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BIOSYNTHESIS OF MUSTARD OIL GLUCOSIDES: N-HYDROXYPHENYLALANINE, A PRECURSOR OF GLUCOTROPAEOLIN AND A SUBSTRATE FOR THE ENZYMATIC AND NONENZYMATIC FORMATION OF PHENYLACETALDEHYDE OXIME*†

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Abstract—The incorporation of DL-N-hydroxyphenylalanine-2-^{14}C into the mustard oil glucoside glucotropaeolin was demonstrated by plant feeding experiments. The efficiency of conversion of ^{14}C from this acid into the aglycone moiety of glucotropaeolin was higher than that from DL-phenylalanine-2-^{14}C or -3-^{14}C, and was comparable with that observed previously from phenylacetaldehyde oxime-1-^{14}C. These results support the reaction sequence: phenylalanine → N-hydroxyphenylalanine → phenylacetaldehyde oxime → glucotropaeolin. Enzyme preparations were obtained from *Sinapis alba* L., *Tropaeolum majus* L. and *Nasturtium officinale* R.Br. which catalyze the transformation of N-hydroxyphenylalanine to phenylacetaldehyde oxime. Although no absolute requirement for cofactors could be found, an increase in the rate of formation of the aldehyde oxime and consumption of oxygen was observed using FMN and enzyme preparations from *T. majus* and *S. alba*. Using the enzyme from *T. majus* it was demonstrated that the μmoles of phenylacetaldehyde oxime formed and of oxygen consumed were equivalent. In this case FMN appeared to be the prosthetic group and oxygen the physiological hydrogen acceptor. In addition to this enzymatic conversion, an unspecific and nonenzymatic oxidation of the N-hydroxyamino acid to phenylacetaldehyde oxime and phenylacetonitrile was observed.

INTRODUCTION

THE mustard oil glucosides constitute a well-defined class of plant products and detailed reviews have appeared on their chemistry,[1] distribution[2] and biosynthesis.[3] The similarity between the carbon–nitrogen skeletons of natural L-amino acids and some mustard oil glucosides suggests that amino acids may be the precursors of their aglycone moieties. Studies[4–6] on the biosynthesis of glucotropaeolin (III), whose aglycone is structurally related to phenylalanine (I), have confirmed this amino acid to be an efficient precursor of the thioglucoside aglycone. Feeding experiments employing L-phenylalanine-^{14}C-^{15}N have

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† This work is part IX of a series of papers on the biosynthesis of mustard oil glucosides; for part VIII see (8).

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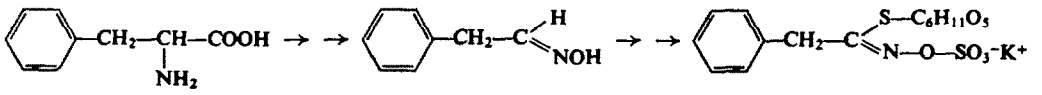
[1] A. KJAER, *Pure Appl. Chem.* 7, 229 (1963).
[2] A. KJAER, in *Chemical Plant Taxonomy* (edited by T. SWAIN), p. 453. Academic Press, New York (1963).
[3] E. W. UNDERHILL and L. R. WETTER, in *Biosynthesis of Aromatic Compounds, Proceedings of the 2nd Meeting of the Federation of European Biochemical Societies*, Vol. 3 (edited by G. BILLEK), p. 129. Pergamon Press, Oxford (1966).
[4] E. W. UNDERHILL, M. D. CHISHOLM and L. R. WETTER, *Can. J. Biochem. Physiol.* **40**, 1505 (1962).
[5] M. H. BENN, *Chem. Ind. (London)*, 1907 (1962).
[6] B. A. TAPPER and G. W. BUTLER, *Arch. Biochem. Biophys.* **120**, 719 (1967).

demonstrated[7] that the carbon and nitrogen skeleton of this amino acid, except C-1, is incorporated as a unit; a result which makes it mandatory that intermediates between L-phenylalanine and the thioglucoside be nitrogenous.

Recently it has been reported[6,8] that phenylacetaldehyde oxime (II) is a more efficient precursor of glucotropaeolin aglycone than phenylalanine. The demonstration of the conversion of phenylalanine to the aldehyde oxime in *Tropaeolum majus* L.[8] further characterized phenylacetaldehyde oxime as a biosynthetic intermediate. In analogous experiments in *Brassica oleracea* 3-indolylacetaldehyde oxime was detected as a product of L-tryptophan metabolism.* Also, the findings that isobutyraldehyde oxime and 3-phenylpropionaldehyde oxime are efficient precursors of the mustard oil glucosides, glucoputranjivin[6] and gluconasturtiin,[8] respectively, confirm a general biosynthetic sequence for the formation of these thioglucosides from amino acids as outlined in Fig. 1 for glucotropaeolin.

This communication deals with the efficient conversion of carbon-14 from N-hydroxyphenylalanine-2-^{14}C into the aglycone of glucotropaeolin. In order to give further support



I II III

FIG. 1. PATHWAY FOR THE FORMATION OF GLUCOTROPAEOLIN.

that N-hydroxyamino acids function as intermediates in natural processes, results of studies using cell-free preparations of *T. majus* L., *Nasturtium officinale* R.Br. and *Sinapis abla* L. are also presented which demonstrate the enzymatic conversion of N-hydroxyphenylalanine to phenylacetaldehyde oxime.

RESULTS

The data in Table 1 summarize the results of three feeding experiments in which labelled compounds were administered to *Tropaeolum majus* shoots. In the first experiment the shoots bore flower buds while those employed in the other experiments had not developed to this stage of maturity. In each of the three feedings there was a somewhat greater efficiency of conversion of carbon-14 into glucotropaeolin aglycone from DL-N-hydroxyphenylalanine-2-^{14}C as compared with that from DL-phenylalanine-2-^{14}C or -3-^{14}C. In agreement with earlier findings in this laboratory[7] only a low conversion of the tracer occurred from sodium 2-oximino-3-phenylpropionate-2-^{14}C in the final experiment.

In preliminary studies of enzyme preparations from several Cruciferae, transformations of L-phenylalanine and DL-2-amino-4-phenylbutyric acid to phenylacetaldehyde oxime and 3-phenylpropionaldehyde oxime were observed to take place in low yields. In order to ascertain more fully whether N-hydroxyphenylalanine functions as an intermediate between phenylalanine and phenylacetaldehyde oxime in the biosynthesis of glucotropaeolin and the involvement, if any, of 2-oximino-3-phenylpropionic acid, we tested these compounds as substrates for various enzyme preparations. The enzyme assay was not based on the disappearance of substrate but rather on the formation of phenylacetaldehyde oxime-^{14}C since preliminary investigations revealed the formation of other radioactive compounds in addition to the aldehyde oxime when DL-N-hydroxyphenylalanine-2-^{14}C was used as substrate.

* H. KINDL, unpublished.

[7] E. W. UNDERHILL and M. D. CHISHOLM, *Biochem. Biophys. Res. Commun.* **14**, 425 (1964).
[8] E. W. UNDERHILL, *European J. Biochem.* **2**, 61 (1967).

TABLE 1. COMPARISON OF ^{14}C-LABELLED COMPOUNDS AS PRECURSORS OF THE AGLYCONE OF GLUCOTROPAEOLIN

Expt.	Compound fed	μC fed	Spec. act. (μC/mmole)	Fresh wt. of shoots (g)	Glucotropaeolin aglycone* Wt. (mg)	Spec. act. (μC/mmole)	% ^{14}C converted
1	DL-Phenylalanine-2-^{14}C	4·13	110	63	58·5	0·90	7·68
	DL-N-Hydroxyphenyl-alanine-2-^{14}C	2·95	82	58	46·8	1·03	9·84
2	DL-Phenylalanine-3-^{14}C	10·73	269	35	29·8	5·31	8·95
	DL-N-Hydroxyphenyl-alanine-2-^{14}C	3·20	82	38	43·3	1·58	12·91
3	DL-Phenylalanine-3-^{14}C	14·12	333	30	17·1	6·82	4·97
	DL-N-Hydroxyphenyl-alanine-2-^{14}C	3·05	82	33	22·6	1·82	8·14
	DL-N-Hydroxyphenyl-alanine-2-^{14}C	2·86	82	37	25·2	1·37	7·28
	2-Oximino-3-phenyl-propionate-2-^{14}C†	12·02	360	46	34·2	0·12	0·20

* Isolated as benzylthiourea.
† Administered as the sodium salt.

The conversion of DL-N-hydroxyphenylalanine-2-^{14}C to labelled phenylacetaldehyde oxime was demonstrated with enzyme preparations (both prior to and following partial heat denaturation) from *Sinapis alba*, *T. majus* and *Nasturtium officinale* whereas no formation of phenylacetaldehyde oxime was detectable using 2-oximino-3-phenylpropionic acid-2-^{14}C. Employing the assay for the detection of labelled phenylacetaldehyde oxime and without addition of cofactors to the incubations the protein was purified as summarized in Table 2. Most remarkable is the great increase in the specific and total activity by heat treatment. It would appear that such increases were due to removal of inhibitor leading to the increase in total activity and removal of denatured protein to increase the specific activity.

TABLE 2. PURIFICATION OF THE ENZYME FROM *N. officinale*

Step	Specific activity (units*/mg) $\times 10^4$	Total activity (units*)
Crude extract	0·3	0·80
Ammonium sulfate fractionation	5·5	0·71
Partial heat denaturation	69	2·30
Sephadex G-100	310	1·80
Ammonium sulfate precipitation	420	1·68

* A unit of enzyme activity is the formation of 1 μmole of phenylacetaldehyde oxime-^{14}C per min at 25° under the defined conditions.

More detailed results could be obtained about the enzyme when crude enzyme preparations were put on a Sephadex G-100 (2·0 × 70 cm) column which was calibrated with dextran blue P-2000 and hemoglobin (Fig. 2 where $V_t = 285$ ml, $V_0 = 85$ ml and K_{av} as defined in Ref. 9). The maximum of enzyme activity was detected corresponding to a molecular weight

of 50,000 to 60,000 for *N. officinale* (Fig. 2) and *T. majus* and of 35,000 to 45,000 in the case of *S. alba*. A major protein peak without enzyme activity had an elution volume corresponding to a molecular weight of about 100,000. In all cases it was obvious that more than one species of protein showed catalytic activity. Furthermore, fractions with a molecular weight lower than 10,000 yielded an increase in the formation of phenylacetaldehyde oxime.

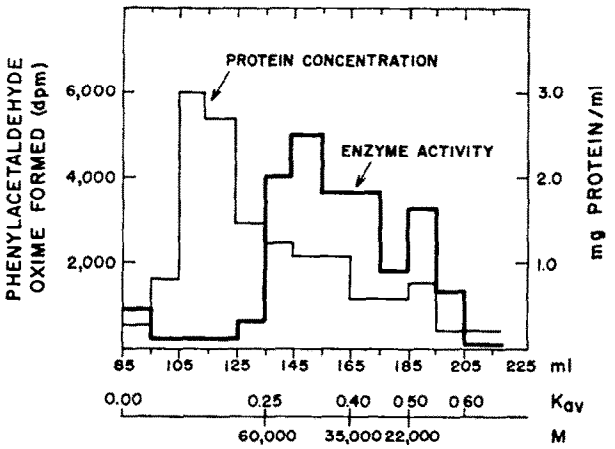


FIG. 2. FRACTIONATION OF AN ENZYME PREPARATION FROM *N. officinale* ON SEPHADEX G-100.

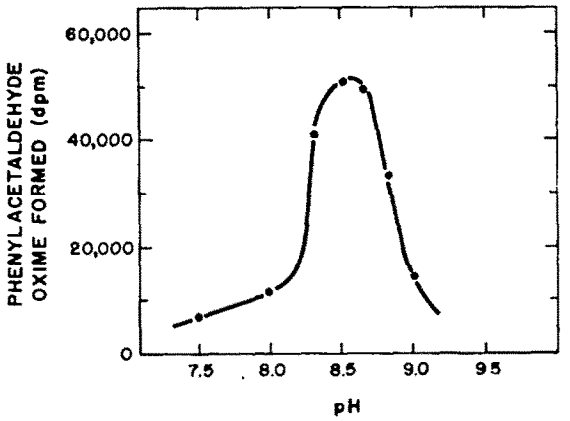


FIG. 3. EFFECT OF pH ON THE FORMATION OF PHENYLACETALDEHYDE OXIME-^{14}C USING AN ENZYME PREPARATION (PURIFIED) FROM *T. majus* L.

Fractions after Sephadex G-100 chromatography exhibited enzyme activities without addition of cofactors and had a pH optimum of 8·5 (Fig. 3). The K_m for N-hydroxyphenylalanine, estimated from the initial velocities of 1·0 to 20 mM solutions, was 7 mM. Substrate inhibition occurred for solutions higher than 8 mM as evidenced by the departure from linearity of the Lineweaver-Burk plot. When N-hydroxyphenylalanine was incubated in the presence of boiled enzyme or in the absence of enzyme small conversions to phenylacetaldehyde oxime were observed and are designated as "background" in Fig. 4 which shows a linear increase in the enzymatic formation of the aldehyde oxime during a period of more than 1 hr. A strict proportionality between the conversion of N-hydroxyphenylalanine to

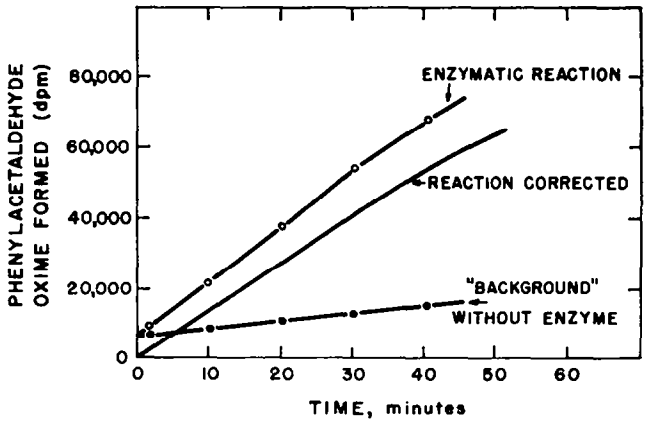


FIG. 4. AMOUNT OF PHENYLACETALDEHYDE OXIME-^{14}C FORMED WITH RELATION TO TIME.

phenylacetaldehyde oxime with enzyme concentration was also obtained. Potassium cyanide and *p*-chloromercuriphenylsulfonate at 0·5 mM did not inhibit the reaction, but the enzyme was almost entirely destroyed by acetone precipitation.

Experiments were undertaken to determine whether the enzymatic formation of phenylacetaldehyde oxime involved a disproportionation[11] of N-hydroxyphenylalanine to give 2-oximino-3-phenylpropionic acid which on decarboxylation would be expected to yield the aldehyde oxime directly (see Fig. 8 under Discussion). According to such a disproportionation mechanism the sum total of 2-nitroso-3-phenylpropionic acid, 2-oximino-3-phenylpropionic acid, phenylacetonitrile and phenylacetaldehyde oxime should be equal to (but not greater than) the amount of phenylalanine formed. However, as shown in Table 3, the amount of phenylacetaldehyde oxime formed from N-hydroxyphenylalanine during 30 min incubation with enzyme was greater than the amount of phenylalanine formed.

TABLE 3. FORMATION OF PHENYLALANINE AND PHENYLACETALDEHYDE OXIME FROM N-HYDROXYPHENYLALANINE DUE TO ENZYMATIC AND SPONTANEOUS REACTIONS

No.	Time (min)	Enzyme	FMN (2 mg)	Formation of Phenylalanine (dpm) $\times 10^{-3}$	Phenylacetaldehyde oxime (dpm) $\times 10^{-3}$	Phenylacetonitrile (dpm) $\times 10^{-2}$
1	10	+	−	46	40	41
2	10	−	−	39	7·6	3·5
3	30	+	−	46	117	40
4	30	−	−	39	29	8·8
5	30	+	+	7	46	410

The incubation mixture (2·5 ml) contained 4 mg protein derived from *T. majus*, 1·55 mg ($1·7 \times 10^6$ dpm) DL-N-hydroxyphenylalanine-2-^{14}C, 0·1 M 2-amino-2-methyl-1,3-propandiol-HCl buffer, pH 8·6, 2 mM EDTA.

[9] T. C. LAURENT and J. KILLANDER, *J. Chromatog.* **14**, 317 (1964).
[10] M. KIESE and M. PEKIS, *Arch. Exptl Pathol. Pharmakol.* **246**, 413 (1964).
[11] I. D. SPENSER and A. AHMAD, *Proc. Chem. Soc.* 375 (1961).

The results obtained in the absence of enzyme indicate the formation of both phenylalanine and phenylacetaldehyde oxime from N-hydroxyphenylalanine. An additional product whose R_f on thin-layer chromatograms corresponded with authentic phenylacetonitrile was also formed, presumably by decarboxylation and dehydration of 2-oximino-3-phenylpropionic acid[12] or by dehydration of phenylacetaldehyde oxime.

On the simplest view, the enzymatic conversion of N-hydroxyphenylalanine to phenylacetaldehyde oxime should take place by removal of a pair of protons and decarboxylation. Although the enzyme did not require cofactors for the formation of phenylacetaldehyde oxime, considerable increases and decreases in the conversion of N-hydroxyphenylalanine to the aldehyde oxime were observed when various natural and unnatural compounds were added to the enzyme incubation mixture. In Table 4 the effects of such additions to the

TABLE 4. EFFECT OF VARIOUS COMPOUNDS ON THE FORMATION OF PHENYLACETALDEHYDE OXIME

Cofactors*	Relative activity
None†	100
FAD, NAD, B_6	91
B_6	88
FAD	125
Phenazine methosulfate (PMS)	320
FMN	265
Dithiothreitol	40

* Each cofactor was added to give a 2 mM solution except for PMS which was 0·5 mM.
† Enzyme was derived from *S. alba*.

enzyme derived from *S. alba* are summarized compared with the incubation without added cofactors. The addition of oxidizing reagents, in particular FMN and PMS (phenazine methosulfate), caused a large increase in the yield of aldehyde oxime whereas the reducing reagent, dithiothreitol, caused a marked decrease. The addition of *o*-iodosobenzoate and Fe^{3+} to incubations both in the presence and absence of enzyme resulted in large increases in the yield of phenylacetaldehyde oxime and the reaction seemed to be entirely due to a nonenzymatic oxidation and decarboxylation of the substrate. Similar results were obtained when the enzyme derived from *T. majus* and *N. officinale* was employed except no increase in aldehyde oxime was observed on addition of FMN to the enzyme preparation from *N. officinale*. Hemoglobin, known to catalyze the oxidation of N-atoms,[10] when incubated in the absence of the enzyme and cofactors also catalyzed the formation of phenylacetaldehyde oxime.

For a better understanding of the oxidation step the uptake of oxygen as measured by the Warburg apparatus was employed. The oxygen uptake was determined for enzyme preparations derived from *T. majus*, *S. alba* and *N. officinale* in the presence and absence of DL-N-hydroxyphenylalanine and of FMN. Also a pH optimum of 8·3 to 8·8 was obtained using this assay. The oxygen uptake in the absence of substrate but in the presence of enzyme alone (derived from each of the three plants) was negligible as was also the case when FMN (5 mM) was incubated with the enzyme and no substrate. It can be concluded from the data

[12] A. AHMAD and I. D. SPENSER, *Can. J. Chem.* **39**, 1340 (1961).

in Fig. 5 that an enzyme reaction involving the uptake of oxygen takes place with N-hydroxyphenylalanine and that the rate of the reaction is increased by the addition of FMN (by approximately the same magnitude as shown in Table 4 for FMN). The K_m for FMN using the protein from *T. majus* was 0·1 mM and using the protein from *S. alba* was 0·6 mM. There was no increase in rate of the reaction on addition of FMN using the protein derived from *N. officinale*. The addition of FAD to the enzyme derived from the above three plants resulted in only a slight increase in rate of oxygen uptake whereas the addition of PMS caused a greater increase in the rate than found when FMN was added (Fig. 6, cf. Table 4).

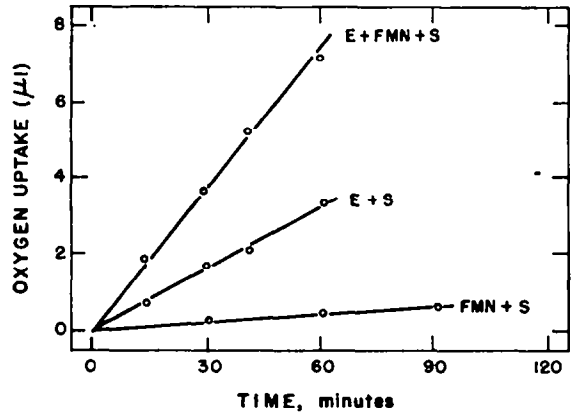


FIG. 5. OXYGEN UPTAKE USING AN UNDIALYZED ENZYME PREPARATION FROM *T. majus* AND DL-N-HYDROXYPHENYLALANINE IN PRESENCE AND ABSENCE OF FMN.
Leaves of *T. majus* (16 g, 3 weeks old) were extracted with 100 ml phosphate buffer, 0·5 M, pH 7·5. The ammonium sulfate precipitated protein (fraction between 0 and 40 g ammonium sulfate per 100 ml final volume) was dissolved in 20 ml 0·1 M 2-amino-2-methyl-1,3-propandiol-HCl buffer, pH 8·6, 2 mM EDTA. DL-N-Hydroxyphenylalanine, 5 mM (S), was incubated with 0·7 ml of enzyme (E) in the presence or absence of FMN (5 mM). Protein concentration was 4·5 mg/ml.

The uptake of oxygen and formation of phenylacetaldehyde oxime was studied simultaneously using the same enzyme preparation from *T. majus* as employed for experiments in Fig 5 and DL-N-hydroxyphenylalanine-2-^{14}C as substrate. Incubations were made in ten

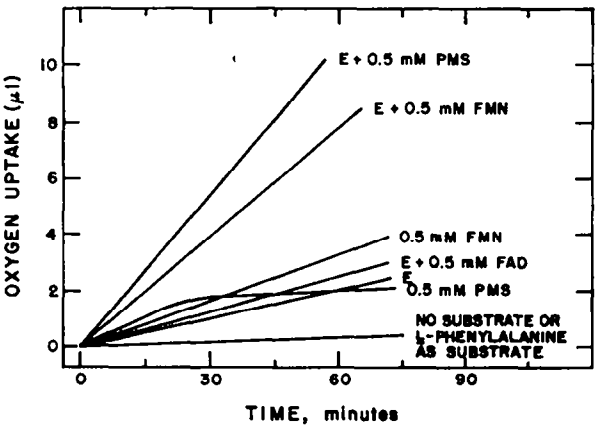


FIG. 6. OXYGEN UPTAKE USING A PURIFIED PROTEIN FROM *T. majus* AND DL-N-HYDROXYPHENYLALANINE AS SUBSTRATE.

Warburg vessels, five in the presence of 5 mM FMN and five without added FMN. At 10 min intervals the mixture from a vessel was worked up for the amount of radioactive oxime formed and the fifth vessel of each series was used for measurement of oxygen consumption (Fig. 7). For each determination the ratio of the μmoles of oxime formed to the μmoles of oxygen consumed was essentially 1:1. With phenylalanine as substrate there was no oxygen uptake (Fig. 6); thus, the above oxidation did not involve the ring.

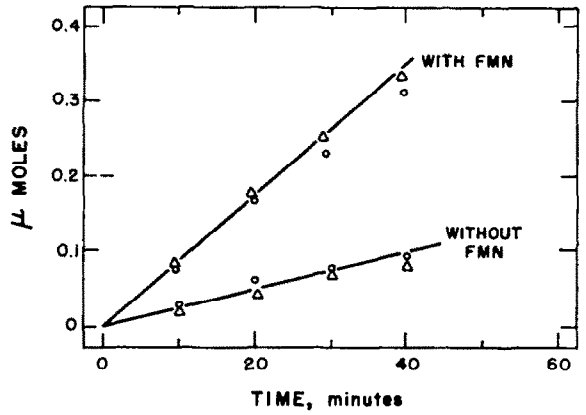


FIG. 7. SIMULTANEOUS MEASUREMENT OF OXYGEN UPTAKE (△) AND PHENYLACETALDEHYDE OXIME FORMED (○) USING ENZYME FROM *T. majus* AND DL-N-HYDROXYPHENYLALANINE-2-^{14}C AS SUBSTRATE.

DISCUSSION

According to theoretical considerations, the most likely intermediates between phenylalanine and phenylacetaldehyde oxime on the pathway leading to the formation of glucotropaeolin seemed to be N-hydroxyphenylalanine and 2-oximino-3-phenylpropionic acid. The results presented in this paper from plant feeding experiments (Table 1) show a greater efficiency of conversion of ^{14}C from N-hydroxyphenylalanine-2-^{14}C into the thioglucoside aglycone as compared with that from DL-phenylalanine-2-^{14}C or -3-^{14}C whereas only an insignificant amount of ^{14}C from 2-oximino-3-phenylpropionic acid was incorporated. The efficiency of incorporation of the N-hydroxyamino acid was comparable to that reported previously from phenylacetaldehyde oxime-1-^{14}C which was shown to be derived from phenylalanine in *Tropaeolum majus*.[8] It would appear to be most unlikely that the ^{14}C from N-hydroxyphenylalanine-2-^{14}C was incorporated via phenylalanine into the thioglucoside as a result of a nonenzymatic or spontaneous disproportionation reaction such as reported by Spenser and Ahmad.[11] Under such circumstances a maximum of one-half of the administered activity would have been converted to phenylalanine with an equal amount of ^{14}C appearing in the other products of the disproportionation: the greater per cent incorporation of the tracer into the glucoside from N-hydroxyphenylalanine-2-^{14}C as compared with that from phenylalanine-2- and -3-^{14}C would appear to rule out such a possibility.

The apparent failure of 2-oximino-3-phenylpropionic acid to serve as a precursor of phenylacetaldehyde oxime and glucotropaeolin is somewhat perplexing, particularly in light of recent investigations on the biosynthesis of cyanogenic glucosides where amino acids,

2-oximino acids[13] and aldehyde oximes[14] are all considered as precursors of this class of glucoside. However, the reaction sequence leading to the formation of the mustard oil glucosides would appear on the basis of the results of the feeding experiments not to involve 2-oximino-3-phenylpropionic acid, but rather the conversions, phenylalanine → N-hydroxyphenylalanine → [?] → phenylacetaldehyde oxime →→ glucotropaeolin. The conversion of phenylalanine to N-hydroxyphenylalanine most likely takes place by an analogous reaction to that reported by Stevens and Emery[15] for the biosynthesis of hadacidin which proceeds by oxygenation of the amino nitrogen of glycine to yield N-hydroxyglycine; a similar pathway but involving ornithine has also been reported for the biosynthesis of ferrichrome.[16]

Some N-hydroxyamino acids have been found in nature, mostly as N-acyl compounds[17] but N-hydroxyphenylalanine itself has not been reported as a natural product. Little detailed information is known about the chemical reactivity of these compounds although Spenser and Ahmad[11] have shown that N-hydroxyphenylalanine disproportionates to phenylalanine and 2-oximino-3-phenylpropionic acid; 2-oximino-3-phenylpropionic acid, which is presumed to be formed from the corresponding 2-nitroso acid by tautomeric shift, may be readily converted to phenylacetonitrile[12] (reaction D, Fig. 8). We could confirm this behavior for N-hydroxyphenylalanine (IV) as at pH 7 and 25° we found phenylalanine (I) and small amounts of phenylacetonitrile (VII) produced. In addition, we have identified phenylacetaldehyde oxime (II) as a further product which may also dehydrate to the nitrile (reaction C). The addition of oxidizing reagents as FMN or *o*-iodosobenzoate caused an increase in the formation of the aldehyde oxime and one must presume that the decarboxylation of one isomer of 2-oximino-3-phenylpropionic acid is spontaneous.

Definite support for the enzymatic conversion of N-hydroxyphenylalanine to phenylacetaldehyde oxime was obtained through studies with cell-free preparations from *T. majus*, *Nasturtium officinale* and *Sinapis alba*. Although the conversion of the N-hydroxyamino acid to the aldehyde oxime with low molecular weight compounds, e.g. FMN, Fe^{3+} and *o*-iodosobenzoate, was observed, an enzymatic conversion was readily demonstrated and would appear to be the physiological process involved in the pathway leading to the formation of phenylacetaldehyde oxime and glucotropaeolin. We have been able to exclude the possibility that our enzyme preparations catalyze the disproportionation reactions A and B (Fig. 8) since in most cases the formation of phenylacetaldehyde oxime was distinctly higher than the amount of phenylalanine formed (Table 3); a result which is incompatible with reactions A and B.

2-Oximino-3-phenylpropionic acid did not act as a substrate for the enzyme, due perhaps to the use of the wrong geometric isomer or to the possibility that it remains enzyme bound in the reaction. In the latter case, reactions E and F could be responsible for the enzymatic conversion to the aldehyde oxime and could account for the finding (Fig. 7) that for each μmole of phenylacetaldehyde oxime formed an equivalent amount of oxygen was consumed. It is evident that the conversion of N-hydroxyphenylalanine to the aldehyde oxime involves an oxidation step but, at present, we have no evidence that 2-oximino-3-phenylpropionic acid is an intermediate in the reaction. On the other hand, it is also possible that the transformation of N-hydroxyphenylalanine to phenylacetaldehyde oxime, involving both a

[13] B. A. TAPPER. Personal communication.
[14] B. A. TAPPER, E. E. CONN and G. W. BUTLER, *Arch. Biochem. Biophys.* **119**, 593 (1967).
[15] R. L. STEVENS and T. F. EMERY, *Biochemistry* **5**, 74 (1966).
[16] T. F. EMERY, *Biochemistry* **5**, 3694 (1966).
[17] J. B. NEILANDS, *Science* **156**, 1443 (1967).

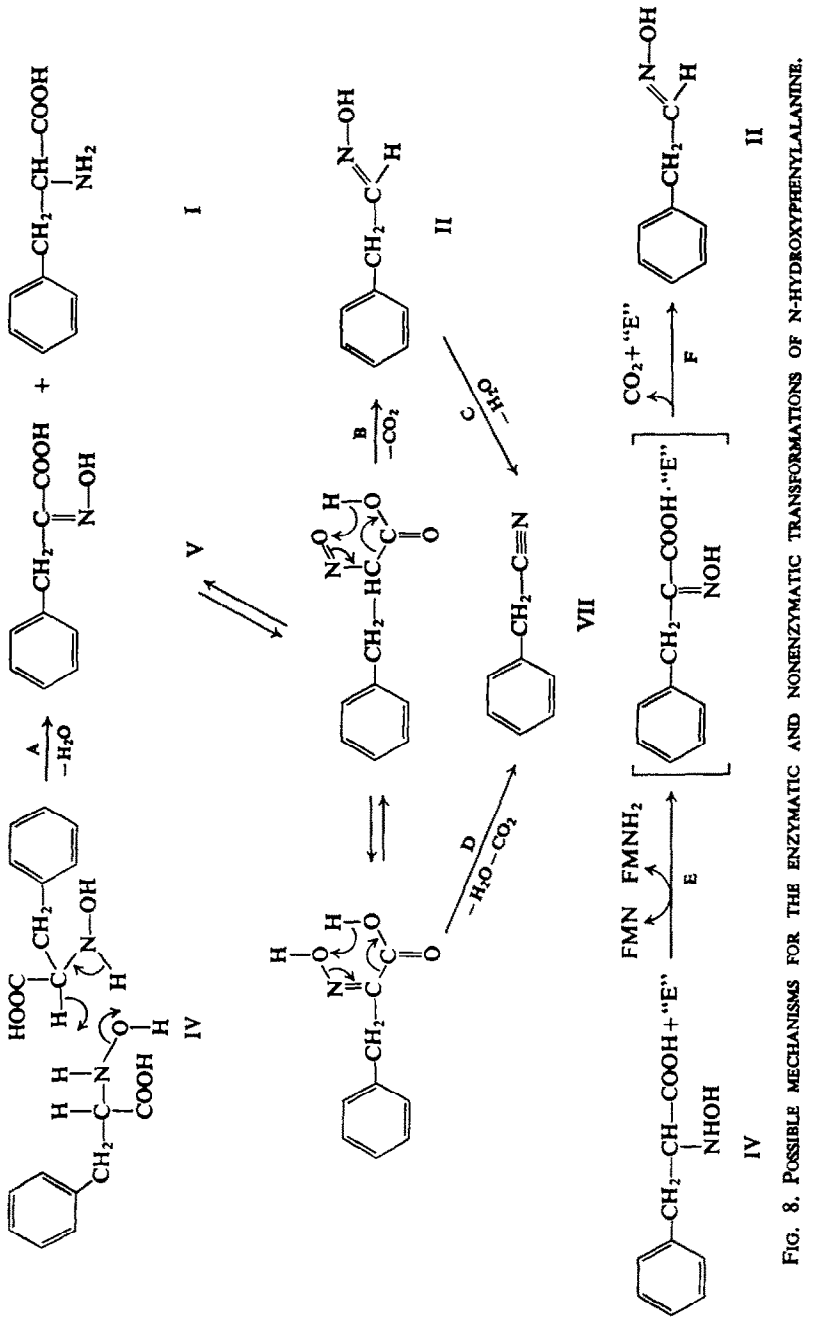


FIG. 8. POSSIBLE MECHANISMS FOR THE ENZYMATIC AND NONENZYMATIC TRANSFORMATIONS OF N-HYDROXYPHENYLALANINE.

dehydrogenation and decarboxylation, may have some parallel with the oxidative decarboxylation of amino acids to the next lower acid amide.[18,19] But the enzyme leading to the phenylacetaldehyde oxime appears to function as an oxidase rather than as a mixed function oxygenase. Analogous reactions may be involved in the formation of various nitro compounds, e.g., β-nitropropionic acid.[20]

Of the natural cofactors employed in these experiments, the addition of FMN to the enzyme preparations from *T. majus* and *S. alba* resulted in the largest increase in the rate of formation of the aldehyde oxime and consumption of oxygen. Since the enzyme functioned without addition of cofactors it is possible that the hydrogen acceptor (FMN?) remained covalently bound to the enzyme throughout its purification.

Owing to the uncertainty of there being one or more enzymes involved in the transformation of the N-hydroxyamino acid to the aldehyde oxime, as well as our present lack of understanding of the mechanism involved, no attempt was made to name the enzyme studied.

EXPERIMENTAL

^{14}C-Labelled Compounds

DL-Phenylalanine-2-^{14}C was purchased from the Radiochemical Centre, Amersham, England, and DL-phenylalanine-3-^{14}C from New England Nuclear Corp., Boston, Mass. 2-Oximino-3-phenylpropionic acid-2-^{14}C was kindly supplied by Mr. M. D. Chisholm of this laboratory.

N-Hydroxyphenylalanine-2-^{14}C was prepared as follows. 2-Bromo-3-phenylpropionic acid was first synthesized according to the procedure of Maimind *et al.*[21]: to an ice-cold solution of DL-phenylalanine-2-^{14}C (495 mg) and KBr (1·2 g) in 5 ml of 3 N H_2SO_4, a saturated aqueous solution of $NaNO_2$ (270 mg) was added dropwise and with stirring over 3 hr. The internal temperature of the reaction mixture was maintained at −10°. The mixture was diluted with 1·5 ml of water and extracted three times with 10 ml of ether and the methyl ester of 2-bromo-3-phenylpropionic acid was prepared using CH_2N_2. The solution of the bromo ester was washed once with 5% $NaHCO_3$ and the ether dried (Na_2SO_4). The yield of methyl-2-bromo-3-phenylpropionate-2-^{14}C was 72%. N-Hydroxyphenylalanine-2-^{14}C was prepared according to the procedure of Buehler and Brown[22] by stirring equimolar quantities of the ^{14}C-labelled α-bromo ester, *anti*-benzaldehyde oxime and Na in anhydrous ethanol overnight and refluxing the resulting substituted nitrone with 3 ml of conc. HCl for 30 min. The α-N-hydroxyamino acid was recrystallized three times from water–ethanol to yield 110 mg of product (20% from DL-phenylalanine-2-^{14}C), m.p. 158–159°, with a radiochemical purity in excess of 95% when chromatographed using *n*-butanol:concentrated NH_4OH:ethanol:benzene (5:3:2:1 v/v),[23] R_f 0·86, and *n*-butanol saturated with 2 N NH_4OH, R_f 0·91. When chromatographed in *n*-butanol: acetic acid: water (4:2:1·8 v/v) there was evidence of the disproportionation reported by Spenser and Ahmad[11] with the appearance of a radioactive ninhydrin positive spot (R_f 0·64) corresponding to phenylalanine in addition to the major radioactive reducing spot[24] at R_f 0·91 with some tailing.

Plant Feeding Experiments

Labelled compounds were administered to *T. majus* L. by immersing the cut end of the plant stem in an aqueous solution of the tracer (2–10 ml). Water was supplied as required for complete uptake of the compounds (*ca.* 2–3 hr) and for the remainder of the metabolic period (22 hr under continuous light). The aglycone moiety of glucotropaeolin was isolated from the plants as benzylthiourea as described previously.[4]

Enzyme Assay

The reaction mixture (2·5 ml) contained 5 mM N-hydroxyphenylalanine, 0·1 M 2-amino-2-methyl-1,3-propandiol-HCl buffer, pH 8·6, 2 mM EDTA and 0·1 to 0·5 units of enzyme (unit of enzyme activity as defined in Table 2). To stop the reaction 0·5 ml of ethanol containing 1–2 mg of inactive phenylacetaldehyde oxime

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was added and heated on a steam bath for 3 min. The reaction mixture was transferred with 20 ml water to a separatory funnel and extracted with 20 ml benzene. The benzene solution was washed with 3 ml water, dried (Na_2SO_4) and an aliquot of 5 ml counted. The main part of the aldoxime could be separated by TLC (thin-layer chromatography) with benzene:ethyl acetate, 7:1 (v/v). R_f values under these conditions were phenylacetaldehyde oxime 0·44, phenylpropionaldehyde oxime 0·55, phenylacetonitrile 0·85, phenylalanine, 2-oximino-3-phenylpropionic acid and N-hydroxyphenylalanine 0·00. The appropriate area was removed from the plates and counted. The amount (μmoles) of phenylacetaldehyde oxime formed was calculated from the ^{14}C activity isolated from the plates and the specific activity of the DL-N-hydroxyphenylalanine-2-^{14}C used in the incubation mixture. To verify that the radioactive area at R_f 0·44 belonged to phenylacetaldehyde oxime two solvents for paper chromatography were used, namely (*a*) paper impregnated with 10% α-bromonaphthalene in $CHCl_3$, then chromatography in water:pyridine, 2:1 (v/v), R_x value to *p*-nitrophenol 0·35 and (*b*) paper impregnated with 20% (v/v) formamide in ethanol, then chromatography in cyclohexane, R_f value 0·27. The aqueous solution from the benzene extraction was put on a column (1 × 10 cm) containing Amberlite IR-4B (OH^-) resin. After elution with 80 ml water and concentration of the effluent, an aliquot was counted and the rest chromatographed on paper using the solvent *n*-butanol:ammonia:ethanol:water, 5:3:2:1 (v/v). The area on the paper chromatogram containing the phenylalanine was cut out and counted.

Purification of the Enzyme

Leaves of *T. majus*, *S. alba* or *N. officinale* (50 g) were ground with liquid N_2 and extracted with 250 m phosphate buffer, 0·5 M and pH 7·5, containing 500 mg EDTA, 50 mg glutathione, 20 mg dithiothreitol and 10 g Polyclar AT. The extract was squeezed through a terylene cloth and centrifuged 15 min at 30,000 × *g*. The supernatant (200 ml) was subjected to $(NH_4)_2SO_4$ fractionation and the protein corresponding to a fraction between 14 to 38 g $(NH_4)_2SO_4$ per 100 ml final solution was used for further purification. The protein in 25 ml Tris-HCl buffer, pH 7·5 and 0·1 M, was heat treated by incubation for 10 min at 45°. After centrifugation, the supernatant sample (10 ml) was put on a Sephadex G-100 column (4·0 × 70 cm) and chromatographed using Tris-HCl buffer, 0·04 M, pH 7·5 and 1 mM EDTA, as solvent. A fraction corresponding to a K_{av}=0·20 to 0·50 was collected (60 ml) and the protein concentrated by precipitation with 30 g of $(NH_4)_2SO_4$. The protein was dissolved in 20 ml Tris-HCl buffer and the solution clarified by centrifugation for 20 min at 30,000 × g. Protein was determined according to Lowry *et al.*[25]

Isotope Analyses

Radioactive areas on paper chromatograms were detected using an Actigraph Chromatogram Scanner (Nuclear Chicago Corp.) or as paper strips suspended in a Butyl-PBD (Ciba Limited, Basle)/toluene scintillator solution (5 g Butyl-PBD per litre of toluene) and assayed in a Nuclear Chicago Mark I scintillation spectrometer. Areas removed from thin-layer chromatograms were suspended using Cab-O-Sil-M-5 (Cabot Carbon of Canada) in Butyl-PBD/toluene. Crystalline lipophilic compounds were counted in the Butyl-PBD/toluene scintillator solution while hydrophilic compounds were counted in Bray's solution.[26]

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