IX. STUDIES ON THE CYCLOCLASTIC POWER OF BACTERIA.

PART II.

A QUANTITATIVE STUDY OF THE AEROBIC DECOMPOSITION OF TRYPTOPHAN AND TYROSINE BY BACTERIA.

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THE method used by Raistrick [1919], in the quantitative study of the decomposition of histidine by bacteria, has been applied with slight modification to the study of the decomposition of the cyclic nuclei of tryptophan and tyrosine. The solution of mineral salts used in these experiments differed slightly however from that previously employed and had the following composition recommended for the purpose by Koser and Rettger [1919]:

Distilled water	1000 cc.
NaCl	5.0 g.
MgSO ₄	0.2 g.
CaCl ₂	0.1 g.
KH ₂ PO ₄	1.0 g.
K ₂ HPO ₄	1.0 g.

I. EXPERIMENTS ON THE DECOMPOSITION OF TRYPTOPHAN.

4 g. of tryptophan were dissolved in 2 litres of the foregoing saline solution. In duplicate portions of 75 cc. the total N[A] was estimated by Kjeldahl's method. 200 cc. of the medium were pipetted accurately into each of nine flasks (250 cc. capacity). To each of three of these 2 cc. of glycerol were added. All the flasks were then plugged with cotton wool and autoclaved at 115° for 20 minutes. Of the nine flasks

(a) One without glycerol was used for the determination of the distribution of N in the medium without precipitation with colloidal iron.

(b) One without glycerol was used for the determination of the distribution of N in the medium, using precipitation with colloidal iron as described later.

(c) Four without glycerol were inoculated with pure cultures of one of the following bacteria:

1. *B. fluorescens*.
2. *B. pyocyaneus*.
3. *B. prodigiosus*.
4. *B. proteus vulgaris*.

(d) The three flasks containing glycerol were inoculated with pure cultures of one of the following organisms:

1. *B. fluorescens*.
2. *B. pyocyaneus*.
3. *B. prodigiosus*.

The inoculated flasks were then incubated at 37° for a specified number of days.

Analysis of medium for distribution of N.

After incubation (or in the case of the control without incubation) 8 cc. *N* acetic acid were added to the 200 cc. medium. Then 25 cc. of colloidal iron (1.25 %) were introduced drop by drop in the cold with constant shaking. The flask was heated on a water-bath to flocculate the iron precipitate, then cooled, and the contents filtered on a small Buchner funnel containing a filter paper covered with a thin layer of kieselguhr. The residual iron precipitate was washed with hot water, dried in a steam oven, and the nitrogen contained in it was estimated by Kjeldahl's method (synthesised N [E]). The combined filtrate and washings were transferred to a litre Claisen distilling flask, and 100 cc. alcohol and 10 cc. of a 10 % suspension of lime were added. The ammonia which was present was distilled *in vacuo* and absorbed in a known amount of standard acid, the excess of acid being titrated with standard thiosulphate (ammonia N [C]). The residue in the Claisen flask was then made just acid with HCl, evaporated to a volume of a few cc., washed out quantitatively and made up to a volume of 25 cc. The amino N content of this was estimated in the usual way by van Slyke's method (amino-acid N [B]).

In this way the following values, expressed as mg. N per 100 cc. medium, were obtained:

- A. Total N.
- B. Amino-acid N.
- C. Ammonia N.
- D. Non-amino N ($= A - [B + C + E]$).
- E. Synthesised N.

From a consideration of these values the following information is obtained:

1. Amino-acid N [B] gives the N contained in the amino-group of the side chain, and hence shows to what extent the amino-group has been broken down.

2. Ammonia N [C] gives the whole of the N, whether originating from the amino-group or from the nucleus, which has been metabolised by the bacteria into ammonia.

3. Non-amino N [D] gives a measure of the degradation of the N contained in the nucleus, and hence a decrease in this indicates that the indole nucleus has been attacked and ruptured.

4. The use of colloidal iron as a precipitant enables one to obtain a measure of the amount of N used for synthesis into complex N compounds. This includes the N contained in the bacterial cell and that in the proteins etc. which may diffuse out of the cell. No amino N arising from the tryptophan is included in the colloidal iron precipitate since it is a well-known fact that colloidal iron does not adsorb amino-acids. This was definitely proved by the comparative experiments, previously mentioned with and without the use of colloidal iron. The results of our experiments are recorded in Table I.

Table I. *Composition of medium in each case expressed as mg. N per 100 cc.*

1. Original Medium					2. Original Medium after treatment with colloidal iron.				
A. Total N ... 26.89					A. Total N ... 26.89				
B. Amino-acid N 14.06					B. Amino-acid N ... 13.95				
C. Ammonia N 0.30					C. Ammonia N ... 0.22				
D. Non-amino N 12.53					D. Non-amino N ... 12.34				
					E. N in colloidal iron precipitate 0.38				
Tryptophan + Glycerol					Tryptophan without Glycerol				
Incubation period, days			Increase of N	Decrease of N	Incubation period, days			Increase of N	Decrease of N
<i>B. pyocyaneus.</i>									
13	Total N ...	26.89	—	—	19	Total N ...	26.89	—	—
	Amino-acid N	3.21	—	10.74		Amino-acid N	0.40	—	13.55
	Ammonia N	0.47	0.25	—		Ammonia N	19.15	18.93	—
	Non-amino N	10.85	—	1.49		Non-amino N	3.00	—	9.34
	Synthesised N	12.36	11.98	—		Synthesised N	4.34	3.96	—
<i>B. fluorescens.</i>									
16	Total N ...	26.89	—	—	27	Total N ...	26.89	—	—
	Amino-acid N	1.47	—	12.48		Amino-acid N	0.02	—	13.93
	Ammonia N	0.03	—	0.19		Ammonia N	16.57	16.35	—
	Non-amino N	7.19	—	5.15		Non-amino N	2.92	—	9.42
	Synthesised N	18.20	17.82	—		Synthesised N	7.38	7.00	—
<i>B. prodigiosus.</i>									
18	Total N ...	26.89	—	—	38	Total N ...	26.89	—	—
	Amino-acid N	9.56	—	4.39		Amino-acid N	5.90	—	8.05
	Ammonia N	0.07	—	0.15		Ammonia N	9.84	9.62	—
	Non-amino N	13.26	0.92	—		Non-amino N	10.10	—	2.24
	Synthesised N	4.00	3.62	—		Synthesised N	1.05	0.67	—
<i>B. proteus vulgaris.</i>									
					33	Total N ...	26.89	—	—
						Amino-acid N	9.15	—	4.80
						Ammonia N	5.19	4.97	—
						Non-amino N	12.05	—	0.29
						Synthesised N	0.50	0.12	—

Discussion of Results.

From a consideration of the results given in Table I, the following conclusions may be drawn:

1. Both in the presence and absence of glycerol, *B. pyocyaneus*, *B. fluorescens* and *B. prodigiosus* can attack and produce ammonia from both the side chain and the indole nucleus of the tryptophan molecule.

2. Whereas with *B. pyocyaneus* and *B. fluorescens* ammonia is produced from the nucleus almost as readily as from the side chain, *B. prodigiosus* produces ammonia from the side chain much more quickly than from the nucleus.

3. With *B. proteus vulgaris* ammonia formation is limited to the side chain nitrogen. The failure to open the indole ring is analogous to the inability of the same organism to rupture the iminazole ring [Raistrick, 1919].

4. The effect of the presence of an alternative source of carbon is seen from the results obtained with *B. pyocyaneus*, *B. fluorescens* and *B. prodigiosus* in the presence and absence of glycerol. Whereas in the absence of glycerol much free ammonia is produced but relatively small amounts of synthesised N, in the presence of glycerol practically no free ammonia is produced, and in two of the three cases the amount of ammonia is actually less at the end of the experiment than at the beginning. This non-formation of free ammonia is coincident with large increases in synthesised N.

These results offer an explanation for a number of observations, made by various bacteriologists [Kendall and colleagues, 1912-1913] during the last few years, on the so-called "protein-sparing" action of carbohydrates. These observers find that, when bacteria are grown in ordinary peptone media containing no carbohydrate, there is progressive production of ammonia, often in considerable amount. In the presence of an available carbohydrate, however, only quite inappreciable amounts of ammonia are formed, although growth is often better in the latter case than in the former. This failure to produce ammonia from carbohydrate-containing media they ascribe to the "protein-sparing" action of the carbohydrates. The first obvious criticism of this interpretation is the fact that these workers regard ammonia formation as an index of protein decomposition, and make no other attempt to estimate the extent to which protein decomposition has taken place. We consider that the best method of measuring protein and amino-acid decomposition is to follow the disappearance of amino-acid N. Our results show that an amino-acid is decomposed by bacteria in the presence of glycerol without the production of a corresponding amount of ammonia, the N of which appears in the form of synthesised N. It seems to us that the probable explanation of the so-called "protein-sparing" action of carbohydrates is the following. Bacteria need for their anabolic processes certain relative amounts of nitrogen and carbon. If the available N is in excess of that amount necessary to combine with the available carbon for these synthetic purposes, this N appears

in the form of ammonia (as shown in the experiments on tryptophan without glycerol, and in the results obtained by other workers on peptone media containing no carbohydrates); if, however, the amount of available carbon is in excess of the amount necessary to combine with the available N for synthesis, no ammonia appears because all the N is used for synthesis (as shown in our experiments on tryptophan with glycerol, and in the results of other workers on peptone media containing carbohydrates). Hence we believe that carbohydrates far from having a "protein-sparing" effect in the sense used by the above authors, actually enable bacteria to utilise more protein or protein products than they would in the absence of carbohydrates.

II. EXPERIMENTS ON THE DECOMPOSITION OF TYROSINE.

2.5 g. tyrosine were dissolved, by the aid of heat, in 2500 cc. of the basal saline solution described in the tryptophan experiments. After cooling, the medium was distributed as follows:

1. 400 cc. were pipetted accurately into each of two flasks (500 cc. capacity). After sterilisation, these were inoculated with *B. pyocyaneus* and *B. fluorescens* respectively.
2. 400 cc. were pipetted accurately into each of two flasks (500 cc. capacity) and 2 cc. of glycerol were added. After sterilisation these were inoculated with *B. pyocyaneus* and *B. fluorescens* respectively.
3. About 500 cc. were sterilised and used for the estimation of the initial distribution of N in the medium.
4. The residual liquid was placed in a flask, plugged with cotton wool, and autoclaved. Along with the inoculated flasks, this was incubated at 37°, and was used as a standard control solution for the estimation of phenolic groups described later.

Analysis of the Medium.

The method used for determining the *distribution of N* was exactly the same as that described in the tryptophan experiments. However, since tyrosine contains no nitrogen in the phenolic nucleus, another method had to be used for following the fate of this nucleus. This was done by using Folin's colorimetric method for the estimation of phenolic groups [Folin and Denis, 1912, 1915]. The details of our methods of procedure are as follows.

After incubating for a specified number of days, 25 cc. of the inoculated medium were pipetted into a graduated 100 cc. flask (the residue being used for the determination of the distribution of N). To this were added 1 cc. of normal acetic acid and 2 cc. of colloidal iron drop by drop. The flask was heated on a water-bath to flocculate the iron precipitate, cooled, made up to 100 cc., and filtered through a dry filter paper. The phenolic groups present in this solution were then estimated by the use of Folin's phosphotungstic-phosphomolybdic colorimetric reagent, using the control tyrosine solution 4 as a standard phenolic solution.

Table II. *Composition of medium in each case expressed as mg. N per 100 cc.*

Original Medium.										
			Total N	...	...	...	7.82			
			Amino-acid N	...	...	...	7.78			
			Ammonia N	...	...	...	0.06			
			Phenolic groups equivalent to				100 mg. tyrosine			
Tyrosine + Glycerol					Tyrosine without Glycerol					
Incubation period, days			Increase of N and phenolic groups	Decrease of N and phenolic groups	Incubation period, days			Increase of N and phenolic groups	Decrease of N and phenolic groups	
<i>B. pyocyaneus.</i>										
45	Total N	...	7.82	—	51	Total N	...	7.82	—	
	Amino-acid N		0.51	—		7.27	Amino-acid N		0.12	—
	Ammonia N	...	0.00	—		0.06	Ammonia N	...	4.78	4.72
	Synthesised N		3.22	3.22		—	Synthesised N		1.61	1.61
	Phenolic groups equivalent to mg. tyrosine...		0.25	—		99.75	Phenolic groups equivalent to mg. tyrosine...		0.14	—
<i>B. fluorescens.</i>										
34	Total N	...	7.82	—	46	Total N	...	7.82	—	
	Amino-acid N		0.30	—		7.48	Amino-acid N		0.00	—
	Ammonia N	...	0.00	—		0.06	Ammonia N	...	3.99	3.93
	Synthesised N		4.98	4.98		—	Synthesised N		2.95	2.95
	Phenolic groups equivalent to mg. tyrosine...		1.90	—		98.10	Phenolic groups equivalent to mg. tyrosine...		0.00	—
100										

Discussion of Results.

From a consideration of these experimental results, the following conclusions may be drawn:

1. As observed in the tryptophan results, relatively large amounts of ammonia but very little synthesised N are formed in the absence of glycerol. In the presence of glycerol, however, the appearance of relatively large amounts of synthesised N is coincident with (in this case) a total absence of free ammonia. This gives further support to our suggested explanation of the so-called "protein-sparing" action of carbohydrates.

2. Both in the presence and absence of glycerol, there is an almost complete disappearance from the tyrosine of phenolic groups giving a colour reaction with Folin's reagent. Since Folin has shown that neither straight chain hydroxy-compounds nor benzene derivatives containing no hydroxyl-group attached to the carbon atoms in the ring give a colour with his reagent, our results may be interpreted in either of the two following ways:

(a) That the benzene ring has been ruptured giving rise to straight chain compounds of some sort.

(b) That the hydroxyl-group has been removed from the nucleus leaving the benzene ring unbroken.

At present we have no experimental evidence to prove which of these possibilities is correct. It is of interest, however, to note that Traetta-Mosca

[1910] isolated from stable manure a bacillus which in cultural characteristics is closely related to *B. pyocyaneus*. This micro-organism decomposes tyrosine and gives rise, in addition to other compounds, to benzoic acid. Thus in this case there is a removal of the hydroxyl-group from the phenolic nucleus, which would harmonise with our second possibility indicated above.

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