BBA 65783

PHENYLALANINE HYDROXYLASE ACTIVITY TOWARDS TWO SUBSTRATES SIMULTANEOUSLY: ENHANCEMENT OR INHIBITION BY PHENYLALANINE, TRYPTOPHAN AND THEIR DERIVATIVES

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(Received April 8th, 1968)

SUMMARY

1. The activity of a rat liver preparation of phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) towards two substrates simultaneously, has been studied.

2. For pairs of substrates chosen from the following, the degree of hydroxylation of each in the presence of increasing amounts of the other was measured; L-phenylalanine, L-tryptophan, 4-fluoro-DL-phenylalanine and 5-fluoro-DL-tryptophan.

3. The hydroxylation of L-phenylalanine and L-tryptophan was increasingly inhibited by increased concentrations of their respective fluoro derivatives. However, even high concentrations of tryptophan and fluorotryptophan only inhibited 30% of the hydroxylating activity towards phenylalanine. The presence of these indolic amino acids enhanced by 70% the hydroxylation of fluorophenylalanine (replacement of -F by -OH), and similarly phenylalanine or fluorophenylalanine enhanced the 5-hydroxylation of tryptophan.

4. These results are reconciled with those of other workers. A model for the phenylalanine hydroxylase system is proposed, with separate hydroxylating sites for benzenoid and indolic substrates, hydroxylation at each site being allosterically modified by binding at the other site.

INTRODUCTION

In 1963, FREEDLAND[1], reported that experiments on the induction and repression of rat liver phenylalanine-4-hydroxylase (L-phenylalanine, tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) and of tryptophan-5-hydroxylase activity showed a very high degree of correlation. He concluded that although these two activities had different activators and inhibitors they must be mediated by a single protein.

In a previous communication[2] we reported some studies of the inhibition of

these activities by 5-fluorotryptophan which led us to suggest that there might be more than one hydroxylating enzyme involved.

In this paper we report the results of more detailed experiments using two of the following substrates simultaneously: L-phenylalanine, L-tryptophan, 4-fluoro-DL-phenylalanine and 5-fluoro-DL-tryptophan. The effects of these substances on the rate of hydroxylation of each other by rat liver preparations are very complex and unusual and in the discussion some suggestions to explain them are made. A preliminary account of this work has appeared[3].

The most stable and highly purified preparation of phenylalanine-4-hydroxylase that has been reported is that of GUROFF AND ITO[4] from *Pseudomonas sp.* ATTC 11299a. However, as we were particularly interested in relating these studies to phenylketonuria we preferred to use the mammalian enzyme. KAUFMAN[5] has reported a partially purified enzyme from rat liver which is moderately stable, but his incubation mixture requires the further addition of sheep liver and beef heart preparations for full activity.

In this work we used 100 000 × *g* supernatant or ammonium sulphate-precipitated fractions similar to those used by MITOMA[6].

Though relatively crude, these preparations had the advantage of considerable reproducibility, which was not found after further purification.

MATERIALS

6,7-Dimethyl-5,6,7,8-tetrahydropterin hydrochloride was obtained from Calbiochem Ltd.

The tyrosine decarboxylase used was a *Streptococcus faecalis* preparation (type I Sigma).

Generally-labelled [^{14}C]phenylalanine was obtained from the Radiochemical Centre, Amersham.

METHODS

(a) The hydroxylating system

The enzyme preparations used for the hydroxylations were either ammonium sulphate-precipitated fractions (Fraction I from 33–40% and Fraction II from 50–60%) from rat liver homogenate, similar to those described by MITOMA[6] or the supernatant fraction obtained after centrifuging a rat liver homogenate (1 part liver to 2 parts 0.15 M KCl at pH 7) at 100 000 × *g* for 1 h.

For the usual incubations, 1 ml of supernatant (or 0.5 ml each of MITOMA's crude Fractions I and II) was incubated with 0.1 ml of 0.015 M NAD, 0.1 ml of 0.1 M ethanol, 0.1 ml of 1.0 M phosphate buffer at pH 6.8, and 0.1 ml of 0.002 M 6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride; appropriate amounts of substrate and inhibitor were added and the volumes made up with water to appropriate minimal volumes (these varied from experiment to experiment due to the different maximum volume of inhibitor solution employed). The tubes were then incubated in a shaking water bath in contact with air at 37°. The incubation times were usually 1 h, though in studies of the inhibition of tryptophan by phenylalanine and fluorophenylalanine the times were reduced to 10 min in some experiments.

In some of the kinetic work, smaller volumes of liver supernatant were used on occasions, to reduce the reaction velocities.

When the products of the reaction were to be fractionated by ion exchange chromatography the reaction was stopped by boiling. When tyrosine was to be determined directly the reaction was stopped by addition of 1 ml of 30% trichloroacetic acid. In both cases the protein was removed by centrifugation.

(b) The decarboxylating systems

(i) 5-Hydroxy-tryptophan to serotonin. The liver preparations used for the hydroxylations contained aromatic amino acid decarboxylase activity[7] which gave very good yields of serotonin (90–100%) under the conditions of the hydroxylase incubation. The decarboxylase was not inhibited by phenylalanine, fluorophenylalanine or tyrosine at the concentrations employed but was inhibited by fluorotryptophan. Therefore in studies using fluorotryptophan as an inhibitor of hydroxylation it was necessary to determine both serotonin and 5-hydroxytryptophan.

(ii) Tyrosine to tyramine. Under the conditions used in these incubations, the liver preparations did not decarboxylate tyrosine to tyramine in detectable quantities and therefore in order to determine the conversion of [^{14}C]phenylalanine to [^{14}C]-tyrosine it was necessary to use *S. faecalis* decarboxylase to convert tyrosine to tyramine which could be the more easily separated from phenylalanine. The yields for this step were variable but of the order of 65%. Phenylalanine itself was not significantly decarboxylated under these conditions. For these incubations 1 ml of the supernatant from the hydroxylation stage *plus* 0.1 ml of 1.0 M acetate buffer at pH 5.0 *plus* 1.0 ml of 2 mg/ml suspension of *S. faecalis* decarboxylase in 0.1 M acetate buffer at pH 5.0 were incubated at 37° for 30 min. The reaction was stopped by boiling and the protein removed by centrifugation.

(c) Separation of products

(i) The separation of tyrosine from high concentrations of fluorotryptophan and tryptophan. These indoles interfered with the determination of tyrosine, and the separation was achieved by ion exchange chromatography.

The deproteinised incubation mixture was applied to a 17.5 cm × 1 cm column of Amberlite CG 50 in the Na^+ form. The amino acids were then eluted with 0.001 M sodium phosphate buffer at pH 6.8. Fractions (5 ml) were collected, and tyrosine which came off in Fractions 3–8 was then determined. The indoles appeared in later fractions. The chromatography column was then regenerated by washing with a further 100 ml of 0.001 M phosphate buffer and reused. The recovery obtained for added tyrosine through this procedure was 80%.

(ii) Separation of serotonin or tyramine from their parent amino acids. The deproteinised incubation mixture was applied to a 3 cm × 0.5 cm Amberlite CG 50 column in the H^+ form. The amino acids were eluted with 30 ml of 0.001 M phosphate buffer at pH 6.8 and the amines were then eluted with 5 ml of 0.1 M hydrochloric acid. The column was regenerated by washing with distilled water to pH 5.0. Recoveries of 5-hydroxytryptophan as serotonin through the incubation and separation step was 60% for incubations of 1 h duration. In Expt. 5 where the incubation time was reduced to 10 min recoveries of 75% were obtained.

Recoveries of tyramine through the decarboxylation step (with *S. faecalis*) followed by the ion exchange procedure, were only of the order of 40% mainly due to the low efficiency of the decarboxylation. This lower and variable recovery for tyramine was, however, acceptable in view of the fact that the recovery was determined separately in every incubation sample of Expt. 2, and therefore no extrapolation was involved.

(iii) Separation of 5-hydroxytryptophan from other indole amino acids. The high concentration of tryptophan and fluorotryptophan used in the incubations quenched the acid fluorescence used to measure 5-hydroxy indoles. It was therefore necessary to purify the 5-hydroxy compounds by ion exchange chromatography. The deproteinised incubation mixture was applied to a 27.5 cm × 1 cm column of Amberlite CG 50 in the Na^+ form and the amino acids eluted with 0.001 M phosphate buffer at pH 6.8. Fractions (5 ml) were collected, 5-hydroxytryptophan appearing in Fractions 5–14. Tryptophan which was originally present in 200 times the concentration of 5-hydroxytryptophan appeared in Fractions 13–40. The amount of tryptophan in Fractions 13 and 14 was sufficiently small as not to interfere with the fluorescence assay. The column was then regenerated by washing with a further 100 ml of dilute buffer to remove tryptophan and 5-fluorotryptophan, and then reused. Recoveries of 5-hydroxytryptophan of the order of 75% were obtained through this procedure.

(d) Determination of products

(i) Tyrosine and tyramine. Tyrosine and tyramine were determined by the nitrosonaphthol fluorescence method of UDENFRIEND AND COOPER[8].

(ii) 5-Hydroxyindoles. 5-Hydroxyindoles were determined by the acid fluorescence method of UDENFRIEND[9].

(iii) [^{14}C]-Tyramine. This was determined by counting samples in a scintillation fluid[10] using a Tricarb liquid scintillation counter.

EXPERIMENTAL

In the Expts., 1–5 below, the amounts of products obtained from the same concentration of one substrate in the presence of various concentrations of a second substrate were determined. The amounts of products recorded are corrected for the recovery of added product through the incubation and purification procedures.

The magnitude of these corrections is indicated by the size of the recoveries quoted in METHODS, but the actual correction employed in each separate experiment was based on recoveries and standards which were always run in parallel with the experiment.

D-Phenylalanine is not a substrate for this enzyme[5], and RENSON, WEISSBACH AND UDENFRIEND[11] have reported that D-phenylalanine only slightly inhibits the hydroxylation of tryptophan. Therefore when DL compounds have been used in the following work their concentrations have been expressed with respect to the L isomer. In the results shown in Figs. 2–7 the amount of hydroxylation expressed as nmoles of product per μmole of substrate at each inhibitor concentration is shown. These relatively simple plots, rather than the more usual double reciprocal plots, were used for the following reasons. Firstly, the enzyme shows substrate inhibition for phenyl-

alanine[5] and also for fluorophenylalanine (Fig. 1). Secondly, in the case of the experiments with tryptophan as substrate the rate of reaction is very slow and thus, long incubations (1 h) are necessary to obtain accurately measurable amounts of product. This made it impossible to determine the initial velocity of the steady state. Furthermore, the results indicate that some of the substrates actually enhance each other's hydroxylation which renders the double reciprocal plot inappropriate.

*Expt. 1. The effect of 5-fluoro-*DL*-tryptophan on the hydroxylation of tryptophan*

In these experiments the concentration of tryptophan was 12.0 mM and that of 5-fluoro-DL-tryptophan varied from 1.5 to 12 mM with respect to the L isomer. Double the amounts of enzyme and cofactors used in the typical incubations (METH-

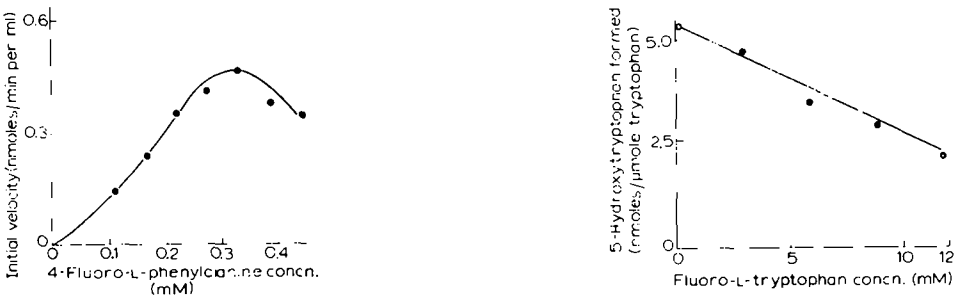


Fig. 1. Substrate/velocity curve for fluorophenylalanine, showing substrate inhibition at concentrations above 0.33 mM. Incubation mixture: 0.5 ml of supernatant with usual amounts of cofactors (see EXPERIMENTAL) in total volume of 3 ml, for 6 min at 37°.

Fig. 2. Effect of various concentrations of fluorotryptophan on the hydroxylation of tryptophan. Tryptophan concentration 12.0 mM. Incubation mixture: double the usual amounts of enzyme and cofactors in total volume of 6 ml, for 1 h at 37°.

ODS) were used in a total volume of 6 ml. This was because, as the 5-fluoro-tryptophan inhibits aromatic acid decarboxylase, it was necessary to measure serotonin and 5-hydroxytryptophan separately. This was achieved by dividing each deproteinised incubate into two parts. One was chromatographed on a 3 cm × 0.5 cm ion exchange column and serotonin recovered and determined. The other was chromatographed on a 27.5 cm × 1 cm column and the appropriate fractions used to determine 5-hydroxytryptophan. Because both fluorotryptophan and tryptophan are substrates for this system they might be expected to competitively inhibit each other. Fig. 2 shows that the inhibition increases with inhibitor concentration over a wide range, which is consistent with competitive inhibition.

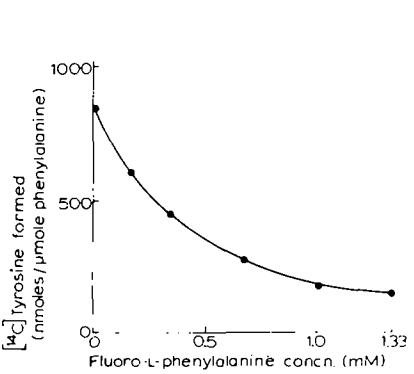
*Expt. 2. The inhibition of the hydroxylation of phenylalanine by 4-fluoro-*DL*-phenylalanine*

The problem here is that 4-fluorophenylalanine is hydroxylated to an appreciable extent and the product of this hydroxylation is tyrosine. It was therefore necessary to be able to distinguish the tyrosine formed from the substrate (phenylalanine) from that formed from the inhibitor (fluorophenylalanine). [^{14}C]phenylalanine was used to facilitate this distinction, but as it is difficult to completely

separate phenylalanine from tyrosine, it was necessary to decarboxylate the tyrosine to tyramine (by added *Streptococcus faecalis* preparation) and then to purify the tyramine.

The molarity of phenylalanine used was 0.3 mM and that of 4-fluoro-DL-phenylalanine ranged from 0.167 to 1.33 mM with respect to the L isomer. Each incubation contained 0.1 μC of [^{14}C]phenylalanine and the total volume of the incubations was 3 ml. The incubations were carried out as described and the reaction stopped by boiling. 1 ml of the deproteinised supernatant was taken and the tyrosine content determined. A further 1 ml of the supernatant was treated with *S. faecalis* decarboxylase, and the *p*-tyramine separated from phenylalanine and tyrosine by ion exchange chromatography. 2 ml of the 5 ml acid effluent from the column was taken and its *p*-tyramine content determined and hence the recovery of tyrosine as *p*-tyramine through this step was calculated. Recoveries of tyrosine as tyramine through this step varied from 30% to 60%, depending mainly on the initial concentration of tyrosine, low concentrations giving rise to low decarboxylation yields. It was the variability which necessitated the determination of recoveries in each separate incubation mixture. A further aliquot of the separated decarboxylated product from this eluate was added to scintillation fluid and counted. From the number of disint./min in this sample and the previously determined recovery of original tyrosine as tyramine in the eluate the amount of tyrosine formed from [^{14}C]phenylalanine in the original incubation mixture could be calculated.

Controls in which the hydroxylase had been inactivated by boiling, and others in which the decarboxylase had been inactivated by boiling, gave almost identical low recoveries of radioactivity, indicating that there was no significant decarboxylation of phenylalanine through this procedure. The results obtained are shown in Fig. 3. As in Expt. 1, the hydroxylation of the non-fluorinated substrate decreases as



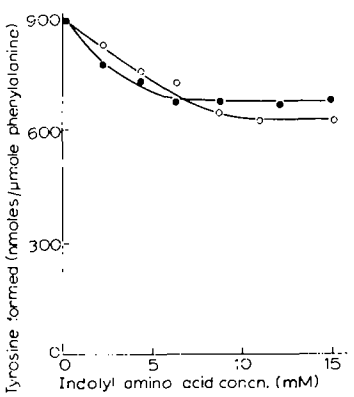


Fig. 3. Effect of various concentrations of fluorophenylalanine on the hydroxylation of phenylalanine. Phenylalanine concentration 0.3 mM. Incubation mixture: usual amounts of enzyme and cofactors in total volume of 3 ml, for 1 h at 37°. Each point represents the mean of 3 experiments.

Fig. 4. Effect of various concentrations of fluorotryptophan (●—●) and tryptophan (○ ○) on the hydroxylation of phenylalanine. Phenylalanine concentration 0.43 mM. Incubation mixture: the usual amounts of enzyme and cofactors in total volume of 2.3 ml, for 1 h at 37°.

the concentration of fluorinated substrate increases. As both are substrates, competitive inhibition is to be expected, and again the results do not rule out this type of inhibition.

*Expt. 3. The inhibition of the hydroxylation of phenylalanine by tryptophan and 5-fluoro-*DL*-tryptophan*

In these experiments the molarity of phenylalanine used was 0.43 mM, that of tryptophan ranged from 2.2 to 15.4 mM with respect to the L isomer, the total volume of each incubation being 2.3 ml. The incubations were carried out and the tyrosine purified on ion exchange columns and determined as described above.

It will be seen from the results, Fig. 4, that the presence of indole amino acids inhibited the hydroxylation of phenylalanine up to 30% for inhibitor concentration of up to 5 mM. Higher concentrations had no further effect which indicates that the inhibition was not competitive over all the range studied.

*Expt. 4. The effect of tryptophan and 5-fluoro-*DL*-tryptophan on the hydroxylation of 4-fluoro-*DL*-phenylalanine*

The experiments were carried out as Expt. 3. The molarity of 4-fluoro-DL-phenylalanine used was 0.33 mM with respect to the L isomer, that of tryptophan ranged from 1.67 to 16.7 mM, and that of 5-fluoro-DL-tryptophan was from 1.67 to

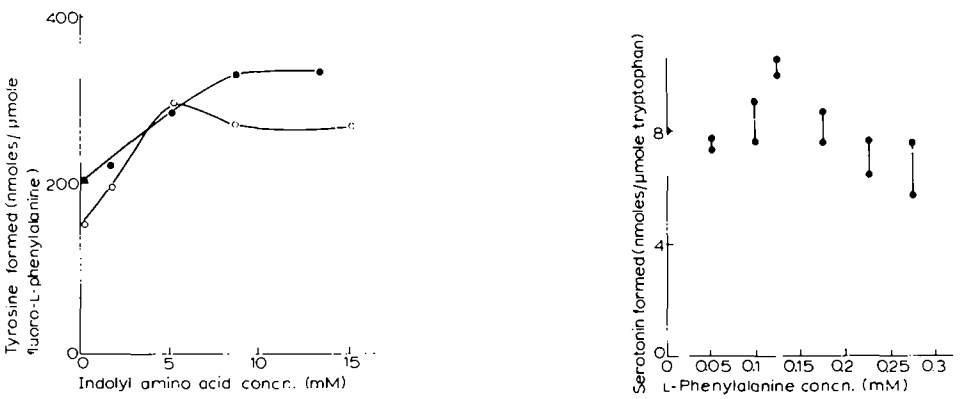


Fig. 5. Effect of various concentrations of fluorotryptophan (●—●) and tryptophan (○—○) on the hydroxylation of fluorophenylalanine. Concentration of fluorophenylalanine 0.33 mM. Incubation mixture: usual amounts of enzyme and cofactors in total volume of 3 ml for 1 h at 37°.

Fig. 6. Effect of various concentrations of phenylalanine on the hydroxylation of tryptophan with 6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride concentration of $0.5 \cdot 10^{-4}$ M. Tryptophan concentration 1.25 mM. The vertical bars represent the range of results obtained from 4 experiments. Incubation mixtures: the usual amounts of enzyme and other cofactors in a total volume of 3 ml, for 10 min at 37°.

12 mM with respect to the L isomer, the total volume of the incubations being 3 ml. The incubations and analyses were carried out as those of Expt. 3. It was found that the presence of indolyl amino acids produced an enhancement of hydroxylation of 4-fluorophenylalanine of about 70% up to concentrations of 5 mM but that further increase in concentration did not have much further effect. These results are shown graphically in Fig. 5.

*Expt. 5. The effect of phenylalanine and 4-fluoro-*DL*-phenylalanine on the hydroxylation of tryptophan*

Initial experiments using incubation times up to 1 h gave results indicating inhibition, but it was felt that shorter incubation times should be used as the phenylalanine was likely to be rapidly used up. Using 1.25 mM tryptophan and phenylalanine levels ranging from 0.05 to 0.25 mM in a total volume of 3 ml, incubation times were reduced to ten min. The results are shown in Fig. 6. These results were extremely variable from one experiment to the next and ranges obtained are shown. There is, nevertheless, clear evidence that some enhancement of hydroxylation had occurred at low phenylalanine concentrations.

Experiments were carried out to test whether tyrosine influenced the hydroxylation of tryptophan, but at the concentrations to be expected in the above incubation it had no detectable effect on either the hydroxylation or decarboxylation step. Furthermore, phenylalanine has no apparent effect on decarboxylation at these concentrations. When the experiments were repeated using a ten-fold higher concentration of 6,7-dimethyl-5,6,7,8-tetrahydropterin hydrochloride it was found that in addition to doubling the rate of tryptophan hydroxylation in the control there was also enhancement of tryptophan hydroxylation in the presence of phenylalanine (see Fig. 7).

Similar results were also obtained with 4-fluorophenylalanine over a similar concentration range (Fig. 7). The results suggest that the hydroxylation of tryptophan by these preparations is markedly enhanced by the addition of either phenylalanine or 4-fluorophenylalanine, and that the apparent inhibition obtained in the

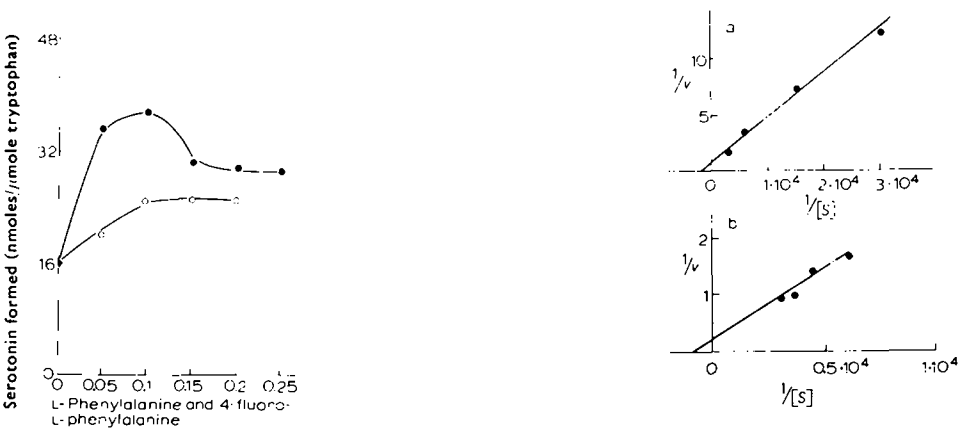


Fig. 7. Effect of various concentrations of phenylalanine (●—●) and fluorophenylalanine (○- -○) on the hydroxylation of tryptophan (concentration 1.25 mM) with 6,7-dimethyl-5,6,7,8,-tetrahydropterin hydrochloride concentration $0.5 \cdot 10^{-3}$ M. Incubation mixture: the usual amounts of enzyme and other cofactors in a total volume of 3 ml, for 10 min, at 37°.

Fig. 8. a. The double reciprocal $1/v$ *vs.* $1/[S]$ plot for phenylalanine. b. The double reciprocal $1/v$ *vs.* $1/[S]$ plot for fluorophenylalanine. Incubation mixture: 0.5 ml of liver supernatant and the usual amounts of cofactors in a total volume of 3 ml, at 37°. Incubation times of 3, 6 and 9 min at each substrate concentration enable rate curves to be plotted from which the initial velocity of the steady state was determined.

earlier experiments is due to the substrates competing for a limited amount of 6,7-dimethyl-5,6,7,8-tetrahydropterin and not for the enzyme.

*Expt. 6. Kinetic studies of phenylalanine-4-hydroxylase activity towards phenylalanine and 4-fluoro-*DL*-phenylalanine*

As a consequence of the results obtained in Expt. 2 it was decided to attempt to demonstrate whether the fluorophenylalanine was indeed a competitive inhibitor of the 4-hydroxylation of phenylalanine. Owing to the analytical problems encountered in Expt. 2, double reciprocal (K_i) plots could not be carried out, so K_m values for each of the two substrates were determined by $1/v$ *vs.* $1/[S]$ plots (Expt. 6 (i)), and then the maximum attainable velocities (v_{max}) for phenylalanine, fluorophenylalanine, and an equimolar mixture of each were determined (Expt. 6 (ii)).

(i) Determination of the K_m values for phenylalanine and 4-fluorophenylalanine. Kinetic studies were carried out using substrate concentrations in the range of 0.033 to 0.33 mM for phenylalanine and 0.11 to 0.33 mM for 4-fluoro-DL-phenylalanine with respect to the L isomer. The incubation mixture was pre-incubated to ensure that the 6,7-dimethyl-5,6,7,8-tetrahydropterin was fully reduced and the reaction started by introduction of the substrate. Incubations were carried out for 3 and 6 min and rate curves drawn for each substrate concentration. From these the initial velocity of the steady state was derived and the double reciprocal plot constructed. These are shown in Figs. 8a and 8b. As will be seen, the K_m for phenylalanine is 0.66 mM and that for 4-fluorophenyl-L-alanine is 1.25 mM.

*(ii) Determination of the maximum attainable velocities for phenylalanine and 4-fluoro-*DL*-phenylalanine.* Incubations similar to those used for the K_m determinations were employed. Concentrations ranged from 0.1 to 0.5 mM for phenylalanine, 4-fluoro-DL-phenylalanine with respect to the L isomer and for an equimolar mixture of the two. In each case the maximum attainable velocity was obtained within this range, and the values were as follows: phenylalanine yielding tyrosine at 0.125 μmole/min; 4-fluoro-DL-phenylalanine yielding tyrosine at 0.042 μmole/min; equimolar mixture yielding tyrosine at 0.066 μmole/min. The general expression:

$$\frac{K_m'}{K_m''} \cdot \frac{v_{max}' - v_{max\ (mixture)}}{v_{max\ (mixture)} - v_{max}''} \qquad (1)$$

described by DIXON AND WEBB[12], applies only if the two substrates are competitive inhibitors of each other. Thus from the data obtained in the experiments just described, it is possible to test whether the phenylalanine and fluorophenylalanine are competitive inhibitors of each other. If this is so, then ratio K_m for phenylalanine/K_m for fluorophenylalanine obtained from the double reciprocal plots (Expt. 6 (i)), should be the same as the value obtained by substituting values obtained in Expt. 6 (ii) in Eqn. 1. As will be seen, the ratio from Expt. 6 (i) = 0.5, whereas that from Expt. 6 (ii) = 2.4.

DISCUSSION

It is clear from the above results that the simple model of one enzyme with different co-factor requirements for the different activities, as postulated by FREED-

LAND[1] is not adequate to explain the behaviour of the enzyme system in the presence of two substrates simultaneously. In an earlier publication[2] we suggested that the inhibition of phenylalanine by the indolic amino acids might be explained by the existence of two enzymes, only one of which is inhibited. However, the effect of added indolic amino acids on the hydroxylation of fluorophenylalanine and the effect of added phenylalanine or fluorophenylalanine on the hydroxylation of tryptophan, does not easily fit this hypothesis.

The results now described require that there are at least two active sites (which may or may not be on the same protein). One of these active sites would be the site at which the benzenoid compounds are hydroxylated, and the other, the site at which the indoles are hydroxylated. The effect of the indoles on the hydroxylation of the benzenoid compounds must be due to them modifying the benzenoid hydroxylating site by binding at some other point. The effects of phenylalanine and fluorophenylalanine on the hydroxylation of tryptophan must also be due to their producing an allosteric modification of the tryptophan hydroxylating site. This hypothesis is further supported by the fact that the maximum inhibition of phenylalanine hydroxylation and the maximum elevation of fluorophenylalanine hydroxylation are reached at approximately the same indole amino acid concentration (see Expts. 3 and 4). If both the hydroxylating sites were on the same protein, the allosteric effects could be produced by the various effectors binding at their own hydroxylation sites; however, we have no evidence for this.

The report by FREEDLAND, WADZINSKI AND WAISMAN[13], and by RENSON, WEISSBACH AND UDENFRIEND[11] that tryptophan hydroxylation was competitively inhibited by phenylalanine must have been due to the two substrates competing for a limited amount of 6,7-dimethyl-5,6,7,8-tetrahydropterin and not for the binding site of the enzyme.

The inhibitory effects of fluorotryptophan on the hydroxylation of tryptophan and of fluorophenylalanine on the hydroxylation of phenylalanine could be due to some sort of direct competition for the appropriate hydroxylating sites. However, from the kinetic studies of Expt. 6 (i) and (ii) it is apparent that fluorophenylalanine is not a true competitive inhibitor of phenylalanine hydroxylation. If there is only one site for the hydroxylation of both phenylalanine and fluorophenylalanine, then these results may be due to the fact that as both substrates exhibit substrate inhibition, the observed maximum velocities are not applicable to the calculation in Expt. 6 (ii). Another factor to be considered is the effect of the presence of fluoro-D-phenylalanine and fluoro-D-tryptophan in these experiments, which must now be investigated. Furthermore, although the hydroxylation site of fluorophenylalanine may be the same as that of phenylalanine, the overall reaction mechanism must be different because the products of the reaction are not analogous, in that a fluoride ion is produced in the hydroxylation of fluorophenylalanine.

These results have revealed an interesting biochemical problem which should form the foundation for further investigation, but one of the important aspects to be considered is the clinical significance of these results. For example, GAL, ARMSTRONG AND GINSBERG[14] have reported that brain tryptophan hydroxylase is enhanced by low concentrations of phenylalanine and inhibited by higher concentrations which indicates an unsuspected similarity between the brain and liver tryptophan hydroxy-

lation. The relevance of this to the biochemistry of phenylketonuria will be a subject of further work.

ACKNOWLEDGMENT

The Spectrophotofluorimeter used in this work was purchased with funds supplied by the Medical Research Council.

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