The Tryptophanase-tryptophan Reaction

7. FURTHER EVIDENCE REGARDING THE MECHANISM OF THE ENZYMIC DEGRADATION OF TRYPTOPHAN TO INDOLE: CRITICISM OF THE THEORY THAT β-o-AMINOPHENYLACETALDEHYDE IS THE INDOLE-FORMING INTERMEDIATE

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(*Received* 14 *January* 1946)

Baker & Happold (1940) deduced from their evidence that the following structural conditions are essential for the breakdown of tryptophan to indole by the tryptophanase enzyme of *Esch. coli*: (1) a free carboxyl group, (2) an unsubstituted α-amino group, (3) a side-chain β-carbon atom capable of oxidative attack. On the basis of these results a tentative scheme for the breakdown mechanism was suggested.

Indole formation from β-o-aminophenylethanol. Krebs, Hafez & Eggleston (1942) have suggested that β-o-aminophenylacetaldehyde is the effective indole-producing intermediate in the breakdown of tryptophan to indole. Certain structural difficulties in accepting this hypothesis have already been briefly summarized (Baker & Happold, 1942); the present communication records the results of further experiments designed with the two-fold object, first, of testing the validity of the mechanism of Krebs *et al.*, and secondly, of supplying further tests of the earlier hypothesis of Baker & Happold.

Data given in the experimental section (2) confirm the findings of Krebs *et al.* that conversion of β-o-aminophenylethanol is effected by the tryptophanase system, but, whereas with tryptophan, indole production is 90% complete in 1–2 hr., with β-o-aminophenylethanol the best results represent only a 22% conversion after 18 hr., thus indicating that a period of adaptation is required before indole is produced from this substrate and that the enzyme system concerned is not simply tryptophanase. Incidentally the figures of Krebs *et al.* indicate that the tryptophanase activity of their cells was small, giving at best only a 7·3% conversion of the β-o-aminophenylethanol after 6 hr. with cells grown for 5 days; but even these preparations effected conversion of tryptophan into indole in 50% of the theoretical amount.

Further evidence of this enzymic adaptation has now been obtained using cells grown in the presence of tryptophan or β-o-aminophenylethanol respectively. The results, detailed in the experimental section (3), show the greater enzymic mobility of the tryptophan-grown cells, their preferential adaptation to tryptophan as substrate, and the fact that those grown in the presence of β-o-aminophenylethanol first produce indole from this substrate.

Action of dimedon in degradation of (a) tryptophan, and (b) β-o-aminophenylethanol. Krebs and his coworkers recognized that the production of indole from β-o-aminophenylethanol via its oxidation product β-o-aminophenylacetaldehyde does not necessarily prove that the latter compound is an intermediate in the degradation of tryptophan to indole, but they prefer to assume that there is a link between the two indole-producing reactions. We do not find that such an assumption is supported by experimental facts. If the aldehyde were an essential intermediate in the tryptophan degradation, production of indole should be completely inhibited by the presence of aldehyde reagents which would react with, and remove, the necessary intermediate. Experiments with reagents like 2:4-dinitrophenylhydrazine were inconclusive, but dimedon (5:5-dimethyl*cyclo*hexane-1:3-dione) was quite without effect on indole production from tryptophan, whereas, as anticipated, its presence completely inhibited the formation of indole from β-o-aminophenylethanol (cf. experimental section 4). The dimedon was added in ethanol solution and, in the study of the effect of ethanol in the control experiments, the unexpected result was obtained that, whereas it had no effect on the indole production from tryptophan, it increased indole production from β-o-aminophenylethanol. The complete inhibiting action of dimedon with β-o-aminophenylethanol confirms the contention of Krebs *et al.* that β-o-aminophenylacetaldehyde is the intermediate in the indole production from the corresponding ethanol, but the absence of any such effect in the tryptophan breakdown is inconsistent with the hypothesis that the aldehyde is also an intermediate in the latter case.

Inhibition of indole formation from β-o-aminophenylethanol by dimedon is not observed (cf. ex-

perimental section 5), however, when the enzyme system is present in cells of *Esch. coli* which were grown in the presence of glucose and which must first metabolize stored carbohydrate (cf. Dawson & Happold, 1943). Possibly this is due to the preferential fixation of dimedon by some carbonyl group arising from the intermediary carbohydrate metabolism, thus enabling the cells to form indole from β-*o*-aminophenylethanol via the corresponding aldehyde, but even this conversion is less rapid than is the production of indole from tryptophan by the same cells.

Effect of N-*methylation in degradation of* (*a*) *tryptophan, and* (*b*) β-o-*aminophenylethanol.* In the earlier work of Baker & Happold (1940) the essential features in their tentative mechanism were: (1) oxidation of the β-CH_2 group of the alanine side-chain; (2) fission of the indolylidene structure which arises from a subsequent prototropic change, which, of necessity, involves the hydrogen atom in the 1-position of the indole nucleus. Such a prototropic change would, therefore, not be possible if this hydrogen atom were replaced by an alkyl group. Accordingly, α-amino-β-(1-methylindolyl-3)-propionic acid was prepared, and the action of tryptophanase upon it was investigated (cf. experimental section 6).

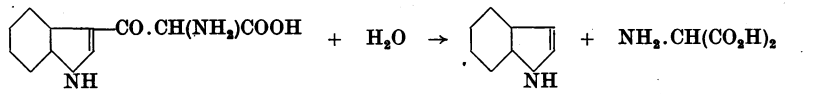
Three separate experiments were carried out using, as comparative substrates, 0·32 mg. of the methylated tryptophan derivative and 0·3 mg. of tryptophan, respectively. The usual quantities of washed cells in a phosphate buffer (pH 7·0) were used as the source of tryptophanase. With tryptophan as substrate, indole formation was 90–95% complete in 1 hr., but *no trace* of *N*-methylindole was formed from the methylated tryptophan. *N*-Methylindole was tested for (*a*) by Ehrlich's reagent, and (*b*) by attempted isolation of the picrate. Both methods gave negative results, although it was shown that synthetic *N*-methylindole added to the media could be correctly estimated by the method applied by Happold & Hoyle (1934) for the estimation of indole. This negative result is to be anticipated on the basis of the mechanism of Baker & Happold and makes it possible to prescribe a fourth essential structural condition for the breakdown of tryptophan to indole by tryptophanase, viz. the presence of an unsubstituted NH group in the 1-position of the indole nucleus. It is difficult to see why alkylation of this group should prevent the oxidation at carbon atom 2 in the indole ring and the subsequent formation of β-*o*-methylaminophenylacetaldehyde and *N*-methylindole required by the mechanism of Krebs *et al.*

Accordingly, β-*o*-methylaminophenylethanol was synthesized and was tested against controls, with both tryptophanase and with a live culture of *Esch. coli* grown in an acid hydrolysate of gelatine medium containing β-*o*-methylaminophenylethanol (cf. experimental section 9). In both cases a slight positive *N*-methylindole reaction was obtained with the Ehrlich reagent after 1–2 days. In the experiments with viable cells the reaction flask, left at room temperature for several months, developed a distinct indole odour, and the light petroleum extract gave a definite positive reaction with the Ehrlich reagent. Since *N*-methylindole treated with picric acid in dry ether solution gives a very characteristic crystalline picrate when examined under the microscope, similar identification of the breakdown product was attempted, but although the formation of a crystalline picrate (in minute quantity) could be observed, it did not have the characteristic crystalline form of *N*-methylindole picrate, and was obviously impure. Thus the most that can be said at the moment is that, whereas methylation of the indole-nitrogen in tryptophan definitely prevents its degradation to (methyl) indole by tryptophanase, the intermediate postulated in the mechanism of Krebs *et al.*, viz. β-*o*-methylaminophenylethanol does produce a basic product which has the odour of and which gives the colour reactions of *N*-methylindole.

Preliminary investigation of β-keto-derivatives of tryptophan. In order to obtain more positive evidence that β-oxidation of the alanine side-chain is involved in the breakdown of tryptophan to indole, an attempt has been made to synthesize α-amino-β-keto-β-3-indolylpropionic acid, in which such β-oxidation, $CH_2 \rightarrow CO$, has already been effected. On the basis of Baker & Happold's mechanism, this compound should be converted into indole by tryptophanase. The preparation of *ethyl β-keto-β-3-indolylpropionate* and its conversion into *ethyl α-bromo-β-keto-β-3-indolylpropionate* is described by one of us elsewhere (Baker, 1946). It has not, so far, been possible to isolate the corresponding α-amino-ester, but when the above α-bromo-ester was kept dissolved in a solution of ammonia in dry methanol (saturated at 0°) for 24 hr., the crude product, obtained after evaporation of the solution *in vacuo* at room temperature, gave slight positive results (by the Ehrlich reagent) for indole formation when acted upon by tryptophanase. This preliminary result (experimental section 7) requires further confirmation by the actual isolation of the pure α-amino-β-keto-acid, but it is interesting as the first example of a synthetic intermediate *containing an intact indole nucleus* which actually yields indole with tryptophanase, and it thus provides some additional support for the suggestion that β-oxidation in the alanine side-chain is actually one step in the degradation of tryptophan to indole by this enzyme system.

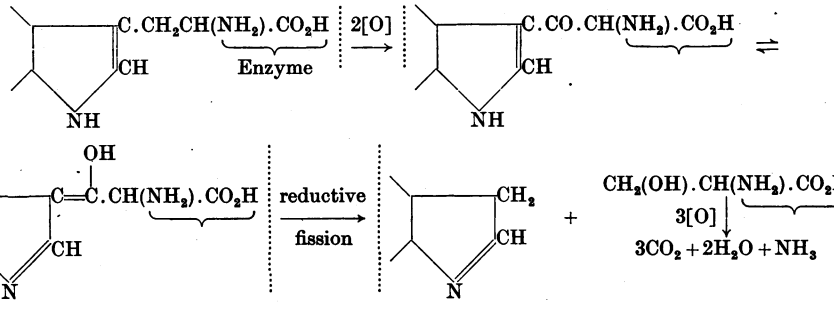
An alternative mechanism to the reductive fission of the indolylidene derivative previously suggested

by Baker & Happold (1942) would be a hydrolytic fission of the β-keto-compound:



This would involve the elimination of the side-chain in the form of aminomalonic acid. It is difficult to understand why the alkylation of the indole nitrogen should inhibit indole production if this mechanism were correct, but aminomalonic acid was synthesized (see experimental sections 1 and 8) and was found to be quite inert towards tryptophanase. It was tested in varying amounts in a phosphate buffer at pH 7·0 in Barcroft respirometers and in parallel with tryptophan. No oxygen uptake was observed with the aminomalonic acid. Thus, whatever the mechanism of fission, aminomalonic acid is not split off as such and then metabolized separately.

A more promising hypothesis may be based on the suggestion of Tatum & Bonner (1944) that, since the tryptophan essential for the growth of a mutant of *Neurospora* may be replaced by a combination of indole and *l*-(−)serine, the degradation of tryptophan to indole may be a reversal of the reaction found in *Neurospora*. On this basis the reductive fission of the indolylidene compound resulting from β-oxidation of the alanine side-chain (Baker & Happold, 1940) could lead to the elimination of the side-chain as serine:



Alternatively, hydrolytic fission might afford the corresponding aldehydo-compound

$$O{:}CH.CH(NH_2).CO_2H.$$

Further experimental tests of these hypotheses are in hand.

EXPERIMENTAL

(1) *Preparation of derivatives. Synthesis of α-amino-β-(1-methylindolyl-3)-propionic acid*

Preparation of N-*methyl-β-indolealdehyde.* The method used by Wieland, Konz & Mittasch (1934) was modified as follows: β-Indolealdehyde (1 g.) was shaken with water (30 ml.) and 10% aqueous sodium hydroxide (25 ml.), whilst dimethyl sulphate (8 ml.) was added in portions during 40 min. More 10% aqueous sodium hydroxide (15 ml. altogether) was added from time to time so as to keep the reaction mixture alkaline. As reaction proceeded, the crystals of β-indolealdehyde softened and yielded a reddish orange oil which solidified on keeping in the ice chest for 2 or 3 hr. The crude product (0·95 g.), m.p. 55–60°, was collected, washed with water, dried on porous plate, and then dissolved in a mixture of methanol (2 parts) and ether (25 parts). The solution was boiled with charcoal, filtered and evaporated to dryness on the steam-bath. The reddish yellow oil so obtained, on stirring with 5 times its volume of ether solidified yielding *N*-methyl-β-indolealdehyde as a pale yellow granular solid, m.p. 70–71°. On recrystallizing from a mixture of ether and a little methanol, *N*-methyl-β-indolealdehyde was obtained as colourless prisms, m.p. 71° (Wieland, Konz & Mittasch (1934) gave the m.p. of the partially purified product as 65°).

Preparation of N-*methyl-β-indolealdehyde*-(p-*chlorophenyl*)-*hydrazone.* *N*-methyl-β-indolealdehyde (0·25 g.) was dissolved in hot methyl alcohol (2 ml.), a solution of *p*-chlorophenylhydrazine (0·25 g.) in hot methanol (5 ml.) and glacial acetic acid (0·5 ml.) added and the mixture boiled for a few seconds and set aside to cool. Nearly colourless needles of the *hydrazone* (0·35 g.), m.p. 196° (decomp.), separated. *N*-methyl-β-indolealdehyde-*p*-chlorophenylhydrazone crystallized from ethanol (charcoal) in glistening colourless needles, m.p. 196° (decomp.). (Found: Cl, 12·6; $C_{16}H_{14}N_3Cl$ requires Cl, 12·5%.)

Condensation of N-*methyl-β-indolealdehyde with hydantoin.* *N*-Methyl-β-indolealdehyde (0·7 g.), hydantoin (0·5 g.) and piperidine (4 ml.) were heated together under reflux for 30 min., a clear orange yellow solution being produced from which no solid separated. After cooling, the liquid was poured into cold water (200 ml.), glacial acetic acid (6 ml.) added, and the yellow precipitate (0·52 g.) was filtered off, washed with water, methanol and then dried. After recrystallizing from glacial acetic acid (charcoal) it had m.p. 310° (decomp.) after darkening at 296°.

Reductive hydrolysis of N-*methylindolalhydantoin.* *N*-Methylindolalhydantoin, m.p. 308° (decomp.), (0·5 g.), 16% A.R. ammonium sulphide solution (6 ml.) and 3% aqueous ammonia (2 ml.) were sealed in a Carius tube and the mixture was heated for 32 hr. at temperatures varying from 120 to 150°. When cold, the tube was opened and the liquid was filtered from a quantity of brown viscous tar. The combined filtrates from two such tubes were evaporated to dryness *in vacuo*, and the residue extracted twice with

hot 3% ammonia (25 ml.). The combined extracts were concentrated to about 20 ml., treated with charcoal, filtered, and after adding 2 ml. of N-NaOH, the filtrate was boiled for a further 10 min. The liquid, after concentrating to about 10 ml. was again treated with charcoal, filtered, and the required amount of N-HCl to neutralize the N-NaOH previously introduced was added to the filtrate. Ethanol (5 ml.) was added to the turbid liquid and it was left overnight. The nearly colourless *N*-methyltryptophan (0·09 g.), m.p. 245–250° (decomp.), was filtered off and was recrystallized from 40% (v/v) ethanol in colourless crystals, m.p. 265–267° (decomp.), and after a second crystallization from 20% (v/v) ethanol, it was obtained as colourless glistening prisms (26 mg.), m.p. 267° (decomp.). (Found: C, 66·5; H, 6·4; N, 12·5. Calc. for $C_{12}H_{14}O_2N_2$; C, 66·1; H, 6·4; N, 12·8%.)

β-o-Methylaminophenylethanol. *β-o*-Aminophenylethanol (0·3 g.) was gently warmed with 0·4 g. of methyl iodide and the reaction mixture was kept at room temperature for 24 hr. The viscous product was extracted with dry ether, and the residual viscous hydriodide was dissolved in a small volume of water and repeatedly extracted with ether to remove all non-ionic material. The aqueous solution was made alkaline with sodium hydroxide and the liberated alcohol was extracted with ether. The residue from the dried, ethereal extract was distilled from a micro-flask under reduced pressure.

β-o-Methylaminophenylethanol distilled at a bath temperature of 130°/0·2 mm. leaving only a trace of residue. (Found: C, 71·5; H, 9·0. $C_9H_{13}OH$ requires C, 71·5; H, 8·6%.) The *picrate*, prepared from ethereal solutions of the base and of picric acid, had m.p. 122° after crystallization from ethyl acetate-light petroleum (b.p. 40–60°). It depressed the m.p. (182°) of the unmethylated compound to 100–150°. (Found: C, 47·0; H, 4·0. $C_{15}H_{16}O_8N_4$ requires C, 47·4; H, 4·2%.) The preparation and characterization of ethyl β-keto-β-3-indolylpropionate and its α-bromo-derivative, have been described elsewhere (Baker, 1946). A small quantity of this α-bromo-derivative was dissolved in a few ml. of dry methanol and the solution saturated with ammonia gas at 0°. It was kept sealed at room temperature for 24 hr. and then evaporated to dryness in a vacuum desiccator at room temperature. The residue without purification was tested with tryptophanase, in the usual manner.

Synthesis of aminomalonic acid. Ethyl aminomalonate prepared by the method of Redemann & Dunn (1940) was treated with concentrated aqueous ammonia in the cold, and afforded aminomalonamide, m.p. 187–188° (decomp.). Aminomalonamide (1 g.) was boiled for 30 min. with a solution of sodium hydroxide (2·4 g.) in water (25 ml.), the solution cooled below 10°, acidified with acetic acid and a slight excess of lead acetate solution added. The white precipitate of the lead salt was filtered off, washed with a little distilled water, suspended in distilled water (20 ml.) and decomposed with H_2S. After filtering off the lead sulphide, the filtrate was allowed to evaporate in a vacuum desiccator at room temperature and yielded colourless crystals of aminomalonic acid which decomposed at 112°. (Found: N, 10·5; $C_3H_5O_4N.H_2O$ requires N, 10·2%.)

(2) *Comparison between tryptophanase action and the production of indole from β-o-aminophenylethanol*

Esch. coli was grown in Roux bottles of tryptic casein digest containing 2% agar for 18 hr. at 37° (longer periods result in a marked decrease in tryptophanase activity). The cells were freshly prepared for each experiment, washed twice with 0·01 M-phosphate buffer, pH 7·0, and filtered twice through glass wool to remove traces of agar and other impurities. The washed suspensions were used at a final concentration of 10^9 cells per ml.

Indole production by these cells at 37° from 0·5 mg. *l*-tryptophan and from variable amounts of *β-o*-aminophenylethanol was studied in parallel series, the total volume of the reactants being 25 ml. so that $2·5 \times 10^{10}$ cells were present.

Indole was determined by the extraction method of Happold & Hoyle (1934). The indole production from *l*-tryptophan was 90% complete after 1–2 hr. in six experiments, but with the *β-o*-aminophenylethanol indole production had not always occurred even after 18 hr., though usually some indole had been produced. A typical positive finding is recorded in Table 1.

Table 1. *Indole formation from β-o-aminophenylethanol*

Reaction time (hr.)	Initial weight of β-o-aminophenylethanol: 0·32 mg.	0·64 mg.	1·28 mg.	1·60 mg.
	Yield of indole (μg.)			
Up to 3	Nil	Nil	Nil	Nil
4	Tr.	Nil	20	Nil
5	Tr.	Nil	40	Nil
12	—	—	30	7·5
18	60	Nil	—	25

(3) *Cell adaptation to tryptophan and to β-o-aminophenylethanol*

This enzymic adaptation can be shown by using cells grown in the presence of tryptophan or *β-o*-aminophenylethanol respectively, suspensions of each type of cell being tested in parallel for indole production from both compounds.

The basal medium used in these experiments was an acid digest of casein broth which was free from tryptophan. Cultures were grown for 18 hr. in the presence of one or other of these two compounds. Indole was invariably produced from the tryptophan, variably from the *β-o*-aminophenylethanol. The cells were now separated, well washed and tested; Table 2 gives the results obtained. One observes the greater enzymic mobility of the cells grown with tryptophan and their preferential adaptation to tryptophan as substrate and also that those grown in the presence of *β-o*-aminophenylethanol first produce indole from this substrate.

(4) *Action of carbonyl reagents*

Experiments with carbonyl group reagents such as 2:4-dinitrophenylhydrazine were inconclusive, but addition of dimedon completely or nearly

Table 2. *Indole formation from tryptophan and β-o-aminophenylethanol by cells adapted to each substrate*

The substrates and cells were suspended in 0·01 M-phosphate buffer, pH 7·0.

	Cells grown in			
	Tryptophan medium		β-o-Aminophenyl ethanol medium	
	Substrates tested			
	dl-Tryptophan (1 mg.)	β-o-Aminophenyl-ethanol (2 mg.)	*dl*-Tryptophan (1 mg.)	β-o-Aminophenyl-ethanol (2 mg.)
Reaction time (min.)	Amount of indole formed (μg.)			
20	75	7	Nil	Nil
40	225	150	Nil	Nil
150	225	180	Nil	Nil
1380	—	—	Nil	20

Table 3. *Action of dimedon on indole production by tryptophanase using tryptophan and β-o-aminophenylethanol as substrates*

Composition of mixtures under tryptophanase action		Indole (mg.) produced after (hr.)			
		2	3½	4	5
Phosphate buffer + 0·5 mg. tryptophan	+ 0·2 ml. satd. sol. of dimedon in ethanol	0·24	—	—	—
Do.	+ 0·2 ml. ethanol	0·25	—	—	—
Do.	—	0·24	—	—	—
Phosphate buffer + 1·28 mg. o-$NH_2.C_6H_4.C_2H_4OH$	+ 0·1 ml. satd. sol. of dimedon in ethanol	Nil	Nil	Nil	Nil
Do.	+ 0·2 ml. satd. sol. of dimedon in ethanol	Nil	Nil	Nil	Nil
Do.	—	Nil	0·02	0·025	0·04
Do.	+ 0·2 ml. ethanol	Tr.	0·045	0·07	0·09

Table 4. *Comparative inhibition of indole production by dimedon acting on cells grown in (a) the presence, and (b) the absence of glucose; tryptophan and β-o-aminophenylethanol as substrates*

Cell concentration + 0·2 ml. of a saturated ethanolic solution of dimedon and phosphate buffer, pH 7·0, to 15 ml. was used as before, tryptophan was used in 0·5 mg. and β-o-aminophenylethanol in 3 mg. amounts.

	(a) Glucose cells + dimedon		(b) Ordinary cells + dimedon	
	+ Tryptophan	+ β-o-Amino-phenylethanol		+ Tryptophan
Reaction time (min.)	Amount of indole formed (μg.)			
40	Nil	Nil	Nil	Complete
90	70	15	Nil	—
120	195	40	Nil	—
150	300	60	Nil	—
600	—	220	Nil	—

completely inhibited the production of indole from β-o-aminophenylethanol, but this reagent had no effect on the tryptophan reaction. Dimedon was added as saturated solution in ethanol and similar amounts of ethanol were added to the controls. Table 3 gives a typical result from one of five concordant experiments.

Attempts to isolate and characterize the small amounts of material which separated in the dimedon β-o-aminophenylethanol experiments were unsuccessful.

(5) *Action of cells grown in the presence of glucose on tryptophan and β-o-aminophenylethanol*

The action of cells grown in the presence of glucose and which contain no free tryptophanase was compared with tryptophanase-containing cells with both substrates and in the presence of dimedon. The varying degrees of conversion into indole are shown in Table 4.

(6) *Action of tryptophanase on α-amino-β-(1-methylindolyl-3)-propionic acid* (*N-methyltryptophan*)

Three separate experiments were made using 0·32 mg. *N*-methyltryptophan + 0·3 mg. tryptophan as substrates respectively. The usual quantities of washed cells in phosphate buffer (pH 7·0) were used as source of tryptophanase. Indole formation was 90–95 % complete in 1 hr. with tryptophan, but no trace of *N*-methylindole was produced from *N*-methyltryptophan. This was tested for by (*a*) using Ehrlich's reagent, and (*b*) by the attempted isolation of the picrate; both these methods gave negative findings although a prepared sample of *N*-methylindole could be estimated when added to media by the method applied by Happold & Hoyle (1934) for the estimation of indole.

(7) *Action of tryptophanase on a β-keto-derivative of tryptophan*

With the assistance of Dr F. W. Chattaway, ethyl α-bromo-β-keto-β-3-indolylpropionate was synthesized and treatment of this with cold methanolic ammonia gave a product (not yet isolated pure) which with the enzyme system gave a slight qualitative reaction for indole. Further attempts in this direction were negative.

(8) *Action of tryptophanase on aminomalonic acid*

This compound was quite inert to tryptophanase. It was tested in varying amounts in phosphate buffer at pH 7·0 in Barcroft respirometers and in parallel with tryptophan. No oxygen uptake was observed with the aminomalonic acid.

(9) *Action of tryptophanase on β-o-methylaminophenylethanol*

About 70–80 mg. of β-*o*-methylaminophenylethanol were available and, having regard to the difficulty experienced in obtaining consistent indole production from β-*o*-aminophenylethanol, a series of five experiments was performed in which 4 mg. portions of the *N*-methyl derivative were incubated with four-fold quantities of enzyme in 0·01 M-phosphate buffer, pH 7. These experiments were carried out on different days and with enzyme preparations of different origin. *N*-Methylindole was sought for in small samples at 1, 2, 3, 6 and 24 hr.; the method used was the extraction method previously mentioned and which can be applied equally to *N*-methylindole and to indole.

Most of the extracts gave characteristic indole colour reactions with Ehrlich's reagent at the 24 hr. period; in two cases where incubation was extended for a further 24 hr. this did not increase. Attempts to isolate a picrate which would assist identification of the compound produced were negative.

An attempt was next made to produce the *N*-methylindole by growing the organism in an acid hydrolysate of gelatine medium to which was added 45 mg. of *N*-methylaminophenylethanol. The basal medium was as follows:

Gelatine hydrolysate, equivalent of 5 g.; lactic acid 0·5 g.; pyruvic acid 0·5 g.; KH_2PO_4 A.R. 4·5 g.; nicotinic acid 0·001 g.; $FeSO_4.7H_2O$ 0·001 g.; $CuSO_4.5H_2O$ 0·0005 g.; $ZnSO_4.7H_2O$ 0·0004 g.; $MnCl_2.4H_2O$ 0·0002 g.; $MgSO_4.7H_2O$ 0·35 g.; pH adjusted with *c.* 25–30 ml. N-NaOH and volume made up to 1 l.

Three 500 ml. Erlenmeyer flasks each containing 200 ml. of the basal medium were taken; to one was added 50 mg. tryptophan, to another 45 mg. of *N*-methylaminophenylethanol, and the third was left as a control. They were all inoculated with *Esch. coli* and incubated at 37°. Good growth occurred in each flank, that containing the tryptophan being the most rapid, and considerable indole formation occurred in this flask after 18 hr. The control was negative whilst the *N*-methylaminophenylethanol flask gave a faint indole-like reaction (order of 20–30 μg.) after 2 days, which did not increase up to 7 days. At no time did control solutions of this compound show any trace of *N*-methylindole formation in the absence of *Esch. coli*.

SUMMARY

1. Contrary to the suggestion of Krebs, Hafez & Eggleston (1942) β-*o*-aminophenylacetaldehyde cannot be an intermediate in the breakdown of tryptophan to indole by tryptophanase. The aldehyde reagent, dimedon, whilst it completely inhibits the formation of indole from β-*o*-aminophenylethanol, is without action in the tryptophan-indole reaction.

2. In support of the earlier mechanism of Baker & Happold (1940) it is found that α-amino-β-(1-methylindolyl-3)-propionic acid is not converted into *N*-methylindole by the action of tryptophanase. This prescribes a fourth essential structural condition for enzymic degradation in addition to the three previously recorded, viz. the presence of an unsubstituted NH group in the indole nucleus of tryptophan.

3. Slight evidence has been obtained of indole production by the action of tryptophanase on a tryptophan derivative in which the β-oxidation $CH_2 \rightarrow CO$ had already been effected.

4. The alanine side-chain is not eliminated as aminomalonic acid, which is inert towards the enzyme.

The authors are indebted to Dr W. C. Evans for assistance in the synthesis of α-amino-β-(1-methylindolyl-3-)-propionic acid. One of us (F.C.H.) is indebted to the Medical Research Council for assistance.

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Comparative Microbiological Assays of Members of the Vitamin B Complex in Yeast and Liver Extracts

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(*Received* 25 *February* 1946)

Although a large number of different micro-organisms have been used for the microbiological assay of members of the vitamin B complex, comparative assays on the same substances do not appear to have been carried out except with micro-organisms belonging to the same genera, e.g. *Lactobacillus arabinosus* and *L. casei* (Skeggs & Wright, 1944). Since it has frequently been reported that yeast and liver extracts contain unidentified growth factors, it seemed particularly desirable in assaying such preparations to confirm the specificity of the response of any organism to any one member of the vitamin B complex by carrying out a comparative assay using another organism, preferably one belonging to a different botanical class. It was also considered of interest to see whether any organisms could be found that responded to these hypothetical growth factors in yeast or liver extracts.

Pantothenic acid can be assayed by means of *Proteus morganii* (Pelczar & Porter, 1941), *Lactobacillus helveticus* (Pennington, Snell & Williams, 1940; Strong, Feeney & Earle, 1941) and *L. arabinosus* (Skeggs & Wright, 1944; Hoag, Sarett & Cheldelin, 1945); pyridoxin by means of an X-ray-induced mutant of *Neurospora sitophila* (Stokes, Larsen, Woodward & Foster, 1943), *Saccharomyces carlsbergensis* (Johnson, 1944; Carpenter & Strong, 1944), and an unspecified yeast (Atkin, Schultz, Williams & Frey, 1943); biotin by means of *L. arabinosus* (Wright & Skeggs, 1944), and a 'choline-less' strain (i.e. one unable to synthesize choline) of *Neurospora crassa* (Beadle, 1944) and by *Saccharomyces cerevisiae* (Woolley, 1941; Jurist & Foy, 1944); and aneurin by *Phycomyces blakesleeanus* (Meiklejohn, 1937; Sinclair, 1938, 1939), an unspecified yeast (Schultz, Atkin & Frey, 1942; Scrimshaw & Stewart, 1944), *Streptococcus salivarius* (Niven & Smiley, 1943) and *Lactobacillus fermenti* (Sarett & Cheldelin, 1944).

Burkholder (1943), Burkholder & Moyer (1943), and Burkholder, McVeigh & Moyer (1944) described the vitamin B requirements of a large number of different species and varieties of yeasts, and used *Kloeckera brevis* for the assay of inositol. Through the kindness of Prof. Burkholder, we have been able to examine a number of these, together with other yeasts obtained elsewhere, with the results recorded in Table 1.

It will be observed that no yeast was found for which either riboflavin or nicotinic acid was essential, whereas according to Burkholder *et al.*, several required nicotinc acid. Many of the yeasts required other members of the vitamin B complex, however, and appeared to be of potential value for use in microbiological assays. In no instance was the response produced by liver extracts plus all seven vitamins markedly greater than with the vitamins alone, so that none of the organisms indicated the presence of any new growth factor in the preparations.

For purposes of microbiological assay, the most interesting species, as already noted by Burkholder, is *Kloeckera brevis*, which requires the addition of six members of the vitamin B complex for optimal growth, namely, aneurin, nicotinic acid, pantothenic acid, pyridoxin, biotin and inositol. It should therefore be possible to assay the other five factors in addition to inositol by means of this organism.