80. THE COLI-TRYPTOPHAN-INDOLE REACTION

3. ESSENTIAL STRUCTURAL CONDITIONS FOR THE ENZYMIC DEGRADATION OF TRYPTOPHAN TO INDOLE

By JOHN WILLIAM BAKER AND FRANK CHARLES HAPPOLD

From the Department of Organic Chemistry and the Biochemical Laboratories, Department of Physiology, The University, Leeds

THE reaction whereby *l*-tryptophan is broken down to indole by *E. coli* has been studied independently by Woods [1935, 1, 2] and by Happold & Hoyle [1935]. Woods worked with washed viable cells of the organism, whereas the latter investigators used suspensions killed in various ways but which, nevertheless, retained the property of catalysing this reaction. By coincidence both investigations, although following different courses, included examination of a similar selection of those indole derivatives which, on the basis of various mechanisms suggested in the literature, should be intermediate stages in the breakdown. Both sets of data (obtained by different techniques) uniformly revealed failure to produce indole from such derivatives and thus effectively disproved the validity of current mechanisms which, after all, had been formulated solely on analogy to other degradations.

Woods [1935, 2] also studied the kinetics of the reaction and showed that the process requires 5 atoms of oxygen, that the conversion of tryptophan into indole goes to completion, and that the rate of indole production is equal to the rate at which the tryptophan disappears. The reaction could be represented

$$C_{11}H_{12}O_2N_2 + 5O \rightarrow C_8H_7N + NH_3 + H_2O + 3CO_2$$

and thus involves an over-all oxidation, deamination and decarboxylation. Whether these processes are effected simultaneously by different enzymes or by a single master enzyme which catalyses simultaneously a number of reactions associated with the fission and oxidative breakdown of the alanine side-chain is not known.

The present communication describes an investigation in which the side-chain of tryptophan was modified systematically in an attempt to determine the structural features which are essential for the degradation of the molecule to indole by tryptophanase. The following derivatives have been tested with active enzyme systems: (CO₂H group modified) *l*-tryptophan methyl ester hydrochloride, *l*-α-amino-β-3-indolylpropionamide; (NH₂ group modified) the phenylurethane, *p*-nitrobenzoyl-, and *N*-methylène-derivatives of *l*-tryptophan, methyl *l*-α-methylamino-β-3-indolylpropionate hydriodide, *r*-α-methylamino-β-3-indolylpropionic acid, methyl-α-dimethylamino-β-3-indolylpropionate methiodide; (whole side-chain modified but still containing groups of the type NH₂ or NHR) β-3-indolylethylamine, 3-indolylacetamide, 3-indolealdehyde semicarbazone, 3-indolylglyoxylamide and, finally *r*-3-indolylglycine, the next lower homologue of tryptophan.

EXPERIMENTAL

Cultures of *E. coli* grown for 18 hr. on nutrient agar in two Roux bottles were each washed off with 40 ml. of normal saline and the cells separated by centrifuging. The deposit was resuspended in a similar amount of saline, the suspension recentrifuged and the deposits were finally suspended in 16 ml. of saline.

Tryptophan or the derivatives used were made up in $M/20$ phosphate buffer pH 7·5 and 1 mg. of the compound tested according to the following scheme.

A. 2 ml. washed cells, 20 ml. phosphate buffer pH 7·5, 10 ml. toluene and 1 mg. tryptophan.

B. 2 ml. washed cells, 20 ml. phosphate buffer pH 7·5, 10 ml. toluene and 1 mg. derivative.

C. 2 ml. washed cells, 20 ml. phosphate buffer pH 7·5, 10 ml. toluene.

D. 2 ml. boiled cells, 20 ml. phosphate buffer pH 7·5, 10 ml. toluene and 1 mg. tryptophan.

These four mixtures were aerated for 18 hr. at laboratory temperature (17–19°), extracted with 2 separate, 30 ml. portions of light petroleum (20–40°) and tested for indole by the method adopted by Happold & Hoyle [1934]. A similar series was incubated without toluene at 37° for periods up to 6 hr.

The toluene has the property of rendering the cells almost immediately non-viable but, since it also extracts the indole and the enzyme system is still appreciably active, the reaction tryptophan → indole goes almost to completion.

PREPARATION OF DERIVATIVES

Modification of the α-amino group

Phenylurethane of l-*tryptophan* was prepared by the method of Abderhalden & Kempe [1907]. Crystallized from methyl alcohol it had M.P. 169° (decomp.) (*loc. cit.*, M.P. 166° (corr.)). (Found: C, 66·9; H, 5·1%. Calc. for $C_{18}H_{17}O_3N_3$: C, 66·8; H, 5·3%.)

p-*Nitrobenzoyl derivative of* l-*tryptophan.* 0·5 g. of l-tryptophan, 2·5 ml. of N NaOH and 0·46 g. of p-nitrobenzoyl chloride dissolved in a little ether were shaken together for 1·5 hr., two further additions, each of 2·5 ml. of N NaOH, being added at intervals. Yellow crystalline material (probably the required derivative) soon separated but dissolved again when the further quantity of alkali was added. The solution was extracted with ether (which removed only a small amount of a brown crystalline material, M.P. *c.* 213°) and the alkaline solution was acidified with a little conc. HCl which precipitated a brown oil. This was extracted with ether. The residue from the dried ethereal extract slowly solidified to a glass-like mass. Crystallization of this from ethyl acetate-ligroin (B.P. 40–60°) gave p-nitrobenzoic acid, M.P. 228–232° (raised to 237° by admixture with a genuine specimen). The red syrup obtained by concentration of the mother liquor was diluted with warm 96% alcohol and seeded with a small crystal which had been obtained in an attempt to crystallize the original mixture from this solvent. The whole mass crystallized: it was triturated with a little cold 96% alcohol and drained on porous pot. Crystallization from 96% alcohol afforded the p-nitrobenzoyl derivative, sinters 114°, M.P. 121° (decomp.) in clusters of slender prisms. The compound seems to contain one molecule of alcohol of crystallization which is not completely eliminated at 120° in a vacuum. (Found: C, 60·0; H, 5·4; N, 10·85%. $C_{18}H_{15}O_5N_3 + C_2H_5OH$ requires C, 60·1;

H, 5·3; N, 10·5 %. Loss in weight when heated at 120°/vac. = 9·6 %. Loss for 1 mol. EtOH = 11·4 %. Found on the residue: C, 60·6; H, 4·3 %. $C_{18}H_{15}O_5N_3$ requires C, 61·2; H, 4·7 %.)

Methylene derivative of l-*tryptophan*. This was obtained by Homer's method [1913]. It was found to be simpler to dissolve the l-tryptophan directly in 40 % formalin solution with the aid of gentle warming. When the solution is kept at 38° ready crystallization of the compound occurs. Contrary to Homer all samples obtained had M.P. 196° (Homer [1913] gives M.P. 235–240°) not raised by washing with boiling acetone. The analysis figures of the compound dried in a vacuum at room temperature, when the specimen remained colourless, or at 110–120° for several hours, when it became slightly yellow, do not agree well with the compositions of the dihydrate and monohydrate, respectively, assigned by Homer. (Found, for a vacuum-dried sample: C, 58·5; H, 6·3; N, 10·0 %. Calc. for $C_{12}H_{12}O_2N_2, 2H_2O$: C, 57·1; H, 6·35; N, 11·1 %. Found for a sample dried at 110–120°: C, 62·3; H, 6·1; N, 12·7 %. Calc. for $C_{12}H_{12}O_2N_2, H_2O$: C, 61·5; H, 6·1; N, 12·0 %.) Such discrepancies raise doubts whether the action of formaldehyde upon l-tryptophan is as simple as the structures assigned by Homer suggest.

Methyl 1-α-*methylamino-β-3-indolylpropionate hydriodide*. A well dried specimen of l-tryptophan methyl ester hydrochloride (0·6 g.) was suspended in dry ether and dry NH_3 was passed into the solution at 0°. The precipitated NH_4Cl was filtered off and the free ester, obtained by evaporation of the solution, was well dried in a desiccator. It was then redissolved in dry ether and 0·24 g. of methyl iodide was added. The stoppered vessel was kept at room temperature for 24 hr. An oil separated on the sides of the vessel and slowly crystallized after standing for several days. The solution was decanted and the hydriodide was repeatedly washed *in situ* with dry ether. Crystallization of the solid residue from a very concentrated solution of dry acetone cooled in a refrigerator gave a small amount of the *hydriodide*, decomp. 192°. Microtitration with $AgNO_3$ gave I 34·8 %. $C_{13}H_{16}O_2N_2$, HI requires I, 35·3 %.

The specimen of r-α-methylamino-β-3-indolylpropionic acid was one synthesized by Miller & Robson [1938], and we wish to express our gratitude to Dr Robson for this generous gift.

Methyl-α-dimethylamino-β-3-indolylpropionate methiodide was obtained by methylation of l-tryptophan as described by van Romburgh & Barger [1913]. After crystallization from boiling water the specimen had M.P. 198° (decomp.). (Found: C, 46·0; H, 5·2 %. Calc. for $C_{15}H_{21}O_2N_2I$: C, 46·4; H, 5·4 %.)

Modification of the carboxyl group and of the side-chain as a whole

l-*Tryptophan methyl ester hydrochloride*, M.P. 214°, was prepared by the method of Abderhalden & Kempe [1907]. (Found: C, 57·0; H, 6·0 %. Calc. for $C_{12}H_{14}O_2N_2$, HCl: C, 56·6; H, 5·7 %.)

l-*Tryptophan amide*, M.P. 165–167° (capillary tube) was a specimen specially synthesized by Prof. C. P. Berg [cf. Baugess & Berg, 1934] to whom we wish to express our deep gratitude for his generous gift and help.

β-*Indolylethylamine hydrochloride* was purchased from Schuchardt.

β-*Indolylacetamide*. Preparation of the amide of β-indolylacetic acid through the acid chloride was found to be unsatisfactory since the action of either thionyl chloride or of phosphorus pentachloride in acetyl chloride on the acid gave amorphous, highly coloured products. It was obtained, however, by thermal decomposition of the ammonium salt. β-Indolylacetic acid (British Drug Houses) was purified by repeated crystallization from dry benzene and a

sample, M.P. 170°, was converted into its ammonium salt by evaporation of a solution of 1 g. of the acid in concentrated aqueous ammonia. The dry ammonium salt was mixed with a little ammonium carbonate and heated in a glycerol bath at 200–210° until gas evolution ceased (15 min.). The cooled residue was extracted with warm, dry benzene. The sparingly soluble amide, M.P. 135–140°, crystallized out when this solution was concentrated. Repeated crystallization from benzene containing a little alcohol gave the *amide*, M.P. 150–151°. (Found: C, 68·6; H, 5·9; N, 15·6 %. $C_{10}H_{10}ON_2$ requires C, 69·0; H, 5·8; N, 16·1 %.) Evaporation of the benzene mother liquors afforded the very soluble skatole, M.P. 90–91°, obtained by decarboxylation of the acetic acid.

Indole-β-aldehyde [Boyd & Robson, 1935] could not be made to give any aldehyde-ammonia compound, but forms a *semicarbazone*, M.P. 220° (decomp.). after crystallization from absolute alcohol. (Found: C, 59·0; H, 5·1. Calc. for $C_{10}H_{10}ON_4$: C, 59·4; H, 5·0 %.)

The synthesis of 3-indolylglyoxylamide, M.P. 252°, and *r*-3-indolylglycine, M.P. 221° (decomp.) have been described by one of us elsewhere [Baker, 1940].

RESULTS AND DISCUSSION

None of the derivatives examined as substrates for tryptophanase gave rise to the formation of indole with the exception of tryptophan methyl ester hydrochloride and tryptophanamide. The former is certainly only an apparent exception, since the data in Table 1 prove conclusively that the indole produced arises from the breakdown of *l*-tryptophan which is first formed by the hydrolysis of the ester in the slightly alkaline medium employed.

Table 1. *Indole formation from l-tryptophan methyl ester hydrochloride at 37°*

Indole formation after	0·5	1·0	2 hr.
(*a*) From fresh ester hydrochloride	0·022	0·070	0·078 mg.
(*b*) From ester hydrochloride previously left for 18 hr. in the medium at pH 7·5	0·070	0·118	0·095 mg.
(*c*) From *l*-tryptophan	0·070	0·118	0·088 mg.

The results obtained with *l*-tryptophanamide are interesting: they are summarized in Table 2.

Table 2. *Indole formation from l-tryptophanamide at 37°*

A. Tryptophanamide just brought to boil in medium pH 7 and left for 2 hr. in medium at 37° before addition of the washed cells.

B. Tryptophanamide previously left for 2 hr. in medium pH 7 at 37°.

C. Tryptophanamide added immediately to cells.

D. *l*-Tryptophan control.

Indole formed after	0·75	1·5	3 hr.
A	0·003	0·09	0·10 mg.
B	0·005	—	0·110 mg.
C	Faint trace	0·042	0·089 mg.
D	0·04	0·15	0·15 mg.

From these results it can be concluded:

(*a*) That the production of indole from the amide occurs very much more slowly (C) than from tryptophan itself (D) especially in the early stages of the reaction.

(b) That pre-treatments (A and B) expected to effect some preliminary hydrolysis of the amide, increase the initial rate of indole production, which, however, is still much less than that from tryptophan itself (D).

(c) That the reaction which produces indole from the amide is autocatalytic. Thus after 3 hr. (C) has given 0·089 mg. of indole which approximates closely to the amount (0·09 mg.) obtained after $2+1·5 = 3·5$ hr. in (A).

It would thus appear probable that indole production again really occurs through the breakdown of tryptophan itself, but that part of the enzyme complex may be able to adapt itself to the enzymolysis of the amide to trypto-phan, thus enabling the tryptophanase reaction to proceed. It has been reported that the coli bacillus possesses deamidases [Uttjino & Imazumi, 1938] so that such a preliminary enzymic deamidation would readily account for these results.

The side-chains which, when attached to the 3-position in the indole nucleus, have now been shown not to undergo degradation to indole with tryptophanase, are summarized in Table 3.

Table 3. *Modifications of the alanine side-chain in l-tryptophan which fail to undergo degradation to indole*
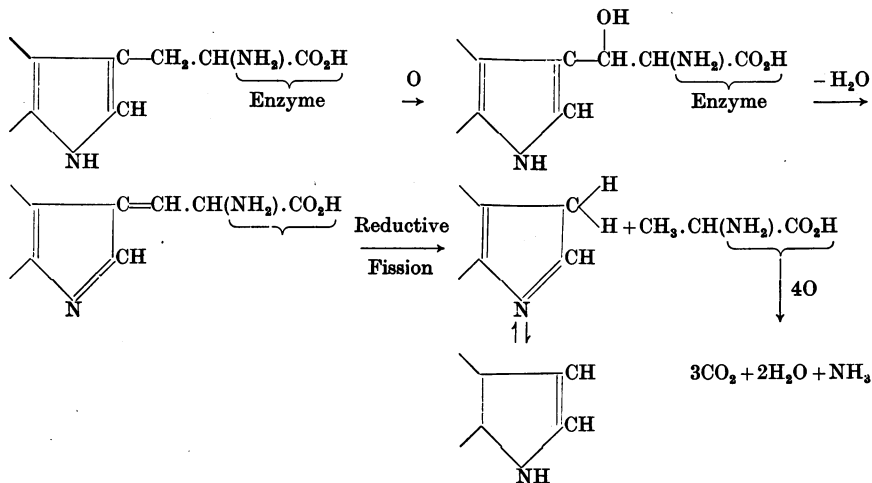
	Side-chain	Reference
I. α-NH_2 group modified	.CH_2.CH(NHMe).CO_2H	B.H.
	.CH_2.CH(CO_2H).N^+Me_3)I$^-$	,,
	.CH_2.CH(CO_2H).N:CH_2	,,
	.CH_2.CH(NHR).CO_2H	,,
	(R = .CO.NHPh and .CO.C_6H_4.NO_2 − p)	
II. CO_2H group modified	.CH_2.CH(NH_2).CO_2CH_3	B.H.
III. Whole side chain modified	.CH_2.CH_2.CO_2H	W., H.H.
	.CH:CH.CO_2H	,,
	.CH_2.CH(OH).CO_2H	,,
	.CH_2.CO.CO_2H	,,
	.CH_2.CO_2H	,,
	.CH_2.CO.NH_2	B.H.
	.CO_2H	W., H.H.
	.CHO	,,
	.CH:N.NH.CO.NH_2	B.H.
	.CH_2.CH_2NH_2	B.H., Frieber
	.CH(NH_2).CO_2H (dl-)	B.H.
	.CO.CO.NH_2	,,

B.H. = this communication.
W. = Woods [1935, 1, 2].
H.H. = Happold & Hoyle [1935].
Frieber [1922].

These data would seem to afford fairly conclusive evidence that the integrity of both the α-amino and carboxylic acid groups in the alanine side-chain is essential for the enzymic breakdown of tryptophan to indole.

The experimental exclusion of indole-3-carboxylic acid as the penultimate stage in this production of indole suggests that the latter may be produced by the elimination of the alanine side-chain as a whole. It is significant that the lower homologue of tryptophan, r-3-indolylglycine, does not give indole. This suggests that the breakdown mechanism may involve oxidation at the β-carbon atom of the alanine side-chain. The presence of a suitable oxygen substituent on this β-carbon renders possible prototropic change in the indole ring with consequent conversion into the indolylidene structure. If a parallel reductase system were present in the enzyme indole might then be formed by the direct reductive fission of this indolylidene structure. The essential integrity of the

α-amino and carboxylic acid groups might be needed to "fix" an amphoteric enzyme system to the tryptophan molecule, and in this connexion, it is perhaps not without significance that the optimum production of indole occurs in a medium of approximately pH 7·5. The following scheme is illustrative of the general type of breakdown tentatively suggested:

$$\text{C--}CH_2.CH(NH_2).CO_2H \quad (\text{Enzyme}) \quad \xrightarrow[\rightarrow]{O} \quad \text{C--}\overset{OH}{CH}.CH(NH_2).CO_2H \quad (\text{Enzyme}) \quad \xrightarrow{-H_2O}$$

$$\text{C=}CH.CH(NH_2).CO_2H \quad \xrightarrow[\text{Fission}]{\text{Reductive}} \quad \text{C}\overset{H}{<}\overset{H}{} + CH_3.CH(NH_2).CO_2H$$

$$\xrightarrow{} 3CO_2 + 2H_2O + NH_3$$

$$\downarrow 40$$

It is recognized that such reductive fission is a very unusual reaction and has no known analogy, nor must it be assumed that the fission occurs directly in one stage.

Amongst the derivatives examined, only in four (with the side-chains —CH:O, .CH:N.NH.CO.NH$_2$, —CO.CO.NH$_2$ and .CH:CH.CO$_2$H) is prototropic conversion into an indolylidene form structurally possible. None of these derivatives retains, in addition, the essential integrity of both the carboxyl and α-amino groups, and in no case is indole produced.

The next step in the experimental testing of the very tentative mechanism proposed is obviously to ascertain whether replacement of the mobile hydrogen in the indole ring by an alkyl group is, alone, sufficient to prevent the breakdown to indole; but since present circumstances make it probable that further attack on the problem might have to be postponed, the results already obtained have been placed on record.

SUMMARY

The action of tryptophanase on a large number of new tryptophan derivatives in which the alanine side-chain has been systematically modified has been studied.

The results obtained suggest that the breakdown to indole requires, *inter alia,* the following structural features:

    (a) a free carboxyl group;
    (b) an unsubstituted α-amino group;
    (c) a β-carbon atom capable of oxidative attack.

A tentative mechanism for the breakdown is suggested.

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