THE INHIBITION BY VARIOUS AGENTS OF THE LYSIS OF *BACTERIUM COLI* BY GLYCINE

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In view of the work of Maculla & Cowles (1948) on the lysis of bacteria by glycine, and of our subsequent work (Gordon, Hall & Stickland, 1951*a*) dealing with the kinetics of the reaction, we thought fresh light might be thrown on the mechanism of the reaction by a study of its inhibition.

This mechanism might conceivably be either a physical one, bringing about rupture of the bacterial membrane by for instance osmotic effects, or a chemical one involving an interaction between glycine and the protein or between the glycine and some enzyme concerned in the lytic process.

Although the exact mode of action is not known, the high temperature coefficient together with the pH relationship of the process, suggests that the action is essentially chemical, presumably enzymic, and that a merely physical action of the high amino-acid concentration on the bacterial membrane is of less, or no, significance. Some evidence was presented also by Gordon, Hall & Stickland (1951*b*) to show that osmosis alone is not enough to account for the lysis by glycine. Many substances are known which can bring about changes in the bacterial surface as was demonstrated by Gordon & Thompson (1936, 1937) and Gordon & Atkin (1938) who showed that tanning agents acted like artificial opsonins in that they affected the surface of bacteria in such a way that they could be phagocytosed by leucocytes. Such substances were inorganic tanning agents such as ferric chloride, chrome alum, and potash alum, and various organic tanning agents such as tannic acid, quebracho, and mirabilans. A study of these tanning agents, and of other substances of varied chemical nature which are known as enzyme inhibitors, might serve to differentiate further the two possible mechanisms of the lysis reaction.

EXPERIMENTAL

Strains used. The strains of *Bacterium coli* used were isolated in the laboratory and identified by their biochemical reactions.

Preparation of suspensions. The bacteria were inoculated on to peptone bouillon agar in Roux bottles and incubated for 16 hr. at 37° C. The growth was suspended in distilled water, filtered through glass wool, centrifuged and resuspended in water, centrifuged again and finally suspended in water at a concentration of between 10 and 20 mg. of dry weight per ml.

Measurement of degree of lysis. 0·5 ml. of the bacterial suspension was measured into each of a series of tubes, and 0·5 ml. of water or of the solution of the substance under test was added to it. One hour later 1·0 ml. of 2 M-glycine, previously adjusted to pH 7·5 by the addition of NaOH, was added and, after mixing of the solutions, the tubes were incubated at 37° C. overnight for 16 hr. The tubes were

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then spun at 3500 r.p.m., or 2500 g. for 20 min. to remove the bacteria, and the clear or opalescent supernatant fluid was poured off and 25 % trichloracetic acid added till a concentration of 5 % was reached. The precipitated protein was washed with 5 % trichloracetic acid, and its quantity estimated colorimetrically by means of the biuret reaction (Robinson & Hogden, 1940). The total protein content of the original suspension was estimated by the same reaction (Stickland, 1951). The proportion of the total protein liberated into the supernatant was expressed as a percentage and called the 'degree of lysis'.

RESULTS

(1) *The lysis of heated bacteria*

If suspensions of *Bact. coli* were heated at 65° C. for as short a time as 10 min., subsequent lysis by glycine was almost totally suppressed (Table 1). However,

Table 1. *The inhibition of the lysis of suspensions of* Bact. coli *which had been previously heated when incubated for* 16 *hr. at* 37° *C. with* M*-glycine*

Strain	Heat treatment	Lysis (%)		Inhibition (%)
		Control	Heated	
7	2 min. at 100° C.	38	7	82
8		42	6	86
7	5 min. at 100° C.	38	6	84
8		42	3	93
1	2 min. at 100° C.	23	9	61
2		21	9	57
3		22	9	59
1	5 min. at 100° C.	23	10	56
2		21	9	57
3		22	10	54
7	10 min. at 65° C.	42	1	98
8		37	3	92
7	30 min. at 65° C.	42	1	98
8		37	2	95

After the period of heating the bacteria were cooled to room temperature before the addition of the glycine.

heating a suspension of *Bact. coli* to 100° C. and then incubating with glycine produced some lysis, as judged by the presence of soluble protein in the supernatant fluid. This result at first led us to believe that the inhibition of lysis by glycine caused by heating bacteria was only partial, but later experiments (Table 2) showed that the protein found in the supernatant fluid from bacterial suspensions previously heated at 100° C. was liberated not as a result of the action of glycine, but as a result of the heating. Glycine can produce no lysis of bacteria which have been heated at 65° C. or at higher temperatures.

(2) *The effect of metals on lysis by glycine*

For these experiments the suspension of bacteria (0·5 ml.) was treated with the solution of the salt of the metal to be tested (0·5 ml.) and the mixture left at room temperature for 1 hr. Then 2 M-glycine (1·0 ml.) was added and, after thorough

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mixing, the tubes were incubated at 37° C. overnight. The concentrations given are those in the final mixtures. In some experiments the metal was removed by washing the bacteria before the addition of the glycine. The bacteria were deposited by spinning for 20 min. at 3500 r.p.m., the supernatant solution was poured off, the bacteria were then resuspended in about 5 ml. of distilled water, redeposited and finally resuspended in 1 ml. of water. To this suspension 1 ml. of 2 M-glycine solution was added and the mixture incubated. It was estimated that this treatment reduced the metal concentration in the final mixture at least a hundred-fold compared with the unwashed specimen.

Table 2. *The liberation of soluble protein by suspensions of* Bact. coli *heated at* 100° C.

		Protein present (% of total bacterial protein)			
		Heated bacteria		Unheated bacteria (control)	
Strain	Heat treatment	Before incubation in glycine	After incubation in glycine	Before incubation in glycine	After incubation in glycine
A	5 min. at 65° C.	0	0	0	53
B		0	0	0	47
C		0	0	0	53
D		0	1	0	40
A	30 min. at 65° C.	0	0	0	53
B		0	1	0	47
C		0	1	0	53
D		0	1	0	40
A	5 min. at 100° C.	2	6	0	53
B		2	4	0	47
C		2	3	0	53
D		7	6	0	40
A	60 min. at 100° C.	10	9	0	53
B		5	10	0	47
C		7	8	0	53
D		8	7	0	40

The incubation was in M-glycine solution for 16 hr. at 37° C.

(*a*) *The effect of ferric salts* (Table 3). Ferric salts at a concentration of 0·0033 M caused almost complete inhibition of lysis. This inhibition was only very slightly prevented by removing the ferric salt by washing, before the addition of the glycine. In these experiments the bacteria were instantaneously and completely agglutinated by the ferric salt and, even after washing, were brown in colour and did not resuspend to give the usual even suspension.

(*b*) *The effect of chromium salts* (Table 4). Chromic salts also completely inhibited the lysis by glycine at concentrations down to 0·002 M. At 0·0008 M the inhibition was not quite complete but, even at this level, washing the salt from the bacteria did not remove the inhibitory action. Chromium salts caused agglutination of the organisms, though not so markedly as ferric salts.

(*c*) *The effect of mercuric salts* (Table 5). The bactericidal action of low concentrations of mercuric salts occurs because the bacteria bind the mercury, probably by

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Table 3. *The inhibitory action of ferric salts on the lysis of* Bact. coli *by glycine*

Expt. no.	Strain	Concn. of Fe	Lysis (%) Untreated	Lysis (%) Treated	Inhibition (%)	Remarks
1	7	0·02 M	36	0	100	—
	8		34	0	100	—
2	1	0·0016 M	23	11	52	—
	2		21	8	62	—
	3		22	9	59	—
2	A	0·0033 M	45	1	98	—
	B		45	4	91	—
	C		42	2	95	—
	D		51	6	88	—
	A		45	13	71	Bacteria washed twice before addition of glycine
	B		45	9	80	
	C		42	9	74	
	D		51	16	69	

The ferric salt was added to the bacterial suspension and the mixture left at room temperature for 1 hr. after which an equal volume of 2 M glycine solution was added and the mixture incubated at 37° C. overnight.

The concentration given is that present in the final mixture, after the addition of the glycine. This concentration was twice as high during the preliminary hour at room temperature.

In Expts. 1 and 2 the salt used was iron alum, in Expt. 3, $FeCl_3$.

Table 4. *The inhibitory action of chrome alum on the lysis of* Bact. coli *by glycine*

Expt. no.	Strain	Concn. of Cr (M)	Lysis (%) Untreated	Lysis (%) Treated	Inhibition (%)	Remarks
1	7	0·002	44	2	95	—
	8		46	2	96	—
	7		44	0	100	Bacteria washed twice before addition of glycine
	8		46	2	96	
2	7	0·004	36	2	94	—
	8		34	0	100	—
	7		36	0	100	Bacteria washed five times before addition of glycine
	8		34	0	100	
3	1	0·0025	23	0	100	—
	2		21	0	100	—
	3		22	0	100	—
4	A	0·0025	45	0	100	—
	B		45	0	100	—
	C		42	0	100	—
	D		51	0	100	—
	A		45	4	91	Bacteria washed twice before addition of glycine
	B		45	5	89	
	C		42	6	86	
	D		51	3	94	
5	A	0·0008	48	17	65	—
	C		54	11	80	—
	A		48	12	75	Bacteria washed twice before addition of glycine
	C		54	10	81	

For details of the experimental procedure see Table 3.

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their —SH groups, consequently the lethal effect may be removed by H_2S and by other substances containing —SH groups (Fildes, 1940).

Lysis of *Bact coli* by glycine was strongly inhibited by mercury salts. Our experiments demonstrated the binding of the mercury by the bacterial cells and the reversal of the inhibition of lysis by H_2S. (Table 5). The concentration of Hg^{++}

Table 5. *The inhibitory action of mercuric salts on the lysis of* Bact coli *by glycine*

Expt. no.	Strain	Concn. of Hg (M)	Lysis (%) Untreated	Lysis (%) Treated	Inhibition (%)	Remarks
1	A		45	0	100	—
	B		45	0	100	—
	C		42	0	100	—
	D	0·001	51	0	100	—
	A		45	0	100	Bacteria washed twice before addition of glycine
	B		45	0	100	
	C		42	0	100	
	D		51	0	100	
2	1		23	19	17	—
	2	0·0001	21	17	19	—
	3		22	21	5	—
3	7	0·0001	42	6	86	—
	8		37	3	92	—
	7	0·00003	42	31	26	—
	8		37	27	27	—
4	7	0·00003	44	25	43	—
	8		46	27	42	—
	7	0·0000125	44	44	0	—
	8		46	42	9	—
5	A		48	5	90	—
	C		54	5	91	—
	A	0·00003	48	3	94	Bacteria washed twice before addition of glycine
	C		54	5	91	
	A		48	27	44	Bacteria washed twice and treated with H_2S before addition of glycine
	C		54	29	46	

For details of the experimental procedure see Table 3.

which is 'toxic' to *Bact. coli* in peptone solution is 10^{-5} M (Hotchkiss, 1923), while that which inhibited the lysis of dense suspensions of the organism was between 3×10^{-5} and 10^{-3} M. Washing the bacteria did not remove the inhibitory effect of Hg^{++}, but treatment of the bacteria with H_2S, after freeing them from surplus Hg^{++} by washing, did largely remove the inhibition. The treatment with H_2S which was shown to have no adverse effect on the lysis of normal bacteria by glycine also demonstrated, by the blackening of the bacteria, the presence even after washing of large amounts of bound mercury in the organisms.

(*d*) *The effect of cupric salts* (Table 6). Copper salts at concentrations down to 0·001 M caused complete, or nearly complete, inhibition of the lysis. Removal of the excess copper by washing did not remove the inhibition, which was, to a slight extent, removed by treatment with H_2S. The blackening of the washed bacteria

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by H_2S indicated again that some of the metal had been bound by the bacterial protein. As with mercury salts, no agglutination of the bacteria took place at these concentrations of copper salts.

(*e*) *The effect of various other salts* (Table 7). Aluminium (0·02 M-potash alum) inhibited lysis completely, as might be expected by comparison with ferric and chromium salts. Of the other metals which are roughly similar to mercury and copper in their behaviour to proteins, cadmium and lead at 0·0025 M showed smaller degrees of inhibition, ranging from 40 to 90 %.

Table 6. *The inhibitory action of copper salts on the lysis of* Bact. coli *by glycine*

Expt. no.	Strain	Concn. of Cu (M)	Lysis (%) Untreated	Lysis (%) Treated	Inhibition (%)	Remarks
1	1	0·0025	23	0	100	—
	2		21	0	100	—
	3		22	0	100	—
2	A	0·001	45	2	96	—
	B		45	2	96	—
	C		42	0	100	—
	D		51	3	94	—
	A		45	1	98	Bacteria washed twice before addition of glycine
	B		45	1	98	
	C		42	0	100	
	D		51	3	94	
3	A	0·001	48	11	77	—
	C		54	12	78	—
	A		48	8	83	Bacteria washed twice before addition of glycine
	C		54	10	82	
	A		48	16	67	Bacteria washed twice and treated with H_2S before addition of glycine
	C		54	20	63	

For details of the experimental procedure see Table 3.

Three other metals, Co, Ni and Ag, which are devoid of tanning properties, were also strongly inhibitory of lysis, Co^{++} and Ni^{++} causing a high degree of inhibition at 0·0025 M concentration and Ag^{+} at 0·00025 M. In all these experiments no reversal of the inhibition could be obtained by washing the bacteria free from the metal before the addition of the glycine.

(3) *The effect of phenol and formaldehyde on lysis by glycine*

At concentrations of phenol down to 0·125 M (1·2 %) the lysis of *Bact. coli* by glycine was completely inhibited. This inhibition was, however, partially (in one experiment almost completely) removed by washing off the phenol.

Formaldehyde also showed full inhibition at 0·1 M (0·3 %). The testing of formaldehyde requires careful attention, as this substance reacts with glycine with the production of an equivalent of acid. The resulting acidity would be enough to bring the pH below the optimum for lysis (Gordon *et al.* 1951*a*). Consequently, after the formaldehyde had acted on the bacteria, the glycine solution added to cause the lysis was adjusted to a more alkaline pH (about 8·5), such that after

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mixture with the formaldehyde-treated bacteria the final pH was 7·5. The actual concentration of free formaldehyde in the resulting mixture was very much lower than that shown in the table, as by far the greater part would have reacted with the glycine. In the experiments in which the formaldehyde was removed by washing, before the addition of glycine, the usual glycine solution at pH 7·5 was of course used. In such experiments the inhibition by formaldehyde was to a large extent removed.

Table 7. *The inhibitory action of salts of various metals on the lysis of* Bact. coli *by glycine*

Expt. no.	Strain	Salt used	Concn. (M)	Lysis (%) Untreated	Lysis (%) Treated	Inhibition (%)	Remarks
1	7	Potash alum	0·02	36	1	97	—
	8			34	1	97	—
2	1	$CdSO_4$	0·0025	23	6	74	—
	2			21	6	62	—
	3			22	8	64	—
3	A	$CdSO_4$	0·0025	48	28	42	—
	C			54	28	48	—
	A			48	28	42	Bacteria washed twice
	C			54	29	46	before addition of glycine
4	1	$CoSO_4$	0·0025	23	5	78	—
	2			21	4	81	—
	3			22	6	73	—
5	1	$NiSO_4$	0·0025	23	5	78	—
	2			21	4	81	—
	3			22	1	95	—
6	A	$PbAc_2$	0·0025	45	22	51	—
	B			45	4	91	—
	A			45	25	44	Bacteria washed twice
	B			45	3	93	before addition of glycine
7	A	$AgNO_3$	0·00075	48	0	100	—
	C			54	2	96	—
	A			48	0	100	Bacteria washed twice
	C			54	2	96	before addition of glycine
	A	$AgNO_3$	0·00025	48	1	98	—
	C			54	12	78	—
	A			48	0	100	Bacteria washed twice
	C			54	19	65	before addition of glycine

For details of the experimental procedure see Table 3.

(4) *The absence of inhibition of lysis by sodium fluoride, sodium monoiodoacetate, and potassium cyanide*

Sodium fluoride (up to 0·25 M) which is inhibitory of many enzyme systems especially phosphatases, and sodium monoiodoacetate (up to 0·005 M) which at even lower concentrations is inhibitory of enzyme systems depending for their activity on —SH groups, had no appreciable effect on the lysis of *Bact. coli* (Table 9). Potassium cyanide (0·001–0·01 M) also had no inhibitory action.

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Table 8. *The inhibitory action of formaldehyde and phenol on the lysis of* Bact. coli *by glycine*

Expt. no.	Strain	Substance used	Concn. (M)	Lysis (%) Untreated	Lysis (%) Treated	Inhibition (%)	Remarks
1	7	Phenol	0·3	34	4	89	—
	8			29	1	97	—
2	1	Phenol	0·25	23	1	96	—
	2			21	1	95	—
	3			22	1	95	—
3	A	Phenol	0·125	45	1	98	—
	B			45	1	98	—
	C			42	2	95	—
	D			51	0	100	—
	A			45	11	76	Bacteria washed twice before addition of glycine
	B			45	42	7	
	C			42	21	50	
	D			51	16	69	
4	A	Formaldehyde	0·1	45	0	100	—
	B			45	1	98	—
	C			42	0	100	—
	D			51	0	100	—
	A			45	26	42	Bacteria washed twice before addition of glycine
	B			45	7	84	
	C			42	24	43	
	D			51	35	31	

For details of the experimental procedure see Table 3.

Table 9. *The effect of sodium fluoride and sodium monoiodoacetate on the lysis of* Bact. coli *by glycine*

Expt. no.	Strain	Inhibitor	Concn. (M)	Lysis (%) Untreated	Lysis (%) Treated	Inhibition (%)
1	7	Sodium fluoride	0·07	38	48	—
	8		0·07	42	50	—
2	1	Sodium fluoride	0·1	55	53	4
	2		0·1	59	56	5
3	A		0·25	14	28	—
	A	Sodium fluoride	0·1	14	37	—
	B		0·25	16	29	—
	B		0·1	16	37	—
4	A	Sodium fluoride	0·1	29	29	—
	B		0·1	35	46	—
5	A		0·004	14	10	29
	A	Sodium iodoacetate	0·002	14	14	—
	B		0·004	16	15	6
	B		0·002	16	18	—
6	A	Sodium iodoacetate	0·004	55	35	36
	B		0·004	59	36	40
7	7	Sodium iodoacetate	0·002	44	41	7
	8		0·002	46	42	9
8	A		0·01	29	25	14
	A		0·003	29	28	3
	A	Potassium cyanide	0·001	29	29	—
	B		0·01	35	31	11
	B		0·003	35	33	6
	B		0·001	35	35	—

For details of the experimental procedure see Table 3.

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DISCUSSION

The treatments, the effects of which on the lysis of *Bact. coli* by glycine are reported here may be divided roughly into four groups.

(1) Heating the bacteria suppresses lysis by glycine completely. Heat treatment which is sufficient to kill the bacteria is also sufficient to prevent the lysis. This does not help to distinguish between possible mechanisms, as these conditions of heating would suppress all enzyme action and coagulate the bacterial protein.

(2) The treatment of *Bact. coli* with substances which may be supposed to react with the bacterial protein with an effect analogous to 'tanning' such as the metals Cr, Fe, Al, and possibly formaldehyde prevents completely the lysis by glycine.

(3) The treatment of *Bact. coli* with substances which have no 'tanning' action but which would be expected to inhibit enzymes by forming inactive compounds such as the heavy metals, and phenol, cause substantial inhibition of the lysis, some of them acting at very low concentrations.

(4) The treatment of *Bact. coli* with substances which, though known to inhibit many enzymes, yet have no effect on the lysis such as sodium fluoride, sodium monoiodoacetate, and potassium cyanide.

The distinction between (2) and (3) is not perfectly clear, since because heavy metals form insoluble compounds with proteins the effects which they cause may all be due to changes in the structure of the proteins of the bacteria. This, however, seems very unlikely, especially for example with Ag which, at the concentration used, can only be supposed to be acting specifically on some enzyme system. Browning & Mackie (1949) postulate that the antiseptic action of metals like Hg must be attributed to their combination with some enzyme necessary for multiplication of the cells. The specific reaction of Hg with —SH groups, which they suggest, does not appear to apply to lysis by glycine, since monoiodoacetic acid has no inhibitory action.

The alternative theory of the mechanism of lysis, in which some simple physical process such as osmosis is the essential factor, is not supported by this evidence. The action of low concentrations of Ag^+ (0·00025 M), for example, does not seem likely to influence the physical properties of the bacterial membrane sufficiently to account for the complete inhibition of lysis.

SUMMARY

1. The lysis of *Bact. coli* by glycine is inhibited completely by various treatments: (*a*) by heating the bacteria at 65 or 100° C.; (*b*) by the action of Cr, Fe, and Al salts at concentrations which cause agglutination of the organisms, (*c*) by various heavy metals, including particularly Hg and Ag salts, at low concentrations (*d*) by lethal concentrations of formaldehyde and phenol.

2. The lysis is not inhibited by sodium fluoride, potassium cyanide, or sodium monoiodoacetate at concentrations which suppress the action of certain enzymes.

3. This evidence on the whole supports the rejection of the view that a simple physical process is responsible for lysis by glycine, but does not yet enable us to distinguish between the other possible mechanisms of the lysis because the treat-

ments used might equally interfere either with the structural proteins of the bacteria, or with the hypothetical enzymes which might be concerned in producing the lysis.

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REFERENCES

BROWNING, C. H. & MACKIE, T. J. (1949). Muir and Ritchie's *Manual of Bacteriology* 11th ed., p. 35. Oxford.

FILDES, P. (1940). The mechanism of the anti-bacterial action of mercury. *Brit. J. exp. Path.* **21**, 67.

GORDON, J. & ATKIN, W. R. (1938). The artificial opsonization of bacteria, Part III. *Brit. J. exp. Path.* **19**, 204.

GORDON, J., HALL, R. A. & STICKLAND, L. H. (1951*a*). The kinetics of the lysis of *Bacterium coli* by glycine. *J. Hyg. Camb.*, **49**, 169.

GORDON, J., HALL, R. A. & STICKLAND, L. H. (1951*b*). Observations on the resistance of *Bacterium coli* to glycine. *J. Path. Bact.* **63**, 285.

GORDON, J. & THOMPSON, F. C. (1936). The artificial opsonization of bacteria. *Brit. J. exp. Path.* **17**, 159.

GORDON, J. & THOMPSON, F. C. (1937). The artificial opsonization of bacteria, Part II. *Brit. J. exp. Path.* **18**, 390.

HOTCHKISS, M. (1923). Studies on salt action. VI. The stimulating and inhibitive effect of certain cations upon bacterial growth. *J. Bact.* **8**, 141.

MACULLA, E. G. & COWLES, P. B. (1948). Use of glycine in disruption of bacterial cells. *Science*, **107**, 376.

ROBINSON, H. W. & HOGDEN, C. G. (1940). Biuret reaction in determination of serum proteins. *J. biol. Chem.* **135**, 727.

STICKLAND, L. H. (1951). The determination of small quantities of bacteria by means of the biuret reaction. *J. gen. Microbiol.* **5**, 698.

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