CXCIX. THE DECOMPOSITION OF ADENINE COMPOUNDS BY BACTERIA.

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THERE seems to be at present good evidence that adenine compounds play an important role in muscle glycolysis and alcoholic fermentation. It would be of much biochemical interest to show whether they are also connected with the mechanism of bacterial fermentation. In order to approach this problem it was necessary first to study the ability of bacterial fermenters to attack adenine derivatives and to determine the content of adenosinetriphosphoric acid in their cells.

The following experiments were done to determine the exact conditions in which adenine and its derivatives undergo chemical changes by bacterial enzymes.

EXPERIMENTAL.

Organisms and culture. The experiments were first carried out with *Bact. coli*; they were subsequently repeated with other organisms (see p. 1409).

Unless otherwise stated the organisms were grown on tryptic broth agar in Roux bottles. After 20 hours' incubation the growth was washed off with distilled water and centrifuged and washed two or three times; the wet weight was then determined and the whole made up to a known volume in uniform suspension. The preparation was generally used the same day but could be kept 2 or 3 days at 0° without deteriorating. Sterilization of the culture by shaking with toluene and allowing to stand for 15 min. has no effect on the activity of the enzymes studied.

Reactions studied. The action of the bacterial suspensions in decomposing by deamination and/or dephosphorylation of the following substances was quantitatively investigated: adenosinetriphosphoric acid (adenylpyrophosphoric acid), muscle adenylic acid (adenine-9-furanoriboside-5-phosphoric acid), yeast adenylic acid (adenine-9-furanoriboside-3-phosphoric acid), adenosine and adenine; inosine and inosinic acid were also studied. Purified specimens of the compounds were used.

Technique. For each experiment the procedure was as follows: samples containing 1 ml. substrate, containing 0.085–0.120 mg. adenine-amino-nitrogen ($\text{NH}_2\text{-N}$) and 1 ml. bacterial suspension, corresponding to 30–160 mg. wet weight and 1 ml. water, which in some cases was replaced by salt solutions (phosphate, arsenate, fluoride, iodoacetate) were incubated at pH 7.0 in air and at 37°. It was however shown that all the reactions described below proceed as well anaerobically in nitrogen previously passed over heated copper. After a given time of incubation the samples were withdrawn from the incubator with corresponding blanks where the substrate was replaced by water, and 1 ml. of 20% trichloroacetic acid was added.

The deamination was determined on the whole sample by the estimation of ammonia. It was carried out in the Parnas-Heller apparatus by distillation *in vacuo* with saturated borax solution and subsequent nesslerization [Parnas &

Heller, 1924]. The dephosphorylation was determined by phosphorus estimations in the trichloroacetic filtrate using the method of Fiske & Subbarow [1925] as modified by Lohmann & Jendrassik [1926].

Adenosinetriphosphoric acid (ATP). From Table I, 1 and 2, it is seen that in the course of 4 hours this substance is deaminated and dephosphorylated completely. It was found that small concentrations of magnesium (0.2 mg. Mg to 5 mg. ATP-barium salt) markedly increase the rate of breakdown of ATP; the appropriate quantity of $MgCl_2$ was accordingly used with this substance. In experiments (Table I, 3 and 4) where both deamination and dephosphorylation were estimated simultaneously in parallel samples, dephosphorylation appeared to precede deamination. This seems to be also the case with skeletal muscle enzymes [Mozolowski *et al.* 1932; Embden, 1933; Jacobsen, 1933] and excludes the possibility of the formation of inosinetriphosphoric acid. There is, however, an important difference in the behaviour of the enzymes of muscle and of *Bact. coli* towards ATP; in the former this substance is dephosphorylated by the elimination of the pyrophosphoric group only, forming adenylic acid, which is then deaminated to inosinic acid; in the latter case the bacterial enzymes attack not only the pyrophosphoric group but also the phosphorus atom of adenylic acid itself so that probably deamination does not occur till the stage when adenosine has been formed.

The decomposition is optimum at pH 7.2 and is inhibited at pH 5.5 and 8.8. Addition of phosphate slows down the breakdown of ATP, an effect which requires no special explanation as phosphate represents, in this case, a reaction product.

Table I.

No. exp.	ATP containing			Time of incubation pH 7.0 37°	Deamination of ATP		Dephosphorylation of ATP						
	Amino-N mg. N	Pyro-P (2P) mg. P	Total-P (3P) mg. P		Ammonia NH ₃ -N mg.	Of 1 amino-N appears as ammonia-N	Phosphate after 7 min. hydrolysis in N HCl					Of two atoms Pyro-P still unchanged	Of three atoms total-P appear as inorganic P
							Inorganic phosphate (P ₀) mg. P	(P ₇) mg. P	Pyrophosphate-P (expressed as P ₇ -P ₀) mg. P				
1	0.08	0.357	0.535	4 hr.	0.074	0.87	0.530	0.535	0.005	0	3		
2	0.06	0.264	0.398	4 hr.	0.062	1	0.398	0.398	0.0	0	3		
3	0.055	0.243	0.365	30 min.	0.003	0.05	0.004	0.233	0.229	1.87	0.03		
	0.055	0.243	0.365	1 hr.	0.018	0.33	0.170	0.278	0.108	0.88	1.39		
	0.055	0.243	0.365	1 hr. 30 min.	0.024	0.43	0.214	0.300	0.086	0.70	1.75		
	0.055	0.243	0.365	4 hr.	0.052	0.95	0.365	0.365	0.0	0.0	3		
4	0.060	0.264	0.398	30 min.	0.003	0.05	0.085	0.269	0.184	1.39	0.63		
	0.060	0.264	0.398	1 hr.	0.010	0.16	0.114	0.296	0.182	1.39	0.86		
	0.060	0.264	0.398	1 hr. 30 min.	0.025	0.42	0.169	0.307	0.138	1.04	1.28		
	0.060	0.264	0.398	4 hr.	0.061	1	0.398	0.398	0.0	0	3		

A remarkable effect of medium on enzyme production is noticeable in this connexion. When *Bact. coli* was grown on tryptic broth with 0.5 % glucose in a flask instead of on the surface of broth agar, the bacterial suspension when used in the same concentration as in previous experiments or even in twice that amount, repeatedly failed to attack ATP, whereas with other adenine compounds (with the exception of yeast adenylic acid to some extent, see below, p. 1407) no such effects were noticed. This suppression of enzyme formation was shown to be due to the presence of glucose, since it did not occur in cultures grown (1) in broth alone in strictly anaerobic conditions, or (2) in broth with 0.5 % formate. The suppression of the enzyme is, however, not complete as when 4-5 times the amount of bacteria was used (160 mg. instead of 30 mg. wet weight), positive results were obtained (Table II, 1 and 2). The partial suppression of the enzyme

system may possibly be due to the acidity occurring during growth in glucose broth. This, however, does not account for the non-proportionality of the enzyme activity and bacterial concentration. This point needs further study.

Table II.

No. exp.	<i>Bact. coli</i> wet wt. mg.	Grown on	Time of incubation pH 7.0 37° hours	Deamination of ATP (containing 0.085 mg. NH ₂ -N)	
				Ammonia mg. N	% of deamination
1	40	Glucose broth	1	0.0	0
			2	0.0	0
			4	0.0	0
		Broth agar	1	0.013	15
			2	0.027	32
			4	0.072	85
2	50	Glucose broth	4	0.0	0
	80			0.0	0
	160			0.062	70

Muscle adenylic acid. This substance undergoes both deamination and dephosphorylation very rapidly, 2.5 mg. being decomposed by 10–20 mg. of bacteria (wet weight) in 40–60 min. No influence of small amounts of magnesium upon this reaction was found. Dephosphorylation evidently precedes deamination, as can be seen from Table III, 4 and 5. Bearing in mind that inosinic acid is readily dephosphorylated by *Bact. coli* (Table III, 3) and that, of all adenine

Table III.

No. exp.	Muscle adenylic acid containing		Yeast adenylic acid containing		Inosinic acid containing P mg.	Time of incubation pH 7.0 37°	Dephosphorylation expressed as		Deamination expressed as	
	P mg.	NH ₂ -N mg.	P mg.	NH ₂ -N mg.			P mg.	%	NH ₂ -N mg.	%
1	0.21	0.095	—	—	—	2 hr. 30 min.	0.21	100	0.096	100
2	—	—	0.21	0.095	—	2 hr. 30 min.	0.20	95	0.082	85
3	—	—	—	—	0.18	2 hr. 30 min.	0.18	100	—	—
4	0.197	0.089	—	—	—	15 min.	0.144	73	0.025	26
	0.197	0.089	—	—	—	40 min.	0.168	85	0.060	67
	0.197	0.089	—	—	—	1 hr. 20 min.	0.170	86	0.080	89
	0.197	0.089	—	—	—	2 hr.	0.180	91	0.085	94
	—	—	—	—	—	—	—	—	—	—
5	0.21	0.096	—	—	—	15 min.	0.164	78	0.040	41
	0.21	0.096	—	—	—	40 min.	0.188	89	0.070	73
	0.21	0.096	—	—	—	1 hr. 20 min.	0.192	91	0.084	87
	0.21	0.096	—	—	—	2 hr.	0.208	99	0.090	94

compounds, adenosine is the most readily decomposed, not only by *Bact. coli* but also by other bacteria, one may suggest that deamination is conditioned by a preliminary dephosphorylation and occurs at the adenosine stage.

In the case of adenylic acid as well as in that of ATP, the addition of inorganic phosphate decreases the rate of breakdown. This time, however, a most marked effect can be produced by phosphoglyceric acid (3-phosphoglyceric acid) (Fig. 3) but not by hexosediphosphoric acid. Whether this is due to the Parnas-Ostern-Mann reaction [1934, 1, 2], i.e. a synthesis of ATP and a formation of pyruvic acid, will be investigated later. Pyruvic acid could of course not be detected owing to its rapid fermentation by *Bact. coli*.

Yeast adenylic acid. This is decomposed by bacterial enzymes both by dephosphorylation and deamination (Table III, 2). In experiments with deamination of both muscle and yeast adenylic acids, it has been repeatedly found that there is a difference in the rates of deamination of these two substances (Fig. 1). This effect is still more marked if the bacteria are grown on glucose broth (Fig. 2);

thus the presence of glucose in the growth medium affects this enzyme system in a manner similar to that described in the case of ATP, but to a much less marked extent.

It is well known that frog skeletal muscle enzymes are totally inactive towards yeast adenylic acid [Schmidt, 1928], whereas frog heart muscle enzymes act on it, a fact which suggested the existence of specific enzymes for the two substances [Ostern & Mann, 1933]. These experiments furnish further evidence for that possibility, a point to which we return later.

In this, as in all phosphorylated compounds tested, neither $M/60$ NaF nor $M/150$ iodoacetate had any effect.

Fig. 1.

Fig. 2.

Fig. 1. Deamination of muscle adenylic acid and yeast adenylic acid by *Bact. coli*. grown on broth agar. (Average data from 9 experiments.) Abscissae: hours. Ordinates: % of deamination.

Fig. 2. Deamination of muscle adenylic acid and yeast adenylic acid by *Bact. coli* grown on glucose broth. (Average data from 7 experiments.) Abscissae: hours. Ordinates: % of deamination.

Adenosine. As well by *Bact. coli* as by any kind of bacteria studied in this work, adenosine is more rapidly decomposed than other adenine derivatives and than adenine itself. The optimum rate occurs at pH 8.8, whereas for all the other adenine derivatives it lies at pH 7.5. Small amounts of magnesium are without effect, but it is interesting to note that phosphate favours the deamination (Fig. 3). Phosphate can be replaced by arsenate.

Adenine. In this case the deamination depends largely on the presence of phosphate as in its absence the reaction—even on prolonged incubation—never exceeds 20 % of the total amino-nitrogen.

End-products. Hypoxanthine has been found and isolated as picrate of the silver salt ($C_5H_3N_4AgO$, $C_6H_2(NO_2)_3OH$) in the kieselguhr filtrate obtained from incubated samples of the following substances with the usual amount of bacterial suspension: adenosine, adenine, inosine, inosinic acid. The picrate was obtained from samples corresponding to about 5 mg. of hypoxanthine according to the procedure of Bruhns [1890] and identified by silver estimation (23 % Ag, theor. 22.88 %). It was also possible owing to the kind help of Dr M. Dixon to show the presence of hypoxanthine in a biological way using a highly specific preparation of xanthine oxidase [Green & Dixon, 1934; Dixon & Lemberg, 1934].

At the stage at which hypoxanthine is formed, no ribose could be traced by the orcinol test.

Comparison of bacteria studied. In order to get information about the distribution of the group of enzymes concerned, the following organisms were studied: *Bact. dispar*, *Bact. cloacae*, *Bact. lactis aerogenes*, *Str. faecalis*, *Ps. pyocyanea*. The

Fig. 3. Deamination of adenine compounds by *Bact. coli* after 1 hour of incubation.

results are summarized in Table IV, where it can be seen that *Bact. dispar*, *Bact. cloacae* and *Bact. lactis aerogenes* show a behaviour very similar to that described for *Bact. coli*. If *Bact. lactis aerogenes* is grown on glucose broth instead of on agar, there occurs the same effect of depressing the enzyme dealing with ATP and yeast adenylic acid as described for *Bact. coli*, but in a slighter degree, probably owing to the fact that its fermentation products in the culture medium are less acid than those of *Bact. coli*. *Str. faecalis* even if used in large amounts shows very

Table IV.

No. exp.	Organism	Time of incubation pH 7.0 37° hr.	Deamination of adenine compounds									
			ATP		Muscle adenylic acid		Yeast adenylic acid		Adenosine		Adenine	
			NH ₂ -N mg.	% of added	NH ₂ -N mg.	% of added	NH ₂ -N mg.	% of added	NH ₂ -N mg.	% of added	NH ₂ -N mg.	% of added
1	<i>Bact. dispar.</i> 30 mg. wet weight; grown on broth agar	1	0.0	0	0.050	52	0.027	28	0.092	76	0.010	8
		2	0.0	0	0.080	83	0.055	57	0.112	93	0.030	25
		4	0.018	21	0.096	100	0.090	94	0.120	100	0.060	51
2	<i>Bact. cloacae.</i> 30 mg. wet weight; grown on broth agar	1	0.012	14	0.079	82	0.036	38	0.104	86	0.041	34
		2	0.028	29	0.090	93	0.065	67	0.112	93	0.065	54
		4	0.057	58	0.097	100	0.085	88	0.120	100	0.095	79
3	<i>Bact. lactis aerogenes.</i> 30 mg. wet weight; grown on {broth agar glucose broth	1	0.080	94	0.090	94	0.080	83	0.105	87	0.060	50
		2	0.083	98	0.090	94	0.083	86	0.110	92	0.008	66
		4	0.086	100	0.095	100	0.092	95	0.120	100	0.100	83
		2	0.023	27	0.070	72	0.040	42	—	—	—	—
4	<i>Str. faecalis.</i> 100 mg. wet weight; grown on glucose broth	4	0.010	11	0.020	20	0.020	20	0.020	18	0.020	18
5	<i>Ps. pyocyanea.</i> 30 mg. wet weight; grown on broth agar	1	0.010	11	0.010	10	0.015	15	0.060	50	0.060	47
		2	0.015	18	0.020	20	0.015	15	0.115	96	0.105	82

low values. *Ps. pyocyanea* has been found to possess active deaminases but it evidently lacks dephosphorylating enzymes. The data in Table IV show clearly the difference already mentioned in the rates of deamination of muscle and yeast adenylic acids, giving again evidence for specificity of both enzymes.

Scheme. The following scheme has been made to compare the results obtained with those already known for frog skeletal and heart muscles and also for yeast maceration extract.

The content of adenosinetriphosphate in Bact. coli.

Bact. coli were grown on tryptic broth agar for 20 hours in 10 Roux bottles. The cultures were washed twice and centrifuged and weighed. The average wet weight of the cultures from 10 Roux bottles after 20 hours' growth was 10 g. with the average water content of 80 %. The total crop was then suspended in 20 ml. of water and treated in one of two ways. (1) It was heated for 20 min. in a boiling water-bath and then precipitated with trichloroacetic acid and left for 10 hours in ice (Table V, 1, 2, 6). (2) It was ground for 30 min. with very sharp quartz sand (Merck) and trichloroacetic acid in a porcelain mortar, then thoroughly shaken and allowed to stand for 10 hours in ice after which it was again ground (Table V, 3, 4, 5). In both cases the final concentration of trichloroacetic acid was 7 %. The trichloroacetic precipitate was removed either by filtration or by centrifuging and a clear yellowish solution resulted. From this ATP was isolated as barium salt and estimated by deamination following the procedure given by Parnas & Lutwak-Mann [1935].

A known volume of the trichloroacetic extract was placed in a centrifuge-tube; it was made alkaline with 10 % NaOH to pH 8.0, treated with barium acetate 50 % and kept for 30 min. at 0°. The barium precipitate was centrifuged and washed twice with 1 % barium acetate, whilst the supernatant fluid tested with barium acetate was poured off and prepared for the estimation of adenylic acid as will be described below (B). The barium precipitate containing among other substances the total ATP—if present—was completely dissolved in hydrochloric acid, the barium removed with Na₂SO₄, the solution neutralized and made up to a known volume (A). The solution B which might contain adenylic acid was freed from barium and ammonia, neutralized and made up to a known volume. Both solutions A and B were next divided into two parts, one was used

as a blank, the other one treated with preparation of specific deaminase from frog skeletal muscle [see Parnas & Lutwak-Mann, 1935]. All samples were incubated at 37° for 4 hours. Ammonia estimation followed. In some cases a frog heart muscle preparation was used but with no difference in result. It was, of course, impossible to use a suspension of *Bact. coli*, as this enzyme preparation, unlike muscle, contains a great number of different deaminases.

A rather low value (Table V) has been found for the ATP content in *Bact. coli* if compared with that in muscle or yeast [Lohmann, 1928, 1, 2; Lutwak-Mann &

Table V.

No. exp.	Wet wt. of bacteria g.	Content of ATP in <i>Bact. coli</i>	
		NH ₂ -N of ATP in the total mg. N	NH ₂ -N of ATP in 100 g. of bacteria (wet wt.) mg. N
1	10.00	0.150	1.50
2	10.64	0.078	0.73
3	15.60	0.183	1.17
4	9.40	0.098	1.04
5	10.00	0.102	1.02
6	11.70	0.134	1.14

Mann, 1935], about 0.7–1.5 mg./100 g. (wet weight) amino-nitrogen and 4 mg./100 g. P as pyrophosphoric group, as was estimated by Lohmann's hydrolysis method [1928, 1], but a good agreement existed between data from different experiments. For the adenylic acid, however, negative results were obtained, which agrees with findings for muscle tissue [Lohmann & Schuster, 1934; Parnas & Lutwak-Mann, 1935].

Other purine compounds. A few experiments only were carried out on guanine compounds with the result that guanylic acid was found to be dephosphorylated and guanosine deaminated in the presence of phosphate by *Bact. coli*.

SUMMARY.

1. Washed suspensions of *Bact. coli* and other members of the Bacteriaceae decompose the following adenine compounds: adenosinetriphosphoric acid, muscle adenylic acid, yeast adenylic acid, adenosine, adenine, the first three being both dephosphorylated and deaminated, the latter two being deaminated.

2. The reactions occur either aerobically or anaerobically and in fresh suspensions as well as in those treated with toluene.

3. The end-product of decomposition is hypoxanthine. At this stage ribose is absent.

4. Adenosinetriphosphate (but not adenylic acid) is found to be a cell constituent of *Bact. coli*.

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