The Tryptophanase-tryptophan Reaction

8. THE MODE OF FORMATION OF INDOLE

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(Received 16 December 1946)

The work of Woods (1935) and of Happold & Hoyle (1935) had failed to throw light on the intermediate steps in the production by *Escherichia coli* of indole from tryptophan. Baker & Happold (1940) examined a number of tryptophan intermediates in which the alanine side chain had been systematically modified. Their results showed that the breakdown to indole required, *inter alia*, (a) a free carboxyl group, (b) an unsubstituted amino group and (c) a β carbon atom open to oxidative attack. A tentative mechanism for the breakdown of tryptophan to indole by *Esch. coli* was suggested, involving the production of alanine according to the following scheme:

The present communication describes further experiments on the mechanism of the reaction and to test the validity of the above hypotheses (see also Dawes, Dawson & Happold, 1946). The effect of serine and alanine upon indole production has been tested and growth experiments performed. The detection of an intermediate or intermediates produced in the reaction is of crucial importance and since both the above mechanisms involve the fission of an intact side-chain, in each case an amino-acid, the method of partition chromatography on filter paper of Consden, Gordon & Martin (1944) was used. An inherent difficulty in much work of this kind is

Bonner & Tatum (1944), from a study of the way in which a mutant strain of *Neurospora crassa* synthesized tryptophan from indole and serine, postulated that the breakdown of tryptophan by *Esch. coli* was a reversal of this reaction, i.e. hydrolytic fission to yield indole and serine:

In support of this they quoted experiments in which inhibition of indole production was obtained by the addition of serine to tryptophan, and also experiments in which the synthesis of tryptophan by *Esch. coli* from indole and serine was claimed.

that the intermediate is immediately metabolized and so never reaches an appreciable concentration. This difficulty was somewhat alleviated by the observation of Dawson (1946) that under the influence of mepacrine there was no oxygen uptake over an initial 60 min. whereas indole was formed in

diminished quantity. Hence, presumably, fission occurs giving indole and intermediate but the latter cannot be metabolized. There is however the possibility that metabolism of the intermediate might occur through internal hydrogen donation.

METHODS

Washed suspensions of *Esch. coli* were obtained from 18 hr. growth at 37° on tryptic digest of casein solidified with agar. Indole was determined by the method of Happold & Hoyle (1934). For growth experiments involving indole utilization, a modified Fränkel's medium was used, viz.: 5 g. NaCl, 4 g. asparagine, 6 g. ammonium lactate, 2 g. KH_2PO_4 , 0.4 mg. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 4 ml. mineral salt solution; made up to 1 l. with distilled water. The mineral salt solution contained in 50 ml. distilled water: 500 μg . $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 400 μg . $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 200 μg . $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; 0.35 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The method of analysis employed was partition chromatography on filter paper (Consden *et al.* 1944). Whatman's no. 1 filter paper was originally used but changed to no. 4 on the advice of Dr Martin.

RESULTS

Effect of serine on indole production

Two series of flasks were set up with contents:

(a) 20 ml. phosphate buffer pH 7.4 + 1 mg. *dl*-tryptophan.

(b) 20 ml. phosphate buffer pH 7.4 + 1 mg. *dl*-tryptophan + 0.25 mg. *dl*-serine.

These were incubated at 37° for 30 min. and then 0.5 ml. washed *Esch. coli* suspension added and re-incubated. Indole was determined at 15 min. intervals. The effect of the addition of equimolecular proportions of *dl*-serine compared with *dl*-tryptophan is shown in Fig. 1. Marked inhibition of indole production was obtained.

Fig. 1. Effect of serine on indole production by suspensions of *Esch. coli*.

Experiments were also performed using 0.5 mg. *dl*-serine/flask, i.e. double the molecular equivalent of *l*-tryptophan, but in various experiments, using different bacterial suspensions, Bonner & Tatum's (1944) observation that the degree of inhibition was directly proportional to the serine concentration was

not confirmed; we found occasionally that, using different bacterial suspensions, a greater inhibitory effect was produced by equimolecular concentrations of serine than by double that amount. This appeared to be a function of the enzyme concentration; further experiments to test this in direct comparison with the effect of alanine are given below.

Effect of alanine on indole production

Two series of flasks were set up:

(a) 20 ml. phosphate buffer pH 7.4 + 0.5 mg. *dl*-tryptophan.

(b) 20 ml. phosphate buffer pH 7.4 + 0.5 mg. *dl*-tryptophan + 0.25 mg. *dl*-alanine.

As in the case of serine, each flask had 0.5 ml. bacterial suspension added. Fig. 2 shows the effect of alanine to be negligible, indole production being parallel with that of the control flask; but here again in certain experiments a degree of inhibition was detected.

Fig. 2. Effect of alanine on indole production by suspensions of *Esch. coli*.

Effect of serine and alanine on indole production at varying enzyme concentration

To investigate the effect of enzyme concentration, parallel experiments on indole production in the presence of serine and alanine were done using four different concentrations of bacterial suspension, e.g. *x*, 0.75*x*, 0.5*x*, 0.25*x*.

Flasks were set up in three series:

(a) 20 ml. phosphate buffer pH 7.4 + 0.5 mg. *l*-tryptophan.

(b) 20 ml. phosphate buffer pH 7.4 + 0.5 mg. *l*-tryptophan + 0.5 mg. *dl*-alanine.

(c) 20 ml. phosphate buffer pH 7.4 + 0.5 mg. *l*-tryptophan + 0.5 mg. *dl*-serine.

Esch. coli suspension (0.5 ml.) was added to each flask and indole determined at 10 and 20 min. The results of two such experiments are given in Tables 1 and 2.

Table 1. *Effect of serine and alanine on indole production by Esch. coli suspensions*(T = *l*-tryptophan, 0.5 mg.; S = *dl*-serine, 0.5 mg.; A = *dl*-alanine, 0.5 mg.)

Bacterial dry weight (mg.): Amino-acids present:	21.0			15.75			10.5			5.25		
	T+A	T+S	T	T+A	T+S	T	T+A	T+S	T	T+A	T+S	T
Time of contact (min.)	Indole produced (μg.); theory, 285 μg.											
10	200	198	210	207	215	218	—	—	—	80	98	111
20	252	234	248	247	247	255	284	268	272	254	215	241
	Indole produced (μg./mg. dry weight of bacteria)											
10	9.5	9.4	10.0	13.1	13.7	13.8	—	—	—	15.2	18.7	21.0
20	12.0	11.1	11.8	15.7	15.7	16.2	27.0	25.0	26.0	48.8	41.0	46.0

There was a possibility of some loss of indole by volatilization during the experiments.

In the case of alanine, retardation of indole production only occurred at the lowest enzyme concentration after 10 min.; at other concentrations, after 10 min. there was no significant retardation, while there was none at all after 20 min. with any concentration.

With serine, inhibition of indole production was manifest at 10 and 20 min. with the lowest enzyme

presence of serine, these experiments were repeated using serine.

Erlenmeyer flasks were set up in three series with contents:

(a) and (b) 100 ml. Fränkel's medium + 0.002 g. indole.

(c) 100 ml. Fränkel's medium + 0.002 g. indole + 0.002 g. *dl*-serine.

Table 2. *Effect of serine and alanine on indole production by Esch. coli suspensions*(T = *l*-tryptophan, 0.5 mg.; S = *dl*-serine, 0.5 mg.; A = *dl*-alanine, 0.5 mg.)

Dry bacterial weight (mg.): Amino-acids present:	32			24			16			8		
	T+A	T+S	T	T+A	T+S	T	T+A	T+S	T	T+A	T+S	T
Time of contact (min.)	Indole produced (μg.)											
10	225	268	226	213	209	226	143	143	226	118	120	146
20	233	191	220	216	223	219	206	199	196	199	194	218
	Indole produced (μg./mg. dry weight of bacteria)											
10	7.0	8.4	7.0	8.8	8.7	9.4	9.0	9.0	10.5	14.8	15.0	18.2
20	7.3	6.0	6.9	9.0	9.3	9.1	12.8	12.2	12.1	25.0	24.2	27.2

concentration but was not significant at other concentrations where the tryptophanase was not saturated with tryptophan (Table 2). In this experiment, after 10 min., inhibition was produced by both alanine and serine to a slight degree at 24 mg. dry-bacterial weight, more so at 16 mg. and markedly at 8 mg. After 20 min., only in the weakest enzyme system was retardation produced by both alanine and serine. Thus the inhibition appeared to be marked only as saturation of tryptophanase with tryptophan was reached. At high enzyme concentrations, there was no inhibition of indole production by serine.

Indole utilization by *Esch. coli*

The utilization of indole by *Esch. coli* in growth experiments over 7-day periods was demonstrated by Happold & Hoyle (1936). In the light of Bonner & Tatum's (1944) work on indole utilization in the

After autoclaving, flasks of series (b) and (c) were inoculated with a loopful of light *Esch. coli* suspension and then incubated; (a) constituted the uninoculated control. Flasks were originally left for 24 hr. but as no utilization was recorded this was increased to 7 days. Indole was then determined in

Table 3. *Indole utilization by cultures of Esch. coli in the presence of serine*

Time (days)	Indole present (μg./10 ml. modified Fränkel's medium)					
	(a)	(b)	(b)	(b)	(c)	(c)
0	200	200	200	200	200	200
7	76.9	63.6	64.9	66.5	74.6	77.5

(a) 200 μg. indole/10 ml. uninoculated.

(b) 200 μg. indole/10 ml. inoculated.

(c) 200 μg. indole + 200 μg. *dl*-serine/10 ml. inoculated.

There was considerable loss of indole due to volatilization.

10 ml. samples taken from each flask. The results are given in Table 3, from which it will be seen that there was no utilization of indole in the presence of serine in excess of that in the inoculated flasks containing indole only.

Analytical investigation

Since oxygen uptake under the influence of mepacrine began after 60 min., the method adopted was to incubate the reaction flasks for 55 min. and then to work up the contents for analysis. Five flasks were set up with contents: 3.4 ml. phosphate buffer pH 7.4 + 0.8 ml. 1% (w/v) *dl*-tryptophan solution + 0.5 ml. 1% (w/v) mepacrine hydrochloride solution + 0.3 ml. *Esch. coli* suspension. The flasks were incubated for 55 min. and then the enzyme system inactivated by shaking with ether. The contents of the flasks were pooled, the ether distilled off and the residue concentrated to approximately 1 ml. when some unchanged *dl*-tryptophan separated from solution. This was removed by centrifugation and the supernatant used for analysis alongside control mixtures of tryptophan + serine and tryptophan + alanine. The solvent used was a solution of *p*-cresol saturated with water and chromatograms were run for 12–18 hr. The first few experiments gave no evidence of the presence of serine, but a faint coloration with ninhydrin was given in a position which approximated to the R_f value of alanine and which corresponded to the alanine of the control solution. On changing to *n*-butanol—benzyl alcohol saturated with water, alanine was confirmed.

Since the presence of mineral salts has a detrimental effect upon these analyses, a modification was later introduced. The contents of the flasks were shaken up with 'Deminrolit' but it was found that this removed amino-acids in addition to salts and so the method was discarded. Another method tried was the addition of the calculated amount of mercuric sulphate in sulphuric acid to remove unchanged tryptophan. The precipitate was collected on the centrifuge and the supernatant tested for further precipitation. Excess Hg^{++} was removed with hydrogen sulphide and excess SO_4^{--} with barium hydroxide. Finally the phosphate buffer of the medium was replaced by distilled water pH 6.4 and maximum indole production was thereby prolonged to approximately 3 hr. on account of the acid environment. Accordingly the flasks were incubated for 2.5 hr. before concentrating for analysis.

With the salt effect thus eliminated, better results were obtained and with three different solvents the presence of alanine was confirmed.

In later experiments, further modification of the contents of the reaction flasks was introduced. One flask was set up with contents: 20 mg. *dl*-tryptophan + 200 mg. mepacrine hydrochloride + 5 ml. *Esch. coli* suspension; made up to 200 ml. with distilled

water. Improved results were obtained by this technique. In all the analyses performed serine was never detected. Results obtained using different solvents are summarized in Table 4.

Table 4. Summary of analyses for alanine and serine, using partition chromatography, of products of reaction between washed suspensions of *Esch. coli* and tryptophan in the presence of mepacrine

Solvent (saturated with water)	Alanine present (experiments)	No indication of alanine (experiments)
<i>p</i> -cresol	5	3
<i>n</i> -butanol-benzyl alcohol	8	2
<i>n</i> -butanol	1	1

Serine was always absent.

Oxygen utilization in presence of alanine, tryptophan and serine

Barcroft respirometers were set up with contents: 4.7 ml. phosphate buffer pH 7.4 + 0.3 ml. *Esch. coli* suspension to which amino-acids were added as follows: (a) nil, (b) 250 μ g. *l*-tryptophan, (c) 125 μ g. *dl*-alanine, (d) 125 μ g. *dl*-serine. The oxygen utilization of *Esch. coli* on these substrates is given in Table 5. Both alanine and serine were oxidized more rapidly than tryptophan, the serine initially at a faster rate than the alanine. This was not true of many other amino-acids, including glycine, histidine and phenylalanine, which were studied similarly.

Table 5. Oxygen utilization by washed suspensions of *Esch. coli* with serine, alanine and tryptophan as substrates

Time (min.)	<i>l</i> -tryptophan 250 μ g.	<i>dl</i> -alanine 125 μ g. O ₂ uptake (μ l.)	<i>dl</i> -serine 125 μ g.
5	5.85	8.81	5.81
10	15.0	21.0	26.2
15	25.4	22.7	36.5
20	32.7	54.9	40.9
30	41.9	62.7	41.0
40	46.7	62.1	—

DISCUSSION

The evidence of Bonner & Tatum (1944) in support of their serine hypothesis is twofold. Serine exerts an inhibitory effect upon indole production from tryptophan by *Esch. coli*, this inhibition being directly proportional to the serine concentration and a mass action effect is suggested. Our experiments, while confirming this inhibitory effect have shown that the effect of serine is not a simple relationship between tryptophan and serine concentrations but rather of saturation of tryptophanase with tryptophan. The degree of inhibition produced by serine in concentrations of 1 or 2 equiv. in relation to the

tryptophan present is a function of the enzyme concentration and it would appear that the mass-action effect may only be manifest when the tryptophanase is fully saturated with tryptophan. When this condition is not fulfilled, serine does not appear to affect indole production and in some cases may stimulate it. Experiments in which alanine was added to tryptophan have been performed, and also parallel investigations using different enzyme concentrations. Here, as with serine, it was found that when the enzyme was not fully saturated with tryptophan there was no inhibition at all, but when saturation was being approached there was some inhibition, though it was usually less well marked than with serine. Thus the results obtained on the effect of alanine and cited above were cases where saturation of enzyme with tryptophan had not been achieved.

It is possible to explain these results in the manner of Bonner & Tatum but equally, since indole formation is dependent upon the continuing reoxidation of the carrier system of the tryptophanase complex (indole can be produced in anaerobic culture by electron transfer) and since alanine and serine are preferentially oxidized as compared with tryptophan by tryptophan-adapted cells, such inhibition may be explained by an insufficiency of oxygen to complete the reoxidation of the carrier system for both sets of reactions, viz. (a) the initial fission of alanine from the indole nucleus and (b) the oxidation of the amino-acid side chain after fission in the presence of the added alanine or serine.

Secondly, Bonner & Tatum quote experiments in which indole and serine were incubated with *Esch. coli* suspensions. In their own words 'Colorimetric tests for tryptophan after 24 hr. incubation indicated that some tryptophan had been synthesized from indole in the presence of serine', but experimental details and quantitative results are not given. All attempts by us to demonstrate indole utilization in the presence of serine in growth experiments over 7-day periods have failed. While this work was in progress Fildes (1945), using suspensions of *Esch. coli* and *Eberthella typhosa*, reported failure to demonstrate the utilization of indole in the presence of serine or production of tryptophan.

A study of the oxygen uptake of *Esch. coli* on alanine, serine and tryptophan substrates has shown that both alanine and serine are oxidized at a greater rate than tryptophan. This possibly explains why there is no real mass-action effect upon indole production when alanine or serine is added to tryptophan. Studies of oxygen uptakes on other amino-acids including glycine, histidine and phenylalanine showed that this was not true of these compounds.

The crucial evidence to decide in favour of one or the other of the two hypotheses must come, however,

from definite detection of that particular intermediate. The difficulty here is that the breakdown of tryptophan to indole is complete within approximately 45 min., and any intermediate formed is immediately metabolized to carbon dioxide, ammonia and water. By the use of mepacrine as inhibitor, the production of indole still occurs, but in diminished amount, while there is no oxygen uptake over the initial 60 min. A detectable concentration of any intermediates might be built up under these conditions. With this technique we have analyzed solutions after 55 min. incubation with *Esch. coli*, and alanine has been detected in several such experiments whereas serine has never been found. The partition chromatography method of Consden *et al.* (1944) has enabled us to detect the alanine intermediate and the density of the colour reaction developed with ninhydrin has been consistent with the concentration expected from the concurrent liberation of the amount of indole determined in the majority of experiments performed.

Since in each experiment a control mixture of tryptophan and alanine (or serine) was used alongside the solution under investigation, our results were obtained by direct comparison and are not entirely dependent upon calculation of the R_f value, which may be affected by salt concentration and other variables. Nevertheless, in all cases we obtained good agreement with the accepted R_f values for alanine in the solvents employed. Thus the fact that the relative position of the unknown intermediate and *d*-tryptophan is paralleled by alanine and tryptophan in control runs, using three different solvents, places beyond doubt the production of alanine in the tryptophanase-mepacrine-indole system, and consequently by analogy in the tryptophanase-indole system itself.

The criticism usually levelled at the detection of intermediates in an inhibited reaction, namely that the normal reaction course is blocked, giving rise to unusual products, does not hold since mepacrine only slows down the reaction rate. In the presence of mepacrine, indole formation goes to completion, the overall oxygen utilization is the same as for tryptophan alone, but the reaction takes twice as long to reach completion. It is the metabolism of the side chain which is retarded. It is desirable that alanine should be detected in the absence of mepacrine, but all experiments conducted with this object have failed, on account of its rapid oxidation.

These investigations support the suggested hypothesis of Baker & Happold (1940) and alanine has been demonstrated conclusively as an intermediate produced in the breakdown of tryptophan to indole by *Esch. coli*. This does not constitute a criticism of Bonner & Tatum's work and views on *Neurospora*, but only of their application to the tryptophanase system of *Esch. coli*.

SUMMARY

1. Serine and alanine have an inhibitory effect upon indole production from tryptophan by *Esch. coli*. This effect is not a simple relation between the tryptophan and serine concentrations, and only holds when the enzyme is saturated with tryptophan.

2. Fildes's (1945) finding that there is no evidence of tryptophan synthesis from indole and serine using cells of *Esch. coli* is confirmed.

3. Using partition chromatographic analysis and retarding the reaction with mepacrine, alanine has been detected as an intermediate; under similar conditions the presence of serine has never been detected.

The authors wish to thank Messrs Imperial Chemical Industries Ltd. (Blackley) for the gift of mepacrine hydrochloride, and also the Medical Research Council for a grant in aid.

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Studies on Alcaptonuria

1. THE ESTIMATION OF HOMOGENTISIC ACID

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(Received 18 November 1946)

An accurate and specific method for the estimation of homogentisic acid applicable to small amounts of urine and plasma was required in connexion with experiments which are described in subsequent papers (Neuberger, Rimington & Wilson, 1947; Neuberger & Webster, 1947). Since it was intended to feed substances closely related to homogentisic acid, it was important to define clearly the specificity of the methods used. The present paper deals firstly with methods already described in the literature, and secondly with a new method which, it is believed, is both very specific and sensitive for homogentisic acid.

Baumann (1892), who established the structure of homogentisic acid, also described a method for its estimation in the urine, based on the reduction of silver nitrate in the presence of ammonia. The method is not specific and it is known that normal constituents of urine such as uric acid also reduce silver ions in an alkaline medium (Garrod & Hurtley, 1905). But in spite of this lack of specificity the many important results on human alcaptonuria obtained by the method of Baumann can be considered substantially correct, for the concentration of homogentisic acid in the urine of alcaptonurics is very high compared with that of other substances having similar reducing properties. For our purpose, however, the method was unsuitable and we have not investigated it further.

A more convenient method which makes use of the reduction of phosphomolybdic acid by hydroquinone and its derivatives in acid solution has been described by Briggs (1922). This method, which has been widely used in America, has considerable disadvantages if other substances arising from the metabolism of aromatic amino-acids are present. Thus Medes (1932) found that both *p*-hydroxyphenylpyruvic acid and 3:4-dihydroxyphenylalanine reduce the phosphomolybdate reagent in an acid medium, though the latter only very slowly. A similar experience was reported by Sealock & Silberstein (1940) who tried to differentiate between the two acids by reading the colour 5 min. after the addition of the final reagent, making allowance for the amount of colour produced in that period by the keto acid. Since similar difficulties were anticipated in our work, we have studied the rate of development of the colour with different concentrations of homogentisic acid in some detail, and have also investigated the validity of Beer's law and the specificity of this reaction.

Another method for the estimation of homogentisic acid is based on an observation by Wieland (1910) who found that hydroquinone is quantitatively oxidized to *p*-quinone by iodine in the presence of sodium bicarbonate without any substitution taking place in the benzene ring. On acidification the quinone reacts with the iodide ion, and is itself reduced again to hydroquinone and a corresponding amount of iodine is liberated which can be titrated with