibition of a generally competitive nature is far in excess of physiological amounts, and the effects can be minimized when an adequate amount of pteridine cofactor is present. Thus, the lowered activity of hydroxylase measured in experimental phenylketonuria may be attributed to a real suppression of enzyme synthesis, rather than inhibition by catabolites.

1. Wang, H. L., Waisman, H. A., Proc. Soc. Exp. Biol. and Med., 1961, v108, 332.
2. Woods, M. N., McCormick, D. B., ibid., 1964, v116, 427.
3. Udenfriend, S., Cooper, J. R., J. Biol. Chem., 1952, v194, 503.
4. Ross, S. B., Haljasmaa, O., Life Sci., 1964, v3, 579.
5. Kaufman, S., Proc. Nat. Acad. Sci. U. S., 1963, v50, 1085.
6. Gal, E. M., Chatterje, S. K., Marshall, F. D., Biochim. Biophys. Acta, 1964, v85, 495.
7. Kenney, F. T., Reem, G. H., Kretchner, N., Science, 1958, v127, 86.
8. Kaufman, S., in Colowick, S. P., Kaplan, N. O., ed., Methods in Enzymology, Academic Press, Inc., 1962, v5, 814.
9. Freedland, R. A., Krakowski, M. C., Waisman, H. A., Am. J. Physiol., 1962, v202, 145.
10. Udenfriend, S., Cooper, J. R., J. Biol. Chem., 1952, v196, 227.
11. McCormick, D. B., Young, S. K., Woods, M. N., Clin. Chim. Acta, 1964, in press.

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Effect of Estradiol Upon Oxytetracycline Utilization as Related to Blood Calcium.* (29777)

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Estradiol, an estrogenic hormone, has been shown to increase blood calcium in young chicks(1). It was later found that injecting chicks with estradiol increased the oxytetracycline (OTC) blood serum level(2). Elevated OTC blood serum levels were obtained in laying hens which were injected with 3 mg of estradiol for 3 consecutive days prior to taking blood(3). Hens which received estradiol injections were also found to have higher blood calcium levels than non-injected hens.

The purpose of this work was to examine further the possible activity of estradiol as an antibiotic potentiator and correlate its

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