Nicotinamide Biosynthesis by Intestinal Bacteria as Influenced by Methyltryptophans

By P. ELLINGER and M. M. ABDEL KADER, *Lister Institute of Preventive Medicine, London*

(*Received* 8 *October* 1948)

In recent papers (Ellinger & Abdel Kader, 1947, 1948, 1949*b*) it has been shown that *Bacterium coli* (*Escherichia coli*) was able to synthesize nicotinamide from ornithine, but not from tryptophan, and that mixed cultures from faeces or caecum contents could synthesize nicotinamide also from tryptophan. It was suggested that in the conversion of tryptophan to nicotinamide, ornithine was formed as intermediate by a cleavage of the tryptophan molecule. It was hoped that the utilization by the intestinal flora of methyltryptophans with the methyl group in various positions, i.e. in the alanine side chain, in the pyrrole and in the benzene nucleus, might throw some light on this theory. This was not so for reasons to be discussed later, but the experiments provided other results which are to be presented in this paper.

METHODS

The experiments were carried out *in vitro* with mixed cultures from rat caecum contents, consisting mainly of coliform bacteria, staphylococci, *Streptococcus faecalis*, plenty of an unidentified coccus growing in very small colonies and pure cultures of *Bact. coli* 4*c* (Type I faecal; Ministry of Health, 1939). The technique used was mainly that described before (Ellinger & Abdel Kader, 1949*b*). Growth was measured by assessing opacity with the Brown scale.

The following compounds were tested: DL-ornithine, DL-tryptophan, DL-2-methyl-, DL-4-methyl-, DL-5-methyl-, and DL-7-methyl-tryptophans,* and L-α-methylamino-β-(3-indolyl) propionic acid (abrin). The 2-, 4-, 5- and 7-methyltryptophans were kindly supplied by Dr H. N. Rydon who described their preparation and properties (Rydon, 1948). Abrin was prepared from the seeds of *Abrus praecatorius* by a method similar to that of Hoshino (1935). The seeds were cracked and the husks removed; the cotyledons were then ground to a fine powder, dried *in vacuo* over P_2O_5 and extracted with ether to remove as much as possible of a yellow oil which interferes with the precipitation of the abrin. The powder was filtered off and extracted to exhaustion with methanol in a shaking machine at room temperature (5–6 hr.). The powder was filtered off and washed with methanol. Filtrate and washings were evaporated to dryness at reduced pressure. When the dry residue was washed with a little cold water the abrin separated as a white powder which was purified by further washings with cold water on the centrifuge. It was then recrystallized several times from hot water, m.p. 292° (decomp.)

Media of ammonium lactate (Fildes, 1938) containing one of the compounds mentioned in 2 mM concentration were incubated with suspensions of mixed cultures of caecum contents of rats. In another series the same media containing ornithine or tryptophan alone, or ornithine plus either tryptophan or one of the various methyltryptophans, were incubated with a pure culture of *Bact. coli* 4*c*. In a third series ammonium lactate containing ornithine and one of the five methyltryptophans in concentrations from 0·01 to 2 mM was incubated with *Bact. coli* 4*c* for 72 hr. In all three series growth and nicotinamide formation were estimated as before (Ellinger & Abdel Kader, 1949*b*).

The effect of the methyltryptophans on acid production by *Lactobacillus arabinosus* had to be tested, since it was the basis for the assay of nicotinamide. For this purpose the Barton-Wright (1944) media containing one of the methyltryptophans in 2 mM concentration, the concentration present in the assay, were inoculated with a suspension of *Lb. arabinosus* and the acid formed was estimated after incubation for 72 hr. at 37°.

In order to obtain information about the effect of the different methyltryptophans on the growth of the various intestinal bacteria, agar plates were inoculated with equal amounts of the 24 hr. cultures using standardized Pasteur pipettes and incubated for 48 hr. Separate counts were made for the most frequent bacteria. They were compared with similarly made plates from cultures in pure ammonium lactate.

RESULTS

In pure cultures of *Bact. coli* 4*c* growth was very little affected by any of the compounds tested (Table 1). Nicotinamide formation from ammonium lactate was slightly inhibited by tryptophan and increased in the usual way by ornithine (Ellinger & Abdel Kader, 1949*b*). The nicotinamide formation from ornithine was slightly inhibited (by 6%) by tryptophan and by abrin (by 14%) and completely inhibited by 2-, 4-, 5- and 7-methyltryptophans in 2 mM concentration. In smaller concentrations (Table 2) growth was very little affected, and, if so, slightly increased; only once a slight inhibition (by 5%) was observed with one concentration of the 7-methyl derivative; this was within the limits of error of the method. Nicotinamide formation was inhibited to different degrees, rising with the concentrations of 2-, 4-, 5- and 7-methyltryptophans, and hardly inhibited by abrin.

In the experiments (Table 3) using mixed cultures from the caecum content, growth was not markedly affected by any of the compounds tested. The viable counts from 24 hr. cultures showed an undiminished growth of coliforms. Nicotinamide production was stimulated by ornithine and tryptophan and by

* The numbers 2, 4, 5 and 7 refer to the positions in the indole nucleus, the usual convention being followed.

Table 1. *Effect of ornithine, tryptophan and ornithine plus tryptophan or one of five methyltryptophans on growth and nicotinamide formation by* Bacterium coli 4c *in ammonium lactate medium*

Compound tested (2 mM)	Growth (% of control)	Nicotinamide formed (mμg./ml. medium)	Nicotinamide formed (% of ornithine control)
None	100	10	29
DL-Ornithine	100	35	100
DL-Tryptophan	100	8	23
DL-Ornithine + DL-tryptophan	100	33	94
DL-Ornithine + DL-2-methyltryptophan	100	0	0
DL-Ornithine + DL-4-methyltryptophan	100	0	0
DL-Ornithine + DL-5-methyltryptophan	100	0	0
DL-Ornithine + DL-7-methyltryptophan	100	0	0
DL-Ornithine + L-abrin	100	30	86

Cell content of inoculum: about 6×10^5/ml.; of cultures after 48 hr. growth, about 380×10^6/ml.

Table 2. *Effect of various concentrations of* 2-, 4-, 5- *and* 7-*methyltryptophans and of abrin, on growth and nicotinamide formation by* Bacterium coli 4c *in ornithine-ammonium lactate medium*

Concentrations of methyltryptophans (mM)	2-Methyltryptophan			4-Methyltryptophan		
	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)
0	100	29	100	100	26	100
0·01	125	20	69	100	18	69
0·03	125	20	69	100	15	58
0·1	125	15	52	100	14	54
0·3	125	6	21	100	6	23
1·0	100	25	9	100	5	19
2·0	100	0	0	100	0	0

Concentrations of methyltryptophans (mM)	5-Methyltryptophan			7-Methyltryptophan			Abrin		
	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)	Growth (% of control)	Nicotinamide formed (mμg./ml.)	Nicotinamide formed (% of control)
0	100	29	100	100	26	100	100	29	100
0·01	125	28	96	100	14	54	100	29	100
0·03	125	27	93	100	13	50	100	29	100
0·1	125	23	79	100	6	23	150	28	96
0·3	125	14	48	100	3·7	14	150	27	93
1·0	100	6	21	95	2·5	10	100	26	89
2·0	100	0	0	100	0	0	100	25	86

Inoculum for 2- and 5-methyltryptophans and for abrin, 62×10^4 cells; and for 4- and 7-methyltryptophans 61×10^4 cells; growth of control after 72 hr., 76×10^6 cells/ml.

Another similar experiment gave similar results.

Table 3. *Effect of ornithine, tryptophan and five methyltryptophans on growth and nicotinamide formation by mixed cultures from rat caecum contents in ammonium lactate medium*

Compound tested (2 mM)	Growth (% of control)		Nicotinamide formed (mμg./ml. medium)		Nicotinamide formed (% of control)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
None	100	100	10	6	100	100
DL-Ornithine	100	64	22	56	220	933
DL-Tryptophan	100	100	18	27	184	450
DL-2-Methyltryptophan	100	100	6	0	60	0
DL-4-Methyltryptophan	100	100	6	0	60	0
DL-5-Methyltryptophan	155	100	6	0	60	0
DL-7-Methyltryptophan	155	100	8	0	80	0
L-Abrin	100	100	19	29	186	483

Four more experiments showed complete inhibition in one case and partial inhibition in three cases by 2-, 4-, 5- and 7-methyltryptophans.

abrin to the same extent as by tryptophan. In Exp. 1, Table 3, it was inhibited to a considerable but varying degree, and in Exp. 2 completely, by the 2-, 4-, 5- and 7-methyltryptophans in concentrations of 2 mM.

Table 4. *Effect of the five methyltryptophans on the acid production by* Lactobacillus arabinosus *in Barton-Wright medium*

Compound tested (2 mM)	Acid produced/ml. medium (ml. 0·1 N-NaOH)	(% of control)
Control	0·672	100
DL-2-Methyltryptophan	0·680	101
DL-4-Methyltryptophan	0·684	102
DL-5-Methyltryptophan	0·684	102
DL-7-Methyltryptophan	0·254	37
L-Abrin	0·692	103

Growth of *Lb. arabinosus* in the Barton-Wright medium was inhibited by 7-methyltryptophan and unaffected by the others (Table 4). This allows the use of the bacterium for quantitative assay for all but the 7-methyl derivatives. Since the inhibition of *Lb. arabinosus* by this compound was incomplete, it could be concluded that the 7-methyltryptophan also interfered with the nicotinamide synthesis by *Bact. coli*.

The viable counts of the mixed cultures from the caecum contents did not show any marked diminution of the main representatives of the intestinal flora by any of the methyltryptophans in 2 mM concentration.

DISCUSSION

Inhibition of growth by the various methyltryptophans with methyl groups in the indole nucleus has been observed for *Bacterium typhosum*, for which tryptophan is an essential nutrient, by Fildes & Rydon (1947). *Bact. coli* does not need tryptophan for growth, and, as would be expected, the methyltryptophans in concentrations up to 2 mM did not interfere markedly with the growth of *Bact. coli*, although Anderson (1945) observed complete inhibition of growth of a certain strain by 5-methyltryptophan (*Bz*-3-methyltryptophan) in concentrations of 18 μM. The difference might be due to the strain, the nature of which is not described by Anderson. The non-utilization of the 2-, 4-, 5- and 7-methyltryptophans by *Bact. coli* made it impossible to draw any conclusions on the chemical mechanism of the tryptophan-nicotinamide conversion. The similarity of the action of abrin and that of tryptophan is paralleled by the findings of Gordon & Jackson (1935) that growing rats fed on a tryptophan-free diet can utilize abrin and suggests that in both cases a demethylation of the α-N atom takes place. The 2- and 5-methyltryptophans seemed to have an action antagonistic to tryptophan in Gordon & Jackson's experiments. The fact that, in the experiments with mixed cultures, nicotinamide synthesis was entirely inhibited in only two cases and partly in the four others might be due to the fact that, in the mixed culture, part of the methyltryptophans might have been used up by non-coliform bacteria.

In lower concentrations small differences in inhibition of nicotinamide synthesis by the various methyl derivatives were observed; but they were too small to justify the drawing of any conclusion on the relation between degree of inhibitory action and molecular structure, as discussed by Fildes & Rydon (1947) for *Bact. typhosum*. The inhibition of the nicotinamide formation by the 2-, 4-, 5- and 7-methyltryptophans, in the light of the theory of the action of metabolite antagonists as formulated by Fildes (1940), suggests that tryptophan is normally involved in this process. The mode of this action is obscure and needs further investigation. In a recent paper Heidelberger, Gullberg, Morgan & Lepkowsky (1948) have shown that after administration of DL-tryptophan, labelled with ^{14}C in the β position, to rabbits, dogs and rats the urine contained labelled kynurenine and kynurenic acid. The nicotinamide methochloride isolated from the urine of all three species was not radioactive. It was surprising that this compound was isolated from the urine of rabbits since these animals are known not to synthesize this compound in normal circumstances (for literature, see Ellinger & Abdel Kader, 1949*a*). These findings, combined with the findings of this paper, suggest the possibility that the nicotinamide-saving action of tryptophan is not due to a direct conversion, but to a stimulating effect of tryptophan on the biosynthesis mechanism. If this supposition should prove to be correct it would entirely change our conception of the nicotinamide-saving action of tryptophan, and attribute to tryptophan a catalytic coenzyme-like action on the enzyme system involved in the synthesis of nicotinamide.

The difference in the action of tryptophan on the nicotinamide synthesis by mixed cultures of intestinal bacteria on one hand and that by pure cultures of *Bact. coli* on the other (Ellinger & Abdel Kader, 1949*b*) could be explained in the following way: pure *Bact. coli* produces sufficient tryptophan to maintain the optimum rate of nicotinamide synthesis, while in mixed cultures non-coliform organisms consume so much tryptophan that extra tryptophan is needed for a maximum formation of nicotinamide. The inhibition of the growth of *Lb. arabinosus* by 7-methyltryptophan alone of all methyltryptophans examined is also obscure. The tryptophan-like action of abrin might be due to the greater instability of this compound which is probably demethylated easily to tryptophan.

SUMMARY

1. The effect of 2-, 4-, 5- and 7-methyltryptophans and of abrin on growth and nicotinamide formation by pure cultures of *Bacterium coli* (*Escherichia coli*)

and mixed cultures of rat caecum contents and on the acid production by *Lactobacillus arabinosus* has been studied.

2. Growth of *Bact. coli* or of mixed cultures was not markedly affected by any one of the methyltryptophans in the concentrations used; that of *Lb. arabinosus* was considerably inhibited by the 7-methyl compound.

3. Nicotinamide synthesis from ornithine or ammonium lactate by *Bact. coli* was completely inhibited by 2 mM and correspondingly less by lower concentrations of 2-, 4-, 5- and 7-methyltryptophans, but not by abrin. Nicotinamide synthesis by mixed bacteria from the rat caecum contents was either completely or partly inhibited by the former at 2 mM and not affected by the latter compound. These findings suggest that tryptophan is involved in the bacterial biosynthesis of nicotinamide.

The authors are greatly indebted to Dr H. N. Rydon for the supply of samples of 2-, 4-, 5- and 7-methyltryptophans. They wish to express their thanks to Dr A. Emmanuelowa for plating and counting the intestinal bacteria. The work was carried out with a grant for expenses from the Medical Research Council to one of the authors (P. E.).

REFERENCES

Anderson, T. F. (1945). *Science*, **101**, 565.
Barton-Wright, E. C. (1944). *Biochem. J.* **38**, 314.
Ellinger, P. & Abdel Kader, M. M. (1947). *Nature, Lond.*, **160**, 675.
Ellinger, P. & Abdel Kader, M. M. (1948). *Biochem. J.* **42**, ix.
Ellinger, P. & Abdel Kader, M. M. (1949*a*). *Biochem. J.* **44**, 77.
Ellinger, P. & Abdel Kader, M. M. (1949*b*). *Biochem. J.* **45**.
Fildes, P. (1938). *Brit. J. exp. Path.* **19**, 239.
Fildes, P. (1940). *Lancet*, i, 955.
Fildes, P. & Rydon, H. N. (1947). *Brit. J. exp. Path.* **28** 211.
Gordon, W. G. & Jackson, R. W. (1935). *J. biol. Chem.* **110**, 151.
Heidelberger, C., Gullberg, M. E., Morgan, A. F. & Lepkowsky, S. (1948). *J. biol. Chem.* **175**, 471.
Hoshino, T. (1935). *Proc. imp. Acad. Japan*, **11**, 227.
Ministry of Health (1939). *Rep. publ. Hlth med. Subj.* no. 71, revised ed. London: H.M. Stationery Office.
Rydon, H. N. (1948). *J. chem. Soc.* p. 705.

The Linkage of Glutamic Acid in Protein Molecules

By F. HAUROWITZ* and F. BURSA
Department of Biological and Medical Chemistry, University of Istanbul, Istanbul, Turkey

(*Received* 30 *August* 1948)

The experiments presented in this paper were carried out to answer the question as to whether the γ-carboxyl groups of glutamic acid are involved in the formation of bonds in protein molecules. The possible existence of such bonds in proteins is suggested by the fact that γ-linked glutamic acid residues have been found in glutathione, in the capsular substance of *Bacillus anthracis* (Bovarnick, 1942; Hanby & Rydon, 1946) and in folic acid (Boothe, Mowat, Hutchings, Angier, Waller, Stokstad, Semb, Gazzola & Subbarow, 1948). Different modes of combination of the γ-carboxyl groups of glutamyl residues in proteins can be considered: (1) formation of a peptide bond with the terminal amino group of a peptide chain, (2) ester linkages with hydroxyl groups of hydroxy amino-acids and (3) thio-ester linkages with cysteine molecules of a peptide side chain (Chibnall, 1942). Since the γ-substituted glutamyl residues could give rise to branching of the main peptide chain, the problem of γ-substitution is of great importance.

In order to test proteins for the presence of γ-substituted glutamyl residues we subjected them to the following series of procedures: (*a*) partial digestion with trypsin, (*b*) oxidation, (*c*) extraction of the acidified solution with ether (ether extract 1), (*d*) total hydrolysis, (*e*) extraction of the acid hydrolysate with ether (ether extract 2) and (*f*) determination of succinic acid in the last ether extract. Succinic acid found in ether extract 2 was considered as originating from the γ-glutamyl residues. The above mentioned method is based on the fact that the γ-peptide bond of glutathione is more resistant to trypsin than are the normal α-peptide bonds (Grassmann, Dyckerhoff & Eibeler, 1930; Kendall, Mason & McKenzie, 1930). It was expected, therefore, that at least a part of the α- and γ-substituted glutamyl residues (formula I) would be transformed into γ-glutamyl peptides (formula II) by the action of trypsin.

(I) $R^1.CO.CH(NHR^2).CH_2.CH_2.CO.R^3$
Trypsin ↓
(II) $HOOC.CH(NH_2).CH_2.CH_2.CO.R^3$
NaOBr ↓
(III) $HOOC.CH_2.CH_2.CO.R^3$
HCl ↓
(IV) $HOOC.CH_2.CH_2.COOH$

* Present address: Department of Chemistry, Indiana University, Bloomington, Indiana, U.S.A.