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Action of Phenylalanine Metabolites on Glutamic Acid Decarboxylase and γ-Aminobutyric Acid—α-Ketoglutaric Acid Transaminase in Brain

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Several intermediary metabolites of phenylalanine, namely phenylpyruvic, *p*-hydroxyphenylpyruvic, phenylacetic, *p*-hydroxyphenylacetic, and *o*-hydroxyphenylacetic acids have been found to inhibit the glutamic acid decarboxylase of brain *in vitro*. The inhibition is of the competitive type and appears to be localized in the apoenzyme.

No inhibitory effect could be demonstrated when the same metabolites were tested on the γ-aminobutyric acid—α-ketoglutaric acid transaminase system.

The significance of these findings in relation to phenylketonuria is briefly discussed.

In phenylketonuria hydroxylation of phenylalanine to tyrosine is markedly reduced. This results in an excess of phenylalanine in the tissues and an overproduction of intermediary metabolites of phenylalanine catabolism. The major metabolites of phenylalanine found in abnormal amounts in phenylketonuria are phenylpyruvic, phenyllactic and phenylacetic acids [1]. It has been suggested [1] that the mental deficiency is caused by an overproduction of one of these metabolites or by an excess of phenylalanine.

These aromatic metabolites of phenylalanine have recently been found to inhibit dihydroxyphenylalanine decarboxylase of beef adrenal medulla [2] and 5-hydroxytryptophan decarboxylase of guinea-pig kidney [3] *in vitro*. This inhibitory effect has been offered as an explanation of the low plasma levels of adrenaline [4] and 5-hydroxytryptamine [5] in phenylketonuria. Since much argues that both adrenaline and 5-hydroxytryptamine play a rôle in normal brain function [6], it appears that inhibition of these enzymes might reasonably be a contributing cause of mental deficiency.

The reported inhibition of dihydroxyphenylalanine decarboxylase and 5-hydroxytryptophan decarboxylase by aromatic phenylalanine metabolites

prompted us to examine the effects on GAD * and GABA-T of brain of these metabolites as well as of some others also occurring in phenylketonuria, namely *p*-hydroxyphenylpyruvic, *p*-hydroxyphenylacetic, and *o*-hydroxyphenylacetic acids and *o*-tyrosine.

The presence, in brain, of very large amounts of GAD and of ABA — the product formed by its action on GA — suggests that decarboxylation of GA may be a reaction of major importance in the metabolism of the central nervous system [7]. No other tissue has been found to show as high GAD activity as brain. This enzyme requires the presence of a coenzyme, namely pyridoxal-5-phosphate.

In studies on the metabolic fate of GABA evidence has also been obtained for its transamination with KGA in brain to form SSA and GA [7,8]. The transaminase catalyzing this reaction also requires pyridoxal-5-phosphate as coenzyme which, however, appears to be more firmly bound to the apoenzyme than is the case in the decarboxylase [9,10]. The SSA can be oxidized further to succinic acid and so enter the tricarboxylic acid cycle. This latter reaction appears to be catalyzed by a diphosphopyridine nucleotide dependent dehydrogenase [11].

In view of these findings it has been suggested that in brain a shunt operates around the KGA oxidase system of the tricarboxylic acid cycle by which KGA could be withdrawn through formation of GA by transamination with GABA and the carbon chain of GABA could enter the cycle at the succinic acid level. It is of special interest that this step in the tricarboxylic acid cycle has been considered to be the rate limiting reaction of the cycle; thus the whole cycle could be secondarily regulated by the activity of "the GABA cycle".

Recent findings also suggest that GABA may act in the cortex as an inhibitory transmitter substance [12,13]. Interference with its formation and/or metabolism may therefore be an example of a biochemical lesion of the central nervous system.

MATERIALS AND METHODS

Materials. The phenylalanine metabolites used were obtained from commercial sources. Phenylacetic acid from Eastman-Kodak Co.; phenylpyruvic acid, sodium salt, from Hoffman-La Roche & Co. Ltd.; *p*-hydroxyphenylacetic acid from K and K Laboratories, Inc.; *p*-hydroxyphenylpyruvic acid, sodium salt, from Sigma Chemical Co.; *o*-hydroxyphenylacetic acid and DL-a-phenyllactic acid from Light & Co. Ltd.; DL-*o*-tyrosine from California Foundation for Biochemical Research; DL-phenylalanine and L-GA from Merck & Co. Ltd.; GABA from Hoffman-La Roche & Co. Ltd.; KGA from Sigma Chemical Co. Pyridoxal-5-phosphate was generously supplied by Dr. W. W. Umbreit, Merck Institute for Therapeutic Research, Rahway, N. J.

The metabolites obtained as free acids were transformed into their sodium salts before use.

SSA was synthesized from glutamic acid according to Mosbach *et al.*[14] and isolated as 2,4-dinitrophenylhydrazone. After recrystallization from ethanol the product had a melting point of 200°.

Other chemicals used in the experiments were of reagent grade.

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* The following abbreviations have been used: GA, glutamic acid; GABA, γ-aminobutyric acid; KGA, α-ketoglutaric acid; GABA-T, γ-aminobutyric acid — α-ketoglutaric acid transaminase; GAD, glutamic acid decarboxylase; SSA, succinic semialdehyde.

Enzyme preparations. GAD was obtained from either rat or guinea-pig brain. The animals were killed by decapitation and the brains immediately placed in small beakers containing cracked ice. Homogenates containing 500 mg of fresh weight of brain per ml were prepared in ice-cold 0.5 M phosphate buffer pH 5.9.

GABA-T was obtained from mitochondria isolated from either rat or guinea pig brains. For the isolation of mitochondria the brains were disrupted in 9 volumes of 0.25 M sucrose in a Potter-Elvehjelm homogenizer. The homogenate was centrifuged twice at 1 500 × *g* for 5 min to remove nuclear and cellular debris. The final supernatant was then centrifuged at 12 000 × *g* for 20 min to sediment the mitochondria. After being washed once in 0.25 M sucrose the mitochondria were suspended in 0.25 M sucrose and the suspension diluted so that 1 ml corresponded to 5 g of brain. The centrifugations were carried out in an International Refrigerated Centrifuge with multispeed attachment.

All steps in the preparation of the enzymes were carried out at 0 – 4°.

Assay of enzyme activity. The rate of GA decarboxylation was determined with a Warburg apparatus in an N_2 atmosphere at 37°. The main compartment of the flasks contained 1 ml of brain homogenate, 200 μg of pyridoxal-5-phosphate and inhibitor dissolved in phosphate buffer to make a final volume of 2.65 ml. One side arm contained GA solution (pH 7.0 to 7.4) to give a final concentration of 0.03 M. The substrate was added after an equilibration time of 10 min. The other side arm contained 0.3 ml of 3 N H_2SO_4. This was added to stop the reaction and to liberate bound CO_2, for which suitable corrections were made. Carbon dioxide output was determined for 60 mins.

To ascertain whether the formation of CO_2 and GABA took place in stoichiometric amounts under the conditions employed, the quantity of GABA in the incubation fluids was determined in some of the experiments. The GABA was separated by means of high voltage electrophoresis and determined quantitatively by treating the ninhydrin-developed spots with a copper nitrate solution, eluting the red colour of the copperninhydrin-complex with methanol and measuring the colour intensity spectrophotometrically [15].

The formation of SSA and GA was taken as a measure of the GABA-T activity. The assay method was as follows: Incubations were carried out, in air, with shaking in small conical flasks for 2 h at 37°. The incubation mixture contained 1.5 ml of 0.1 M phosphate buffer pH 7.4, 0.5 ml of mitochondria suspension, 0.2 ml of 0.1 M KGA (20 μM), 0.4 ml of 0.2 M GABA (80 μM), and inhibitor (usually in a volume of 0.6 ml). The final volume of the incubation mixture was usually 3.2 ml. At the end of the incubation period 1 ml of 15 % trichloroacetic acid was added to deproteinize the contents which were then centrifuged for 10 min.

For estimation of the SSA formation an aliquot of the supernatant was pipetted into a glass-stoppered test-tube placed in an ice-bath and 3 ml of a 0.5 % solution of 2,4-dinitrophenylhydrazine in 5 N HCl added. After 10 min 5 ml of xylene were added to the tube for extraction of the hydrazone formed. This extraction was repeated once. The pooled extracts were then extracted with 5 ml of a 0.1 M borate buffer pH 10. An aliquot of the borate extract was diluted with borate buffer to a volume of 6 ml and this was made alkaline with 0.5 ml of a 5 % alcoholic sodium hydroxide solution.

Exactly 2 min later the colour density was measured in a Beckman DU spectrophotometer at 420 mμ against a blank consisting of borate buffer and alcoholic sodium hydroxide. The concentration of SSA in the extract was assessed from a calibration curve made up from known amounts of a synthetic sample of SSA-2,4-dinitrophenylhydrazone. Only small amounts of the KGA present in the incubation mixture was found to react with the 2,4-dinitrophenylhydrazine to give a hydrazone under the conditions described, and xylene extracted but little of this hydrazone.

In some of the experiments the presence of SSA-2,4-dinitrophenylhydrazone in the borate extract was confirmed by paper chromatography. For this the extract was acidified to pH 2 with HCl and extracted with a mixture of ethanol and chloroform (20:80). This extract was evaporated to dryness and the residue taken up in a small volume of ethyl acetate and applied to the paper. Ascending chromatography with a mixture of *n*-butanol, ethanol and 0.5 N ammonia (70:10:20) as developing system was used. In this system SSA-2,4-dinitrophenylhydrazone gave two spots having the R_F values 0.09 and 0.43, respectively.

When the inhibitor tested was a carbonyl compound, such as phenylpyruvic and *p*-hydroxyphenylpyruvic acids, the transamination assay could not be based on the for-

mation of SSA since the inhibitors formed hydrazones. Here only the formation of GA was used for the assay.

The GA formed in the transamination was measured in the deproteinized incubation mixture by the high voltage electrophoresis method mentioned above [15].

RESULTS AND DISCUSSION

Several of the phenylalanine metabolites were found to inhibit the GAD. Some of the results of these experiments are given in Table 1. Phenylacetic

Table 1. Inhibition of glutamic acid decarboxylase of guinea-pig brain by phenylalanine metabolites.

Additions	Concentration of inhibitor μmole/l	CO_2 output (μl/60 min)	Inhibition %
Control		174	
Phenylpyruvic acid	25	82	47
	50	76	56
	100	67	61
Control		172	
p-OH-Phenylpyruvic acid	25	150	13
	50	137	20
	100	107	38
Control		119 *	
Phenylacetic acid	25	106	11
	50	60	50
	100	14	96
Control		119 *	
p-OH-Phenylacetic acid	25	55	54
	50	29	76
	100	15	92
Control		173	
Phenyllactic acid	25	169	2
	50	167	3
	100	157	9
Control		130 *	
o-OH-Phenylacetic acid	125	126	3
	50	99	24
	100	64	50
Control		183	
o-Tyrosine	100	178	3
Control		164	
Phenylalanine	100	151	8

* These experiments were performed with rat brain.

Table 2. Equivalence of formation of CO_2 and γ-aminobutyric acid by guinea-pig brain homogenate.

Expt. No.	Incubation time, min.	CO_2 formed μmole	γ-Aminobutyric acid formed μmole
1	60	8	8.3
2	60	6.9	7.1
3	30	3.9	4
4	30	3.6	3.6
5 *	60	2.2	2.4
6 *	30	1.6	1.7

* In experiments 5 and 6 the incubation mixture contained *p*-OH-phenylacetic acid (50 μmole/l).

and phenylpyruvic acids were found to be the most effective inhibitors of the unsubstituted compounds. Phenyllactic acid and phenylalanine had very little activity. Of the *para*-hydroxylated compounds studied, *p*-hydroxyphenylacetic acid proved a more active inhibitor than *p*-hydroxyphenylpyruvic acid. Two *ortho* compounds were also tested. Of these, *o*-hydroxyphenylacetic acid was rather active, while its precursor, *o*-tyrosine, was inactive.

Table 2 gives the results of experiments performed to determine whether the formation of CO_2 and GABA takes place in stoichiometric amounts under the conditions employed. The experiments were performed both with and without added inhibitor. Good equivalence was found and it may be said that, within experimental error, there was a mole for mole formation of CO_2 and GABA.

It may be of interest to compare some of these results with those obtained from similar studies on other decarboxylases. Davison and Sandler [3] in their study of 5-hydroxytryptophan decarboxylase from guinea-pig kidney as well as Hartman *et al.*[16] in their study of dihydroxyphenylalanine decarboxylase from hog kidney obtained comparable inhibitions of their enzyme preparations with phenylacetic and phenylpyruvic acids and less or no inhibition with phenyllactic acid. In Fellman's study of the dihydroxyphenylalanine decarboxylase from beef adrenals phenylpyruvic and phenyllactic acids were the most effective inhibitors while phenylacetic acid was a poor inhibitor [2]. An inhibitory effect of *p*-hydroxyphenylpyruvic acid on dihydroxyphenylalanine decarboxylase has been reported by Hartman *et al.*[16]. Its effect was equal to that of phenylpyruvic acid.

The equations of Hunter and Downs [17] were used for characterizing the type of inhibition observed in the present experiments. If α is defined as the fractional activity, *i. e.* v_1/v, where v_1 is the activity in the presence of inhibitor and v the activity in its absence, with the same substrate concentration, these equations are, for a competitive inhibition,

$$[I]\ \frac{\alpha}{1-\alpha} = K_i + \frac{K_i}{K_m}\ [S]$$

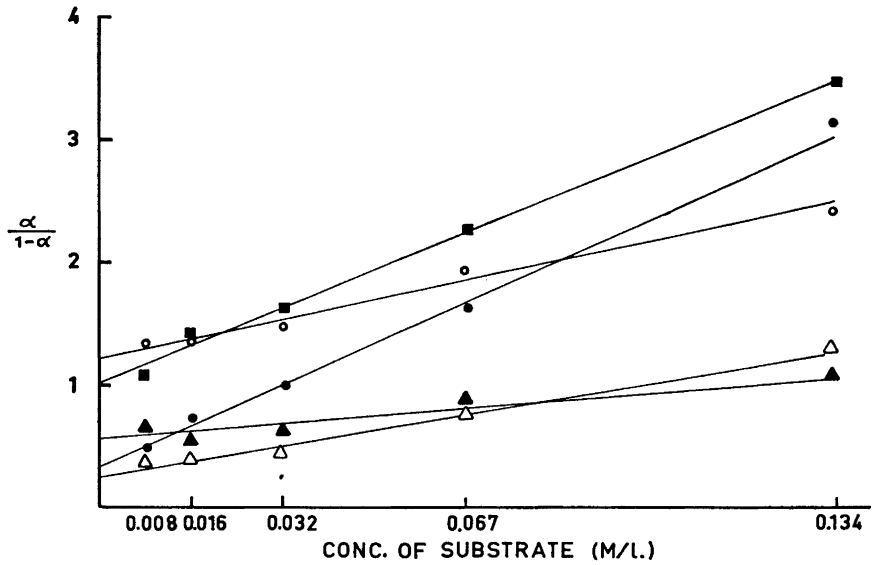

Fig. 1. Inhibition of glutamic acid decarboxylase of brain by phenylalanine metabolites. Experimental conditions as described in text. The results are plotted according to the equation derived by Hunter and Downs [17]: $a/(1-a)$ against substrate concentration. Values of a are determined as the ratio of inhibited velocity to uninhibited velocity with the same concentration of the substrate. Inhibitor concentration 100 μmole/l. Inhibitors: ● Phenylpyruvic acid; ○ *p*-OH-Phenylpyruvic acid; ▲ Phenylacetic acid; △ *p*-OH-Phenylacetic acid; ■ *o*-OH-Phenylacetic acid.

and, for a non-competitive inhibition,

$$[I]\ \frac{a}{1-a} = K_i$$

where [I] is the inhibitor concentration, K_i the dissociation constant of the enzyme-inhibitor complex, [S] the substrate concentration, and K_m the Michaelis constant. By plotting $a/(1-a)$ against [S] one obtains for competitive inhibition a straight line with slope K_i/K_m and intercept K_i; and, for non-competitive inhibition, a straight line of zero slope and intercept K_i. The value of K_i represents the amount of inhibitor required for 50 % inhibition.

When $a/(1-a)$ was plotted against [S] with the different inhibitors the graphs obtained were found to be straight lines with different slopes (Fig. 1). This indicates that the inhibition is dependent of the substrate concentration, [S], *i. e.* it is of the competitive type. The value of K_i can be calculated from each of the lines by multiplying the value of [I] by the value of the intersection point obtained by extrapolating the line to the vertical axis (Table 3).

To ascertain whether the inhibition noted was due to any interference with the coenzyme of GAD, *i. e.* pyridoxal-5-phosphate, determinations were made of the influence of increasing amounts of the latter on systems inhibited by the

Table 3. K_i values of different competitive inhibitors of glutamic acid decarboxylase. (Values of K_i were calculated form $a/(1-a)$ values obtained by the graphical method shown in Fig. 1).

Inhibitor	Inhibitor conc. (μmole/l)	Calculated K_i (μmole/l)
Phenylpyruvic acid	100	30
p-OH-Phenylpyruvic acid	100	120
Phenylacetic acid	100	55
p-OH-Phenylacetic acid	100	25
o-OH-Phenylacetic acid	100	100

various inhibitors. It is clear from Table 4 that increasing concentration of the pyridoxal-5-phosphate produced no demonstrable effect. The inhibition thus seems to be localized in the apoenzyme and not to be of the type caused by hydrazides or certain pyrocatechol derivatives. The former compounds have been shown by Killam and Bain [18] to inhibit the brain GAD, the inhibition being competitively reversed by pyridoxal-5-phosphate, however. Of the pyrocatechol derivatives, primary phenylalkylamines containing a phenolic hydroxy group in *meta* position such as dopamine, noradrenaline, and *m*-hydroxyphenylserine have been found by Holtz and Westermann [19] to inhibit the same enzyme. In this case, however, the inhibition is not of the competitive type but has been interpreted as a reaction of the inhibitor with the coenzyme with formation — *via* Schiff base formation — of tetrahydro*iso*-quinoline derivatives resulting in strong inhibition of the enzyme. If added in excess, pyridoxal-5-phosphate can counteract the inhibition.

When tested on the GABA-T system none of the inhibitors had any effect in the concentrations found to inhibit the GAD. Some of the results of these experiments are shown in Table 5. It appears from the data that there is a formation of about 0.5 μM of SSA for each micromole of GA. This difference in equivalence between the compounds formed in the transamination reaction is probably due to the SSA being utilized by the brain mitochondria and thus

Table 4. Influence of increasing amounts of pyridoxal-5-phosphate on the inhibition of glutamic acid decarboxylase. (The reaction mixture was as described above. Concentration of inhibitor 100 μmole/l. The figures refer to CO_2 output in μl).

	Without inhibitor	Concentration of pyridoxal-5-phosphate (μmole/3.05 ml)				
		1	2	4	6	8
Phenylpyruvic acid	165	62	64	65	66	66
p-OH-phenylpyruvic acid	161	96	92	95	93	94
Phenylacetic acid	176	21	23	25	28	25
p-OH-Phenylacetic acid	177	66	70	74	78	71
o-OH-Phenylacetic acid	156	76	73	78	81	80

Table 5. Influence of different phenylalanine metabolites on the α-aminobutyric acid-α-ketoglutaric acid transaminase. (Reaction mixture as described above. Concentration of inhibitor 100 μmole/l).

Additions	Products formed μmole	
	Succinic semialdehyde *	Glutamic acid
Control	3.4	6.3
Phenylpyruvic acid	— **	6.1
p-OH-Phenylpyruvic acid	— **	5.9
Phenylacetic acid	3.1	6.4
p-OH-Phenylacetic acid	2.8	6.0
o-OH-Phenylacetic acid	3.0	5.9

* Calculated from its hydrazone.
** Addition interfered with method of estimation.

partially disappearing spontaneously. Oxygen uptake of guinea-pig brain slices [20] as well as esterification of phosphate by cat cortex mitochondria [21] have been shown to be supported by SSA and brain homogenates have also been found to catalyze a diphosphopyridine nucleotide dependent oxidation of SSA [11].

It is difficult to say whether these findings are of any significance in the investigation of the cause of the mental defect in phenylketonuria because no figures are available on the concentration of the different phenylalanine metabolites in the central nervous system. It is therefore impossible to decide whether the *in vitro* findings reported here have any counterpart *in vivo*.

In a search for the cause of the mental defect in phenylketonuria, however, it seems reasonable to consider the rôle played by the phenylalanine metabolites as inhibitors of enzymes believed to be important for normal function of the central nervous system. The fact that these metabolites are able to inhibit three different decarboxylases, all producers of possible transmitter substances, *i. e.* adrenaline, 5-hydroxytryptamine and GABA, is not without interest.

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