XXXIX. STUDIES ON THE CYCLOCLASTIC POWER OF BACTERIA.

PART I. A QUANTITATIVE STUDY OF THE AEROBIC DECOMPOSITION OF HISTIDINE BY BACTERIA.

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THE investigation of the products formed during the decomposition of histidine by micro-organisms has led to the isolation of a few compounds of biochemical interest. Ackermann [1910] obtained a 42 % yield of histamine (β -iminazolyethylamine) as a result of the growth of mixed putrefactive bacteria on solutions containing histidine. This discovery became of intense interest when Barger and Dale [1910] showed the remarkable physiological activity of this amine, and identified it as one of the active principles of ergot. More recently, Mellanby and Twort [1912] have observed the formation of this amine by pure cultures of an unnamed bacillus which they isolated from the alimentary canal. Berthelot and Bertrand [1912] also observed that a similar decarboxylation of histidine, resulting in the formation of histamine, is readily accomplished by a bacillus to which they gave the name of *Bacillus aminophilus intestinalis*. In fact, the bacterial method of preparation of histamine is used industrially by Hoffmann, La Roche and Co.

It is stated by Windaus and Opitz [1911] that F. Ehrlich obtained β -iminazolyethyl alcohol by the action of yeast on histidine, but I have failed to find that Ehrlich has published his results.

Of the acids which might arise from histidine, by the action of micro-organisms, only two have been isolated. In the paper previously referred to, Ackermann [1910] obtained, in addition to histamine, a very small amount of β -iminazolypropionic acid. The present writer [1917], working with pure cultures of bacteria of the coli-typhosus group, obtained the unsaturated acid, urocanic acid (β -iminazolyacrylic acid), from histidine. The yield in some cases—in particular with *B. paratyphosus A*—was of the order of 60 % of the histidine employed. Later, in extending this work to organisms of other groups, it was found—more particularly with *B. pyocyaneus*—that, although a considerable amount of ammonia was produced and hence at any rate part of the histidine was decomposed, it was impossible to isolate any iminazole

derivatives, except unchanged histidine, from the culture media. For this reason, it was decided to investigate quantitatively the breakdown of the histidine molecule by bacteria. The results of this investigation are given in this paper.

During this work, the fate of the nitrogen contained in the histidine molecule was the only question studied, since, as will be immediately shown, this enables one to form a good idea not only of the fate of the side chain, but of the iminazole nucleus as well. A consideration of the formula for histidine

indicates that the N (numbered (1)) in the side chain may be easily differentiated from the N (numbered (2) and (3)) in the ring. Van Slyke [1911, 1] has indeed shown that, when histidine is treated with nitrous acid—as in his method for the estimation of amino acid nitrogen—only one-third of the total nitrogen in the molecule is liberated as nitrogen, *i.e.* the amino group in the side chain. .

Using this as the basis of the method employed, the following estimations were performed at stated intervals on media containing histidine dissolved in Ringer's solution, and sown with the organism under investigation.

A. Total Nitrogen (by Kjeldahl) gives the sum of the three nitrogen atoms ((1), (2) and (3)) whether present, as at the commencement of the experiment, as histidine, or, as in the later stages, as products of the breakdown of histidine.

B. Amino Nitrogen (by van Slyke [1912]) gives the nitrogen (1) contained in the amino group of the side chain, thus providing a measure of the rate of breakdown of the amino group.

C. Ammonia Nitrogen gives the whole of the nitrogen, whether of the side chain or nucleus, which is metabolised by the bacteria into ammonia.

By simple calculations the following values can also be determined:

D. Total Non-amino Nitrogen (other than ammonia nitrogen). The difference between the total nitrogen (*A*) and the sum of the amino nitrogen and ammonia nitrogen (*B* + *C*) gives one the *total* non-amino nitrogen present at any stage,

$$\text{i.e. } A - (B + C) = D.$$

In other words, *D* gives one a measure of the rate of degradation into ammonia of the nitrogen contained in the nucleus.

E. Non-amino Nitrogen (other than ammonia nitrogen and the non-amino nitrogen present as unchanged histidine).

Since the ratio of non-amino nitrogen to amino nitrogen in histidine is 2 : 1, the amount of non-amino nitrogen present at any stage as unchanged histidine will be double the amount of amino nitrogen, *i.e.* 2*B*. Thus

$$E = D - 2B.$$

In other words, E gives a measure of the rate of production from histidine of compounds (other than ammonia) containing non-amino nitrogen.

It will be noted that no account is taken of the nitrogen which may be used for synthesis, *e.g.* in the formation of bacterial protein. It is, however, very probable, as is shown from results obtained with *B. proteus*, that the amount of nitrogen used for synthesis by these bacteria is relatively negligible (with the possible exception of *B. pyocyaneus*) and does not vitiate the general conclusions drawn from these experiments.

EXPERIMENTAL.

In all cases, the culture solution was contained in a two litre flask, fitted with a rubber stopper carrying two glass tubes. One of these, bent at an angle of about 60° , was fitted at one end with a plug of cotton wool about three inches long, and the other end was inserted, to a distance of about an inch, through the rubber stopper. The other tube, bent at the same angle, was fitted at one end with a piece of rubber tubing, carrying a small glass tube drawn out to a sealed capillary point. The other end of the tube was inserted through the rubber stopper, and terminated about $\frac{1}{4}$ inch from the bottom of the flask. The apparatus thus closely resembled an ordinary wash bottle, except that the tube carrying the cotton wool plug was bent downwards, to lessen the risk of contamination.

The following solution was then introduced into the flask, and the whole was autoclaved at 120° for an hour.

Distilled water	1550 c.c.
Sodium chloride	16.2 g.
Potassium chloride	0.756 g.
Calcium chloride	0.432 g.
Normal caustic soda	23.2 c.c.

(to neutralise the HCl of the histidine hydrochloride).

In a separate 300 c.c. conical flask, 4.86 g. of pure histidine monohydrochloride was dissolved in 250 c.c. of water, and autoclaved. This separate autoclaving was preferred, as histidine undergoes undesirable changes to a far less extent in acid solution (as the hydrochloride) than in alkaline solution (as the free base).

The contents of the two flasks, after mixing, thus gave a 0.2 % solution of histidine in Ringer's solution, this being the medium previously used [Raistrick, 1917]. In order to introduce the solution of the histidine hydrochloride into the large flask, the following procedure was used. A piece of rubber tubing, fitted with a screw clip, was fixed on the end of the tube containing the cotton wool plug, and, by sucking out a little of the air and then turning the clip, a slight decrease of the pressure of the air inside the flask was obtained. The rubber connection on the other tube was then closed by a clip, and the end of the capillary tube was broken off and carefully sterilised. It was then introduced beneath the surface of the histidine solution which, on opening

the screw clip, passed over into the larger flask. The flask was then sown with an emulsion in sterile Ringer's solution of the organism under investigation, obtained by emulsifying the growth from 20 agar slopes, after 24 hours' incubation. The bacterial emulsion was introduced into the large flask in the same way as the solution of histidine hydrochloride. Samples for analysis were taken from the large flask by creating a slight increase in the pressure of the air in the flask, by blowing gently through the tube containing the cotton wool plug, and then clipping the rubber tube. After closing the rubber connection on the other tube with a clip, the contents of the flask were shaken, and the end of the capillary tube, which was always fused in the Bunsen flame after taking a sample, was broken and sterilised. On opening the clip, the culture media coming over was collected, after rejecting the first 20 or 30 c.c. Care was taken both in sowing the culture media with the organism studied and when samples for analysis were subsequently taken out that no air was allowed to enter the flask except through the cotton wool plug. Bacteriological control showed that, in all the experiments here recorded, there was no contamination. The flasks were incubated at 37°, and, since the tube containing the cotton wool plug was open to the air during incubation, the experiments took place under aerobic conditions.

The following estimations were made on each sample:

I. *Total nitrogen.*

The nitrogen present in 100 c.c. medium was estimated by the Kjeldahl method in the usual way. Since experience showed that there was practically no variation in the total nitrogen during the experiment, determinations were only made at the beginning and end of the experiment. In this, and in the subsequent estimation of free ammonia, the excess of standard acid was always estimated by adding potassium iodide and potassium iodate, and titrating the iodine liberated with standard sodium thiosulphate.

II. *Ammonia and amino acid nitrogen.*

200 c.c. of culture medium was introduced, without previous sterilisation, into a litre Claisen distilling flask, 100 c.c. alcohol, and 10 c.c. of a 10 % suspension of pure lime were added. The free ammonia was distilled off, *in vacuo*, at 40°–45°, and absorbed in an excess of standard *N*/10 acid. The distillation apparatus used was that recommended by van Slyke [1911, 2] for the estimation of the ammonia formed during the hydrolysis of proteins. By this means, one avoids the decomposition into ammonia of readily hydrolysable nitrogen compounds which might be formed by the action of bacteria on histidine (*e.g.* urea). By titration of the excess of acid present, the amount of ammonia formed by the organism studied can be calculated.

After half-an-hour's distillation, the apparatus was disconnected. An excess of glacial acetic acid was added to the Claisen flask, and the solution was concentrated *in vacuo* to a volume of a few c.c. This was then washed out

quantitatively into a 25 c.c. flask, and 2 c.c. of glacial acetic acid were added to prevent any possible action by bacteria which might by this time still be alive, and the whole was made up to 25 c.c. The amino acid nitrogen present was then estimated by van Slyke's [1912] method, 8 c.c. being used for each of the estimations, which were done in triplicate.

All the samples were taken at the same time each day, so that periods represented whole days, and all the estimations on one sample were done on the same day. In the subsequent table, the different forms of nitrogen are expressed as milligrams of nitrogen per 100 c.c. of the medium, and are corrected for impurities due to reagents, etc.

In all the curves given to express graphically the results obtained (pp. 452—455), the following plan has been used:

Curve *B* = ————— represents the amino acid nitrogen (*B*) at different times.

Curve *C* = represents the ammonia nitrogen (*C*).

Curve *D* = - - - - - represents the *total* non-amino nitrogen (*D*).

Curve *E* = — - - — represents the non-amino nitrogen (*E*).

A consideration of the results shows that, with the exception of *B. proteus vulgaris*, the general *type* of curve is very similar in each case. They all show a gradual decrease in amino acid nitrogen (*B*) and total non-amino nitrogen (*D*), along with a gradual increase in the ammonia nitrogen (*C*) and non-amino nitrogen (*E*). Notably with *B. pyocyaneus*, and to a less extent with *B. proteus vulgaris*, the end point of the reaction is reached much more quickly than with the other organisms. A comparison of the relative *rates* of production of ammonia nitrogen (*C*) and non-amino nitrogen (*E*), and of the decomposition of amino acid nitrogen (*B*) and total non-amino nitrogen (*D*) shows the following interesting differences:

(a) With *B. paratyphosus* *A*, and the two experiments with *B. paratyphosus* *B*, the *rates* of each of these reactions increases to a maximum which occurs between the twentieth and thirtieth days, and then decreases.

(b) With *B. faecalis alcaligenes*, *B. pyocyaneus*, and *B. proteus vulgaris*, the *rates* of each of these reactions are all decreasing over the whole course of incubation.

B. proteus vulgaris is sharply differentiated from all the other organisms studied, by the fact that no ammonia is produced from the nitrogen contained in the iminazole nucleus.

A clearer conception of the meaning of the results obtained is gained by comparing the ratios of values obtained by experiment with those which would arise in any hypothetical case. Thus, if the following values are assigned to the letters given:

B = Total decrease in amino acid nitrogen during the course of the experiment, *i.e.* the difference between the initial and final amino acid nitrogen values;

Table I.

Distribution of nitrogen in milligrams per 100 c.c. of medium.

Organism studied	Period of incubation in days	Total nitrogen	Amino acid nitrogen	Ammonia nitrogen	Total non-amino nitrogen	Non-amino nitrogen
		A	B	C	D	E
<i>B. paratyphosus</i> A Expt I (Fig. 1)	—	51.41	16.70	0.59	34.12	0.72
	9	51.41	14.65	3.53	33.23	3.93
	17	51.41	10.82	8.75	31.84	10.82
	24	51.41	7.72	13.51	30.18	15.44
	31	51.41	5.17	18.42	27.82	17.48
	38	51.41	2.58	22.83	26.00	20.84
	45	51.20	1.02	25.49	24.69	22.65
<i>B. paratyphosus</i> B (Birmingham) Expt II (Fig. 2)	—	50.07	16.37	0.51	33.19	0.45
	8	50.07	14.47	3.39	32.21	3.27
	16	50.07	12.02	7.70	30.35	6.31
	23	50.07	8.79	13.29	27.99	10.41
	30	50.07	4.62	20.69	24.76	15.52
	37	50.07	1.35	26.55	22.17	19.47
	43	49.75	1.22	27.55	20.98	18.54
<i>B. paratyphosus</i> B (Schottmüller) Expt III (Fig. 3)	—	51.75	16.63	0.67	34.45	1.19
	5	51.75	15.36	3.11	33.28	2.56
	19	51.75	7.33	18.13	26.29	11.63
	26	51.75	2.83	24.46	24.46	18.80
	38	51.75	2.39	26.76	22.60	17.82
	73	51.06	1.40	27.67	21.99	19.19
<i>B. faecalis alcaligenes</i> (i) Expt IV (Fig. 4)	—	52.21	17.14	0.66	34.41	0.13
	5	52.21	12.29	9.02	30.90	6.32
	8	52.21	10.41	12.12	29.68	8.86
	14	52.21	7.44	18.68	26.09	11.21
	22	52.21	5.04	23.68	23.49	13.41
	30	52.21	4.24	26.41	21.56	13.08
	57	52.05	4.25	27.75	20.05	11.55
<i>B. faecalis alcaligenes</i> (ii) Expt V (Fig. 5)	—	46.44	15.13	0.21	31.10	0.84
	4	46.44	13.45	3.26	29.73	2.83
	9	46.44	12.09	6.03	28.32	4.14
	16	46.44	9.62	10.58	26.24	7.00
	25	46.44	6.69	17.35	22.40	9.02
	38	46.44	4.24	22.57	19.63	11.15
	72	47.76	1.07	31.38	15.31	13.17
<i>B. pyocyaneus</i> Expt VI (Fig. 6)	—	51.60	16.68	0.55	34.37	1.01
	3	51.60	13.62	9.02	28.96	1.72
	7	51.60	8.52	17.49	25.59	8.55
	12	51.60	4.14	27.27	20.19	11.91
	19	51.60	1.50	35.65	14.45	11.45
	25	51.60	1.03	37.04	13.53	11.47
	33	51.51	0.75	37.61	13.15	11.65
<i>B. proteus vulgaris</i> Expt VII (Fig. 7)	—	51.63	16.62	0.66	34.35	1.11
	5	51.63	10.77	6.20	34.66	13.12
	8	51.63	8.67	8.38	34.58	17.24
	15	51.63	7.42	9.44	34.77	19.93
	23	51.63	6.73	9.93	34.97	21.51
	35	51.60	5.87	10.59	35.14	23.40

Fig. 1. Experiment I. *B. paratyphosus* A. Fig. 2. Experiment II. *B. paratyphosus* B (Birmingham)

Fig. 3. Experiment III. *B. paratyphosus* B (Schottmüller) Fig. 4. Experiment IV. *B. faecalis alcaligenes* (i)

Fig. 5. Experiment V. *B. faecalis alcaligenes* (ii) Fig. 6. Experiment VI. *B. pyocyaneus*

C = Total increase in ammonia nitrogen under the same conditions;

D = Total decrease (or increase) in total non-amino nitrogen (as previously defined);

E = Total increase in non-amino nitrogen (as previously defined);

the ratios which these values would have in the following hypothetical cases may be considered:

(i) *Simple de-aminisation of the side chain.* If the amino acid nitrogen in the side chain were converted into ammonia, then

$$\frac{C}{B} = \frac{1}{1}, \quad \frac{D}{B} = \frac{0}{1}, \quad \frac{E}{B} = \frac{2}{1}.$$

Fig. 7. Experiment VII. *B. proteus vulgaris*

(ii) *Simple decarboxylation of the side chain.* If this reaction alone took place, no ammonia would be formed. The amino acid nitrogen would decrease, since the amine grouping $-\text{CH}_2-\text{NH}_2$ does not give any appreciable amount of nitrogen under the conditions employed for estimating amino acid nitrogen.

Then

$$\frac{C}{B} = \frac{0}{1}, \quad \frac{D}{B} = \frac{1}{1}, \quad \frac{E}{B} = \frac{3}{1}.$$

(iii) *Production of ammonia from both side chain and nucleus.* Since there are two nitrogen atoms in the iminazole nucleus, we may assume that either

(a) both nuclear nitrogen atoms and side chain nitrogen would be attacked at the same rate, then

$$\frac{C}{B} = \frac{3}{1}, \quad \frac{D}{B} = \frac{2}{1}, \quad \frac{E}{B} = \frac{0}{1};$$

or (b) only one of the nuclear nitrogen atoms, in addition to the side chain nitrogen, would be attacked, then

$$\frac{C}{B} = \frac{2}{1}, \quad \frac{D}{B} = \frac{1}{1}, \quad \frac{E}{B} = \frac{1}{1}.$$

The ratios obtained for the different bacteria investigated, calculated from the results previously given, are summarised in the following table.

$$\left[\text{Thus e.g. for } B. \text{ paratyphosus } A, \frac{C}{B} = \frac{25.49 - 0.59}{16.70 - 1.02} = \frac{24.90}{15.68} = \frac{1.59}{1}. \right]$$

Table II.

Organism	$\frac{C}{B}$	$\frac{D}{B}$	$\frac{E}{B}$
<i>B. paratyphosus</i> A	$\frac{1.59}{1}$	$\frac{0.60}{1}$	$\frac{1.40}{1}$
<i>B. paratyphosus</i> B (Birmingham)	$\frac{1.78}{1}$	$\frac{0.81}{1}$	$\frac{1.19}{1}$
<i>B. paratyphosus</i> B (Schottmüller)	$\frac{1.77}{1}$	$\frac{0.82}{1}$	$\frac{1.18}{1}$
<i>B. faecalis alcaligenes</i> (i)	$\frac{2.10}{1}$	$\frac{1.11}{1}$	$\frac{0.886}{1}$
<i>B. faecalis alcaligenes</i> (ii)	$\frac{2.22}{1}$	$\frac{1.12}{1}$	$\frac{0.877}{1}$
<i>B. pyocyaneus</i>	$\frac{2.33}{1}$	$\frac{1.33}{1}$	$\frac{0.67}{1}$
<i>B. proteus vulgaris</i>	$\frac{0.985}{1}$	$\frac{0.073}{1}$	$\frac{2.07}{1}$

From a consideration of the results given in Table II, and from the different graphs, it seems justifiable to draw the following conclusions:

(i) *B. proteus vulgaris* alone, of the organisms investigated, cannot produce ammonia from either of the nitrogen atoms in the iminazole nucleus, although it does so from the nitrogen atom in the side chain. This is probably due to one of two causes:

(a) The inability of the organism to open the ring. If this explanation, which seems the more probable, is correct, the reason would appear to be that the enzyme, which enables the other bacteria investigated to open the iminazole ring, is not present in *B. proteus vulgaris*. The evidence offered in this paper seems to point, in fact, to the presence in many bacteria of a specific enzyme (perhaps more than one), which has the power of splitting open the iminazole ring, and producing ammonia from the nitrogen atoms contained in the opened ring. The ease with which this change is brought about by bacteria is in marked contrast to the well-known stability of the iminazole ring towards most chemical reagents.

(b) The possibility, an improbable one, that although *B. proteus vulgaris* can open the iminazole ring it cannot convert the nitrogen contained in the ruptured ring into ammonia.

(ii) None of the bacteria produced simple decarboxylation of the histidine, and it is to be regretted that for the sake of completeness it was impossible

under conditions prevailing at the time, to obtain a culture of one of the well-known decarboxylating bacteria.

(iii) *B. faecalis alcaligenes*, *B. pyocyaneus*, *B. paratyphosus A*, and *B. paratyphosus B* produce ammonia both from the nitrogen in the side chain and in the nucleus. All these bacteria attack the histidine molecule as a whole by a series of reactions which proceed concurrently, and produce a complete breakdown of at least a portion of the iminazole ring. Two of them, *B. faecalis alcaligenes* and *B. pyocyaneus*, produce ammonia from the ring more quickly than they produce it from the side chain. Since the ratio $\frac{D}{B}$ is greater than $\frac{1}{1}$ and since the ratio $\frac{C}{B}$ is greater than $\frac{2}{1}$ with these two organisms, it follows that ammonia is formed by each of them from each of the three nitrogen atoms in the histidine molecule. The fact that the ratio $\frac{E}{B}$ is in the one case $\frac{0.88}{1}$ and in the other $\frac{0.67}{1}$ probably explains the previously described failure to isolate any iminazole compound from the action of *B. pyocyaneus* on histidine.

A comparison of the ratios obtained with *B. paratyphosus A* and *B. paratyphosus B* shows that these two organisms, and more particularly *B. paratyphosus A*, produce ammonia from the side chain more quickly than they do from the ring since the ratio $\frac{D}{B}$ is in each case less than $\frac{1}{1}$. The fact that the ratio $\frac{E}{B}$ for *B. paratyphosus A* is relatively large ($\frac{1.40}{1}$) explains why such a good yield of urocanic acid was obtained as a result of the action of this organism on histidine, since $\frac{E}{B}$ represents the ratio of end products, other than ammonia, to histidine decomposed.

It is of interest to note that though the value of any one of the ratios $\frac{C}{B}$, $\frac{D}{B}$, $\frac{E}{B}$ is different for each of the different bacteria investigated, they show very good agreement for different strains of the same organism (e.g. for two different strains of *B. paratyphosus B* and of *B. faecalis alcaligenes*).

SUMMARY.

The aerobic breakdown of histidine by bacteria (*B. paratyphosus A*, *B. paratyphosus B*, *B. faecalis alcaligenes*, *B. pyocyaneus*, and *B. proteus vulgaris*) was studied quantitatively.

The results obtained showed that:

(i) *B. paratyphosus A*, *B. paratyphosus B*, *B. faecalis alcaligenes*, and *B. pyocyaneus* can attack, and produce ammonia from, the nitrogen atoms in both the side chain and the iminazole nucleus of the histidine molecule, thus proving that they are able to open the iminazole ring.

(ii) With *B. proteus vulgaris*, ammonia is formed only from the side chain nitrogen, and it is very probable that this organism is unable to open the iminazole ring.

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