The Assimilation of Amino Acids by Bacteria

24. INHIBITORS OF INCORPORATION OF GLYCINE IN DISRUPTED STAPHYLOCOCCAL CELLS*

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In previous papers of this series (Gale & Folkes, 1955*a, b*) a description has been given of a preparation of disrupted staphylococcal cells which can be used to study the dependence on nucleic acid of protein-synthesis and amino acid-incorporation reactions. Removal of nucleic acid from the preparation results in marked decrease in the incorporation of certain amino acids; the incorporation can be restored by addition of staphylococcal nucleic acid to the incubation mixture. The action of ribonucleic acid (RNA) in restoring incorporation is not abolished by the action of ribonuclease, and active materials can be separated from the digestion products. In the case of the incorporation of glycine, fractionation of RNA digests has led to the separation of a highly active 'incorporation factor' whose properties are not those of any known nucleotide or nucleoside (Gale, 1957).

In the present paper an investigation of the action of various inhibitors on the incorporation of glycine in the disrupted-cell preparation is reported. The actions of chelating agents and analogues of purines, pyrimidines and growth factors were studied in an attempt to throw light on the possible nature of components of the system, including the incorporation factors. Various antibiotics and antimicrobial drugs were included in the survey, since the disrupted-cell preparation affords a means of studying the action of such selective agents in the absence of penetration barriers which may be largely responsible for their selective nature.

Of the synthetic substances investigated, 8-hydroxyquinoline (oxine) and various derivatives of benzimidazole have proved to be potent inhibitors. The antibacterial action of oxine has been investigated by Albert, Gibson & Rubbo (1953), who concluded that oxine itself is non-toxic but forms toxic chelates with certain heavy metals, iron in particular, and that the toxicity in a given medium is determined by the relative amounts of oxine and metal. Woolley (1944) showed that benzimidazole and certain derivatives thereof inhibited the growth of yeasts and bacteria and that the action could be

antagonized by the presence of purines, guanine being more effective than adenine. 5:6-Dimethylbenzimidazole is part of the structure of vitamin B₁₂; Woolley & Pringle (1952) devised a number of analogues of 4:5-diamino-1:2-dimethylbenzene, a precursor of dimethylbenzimidazole (Woolley, 1950, 1951), which were potent inhibitors of the growth of *Staphylococcus aureus*; it was suggested that these substances acted by inhibiting the synthesis of vitamin B₁₂ and riboflavin. Further elaboration of the benzimidazole structure led to the synthesis of 1:2-dichloro-5-nitro-4-(*p*-nitrobenzenesulphonamido)benzene (DCDNS) by Woolley (1952*a*) and the demonstration that it was effective against mammary cancers in mice (Woolley & Schaffner, 1954). Gillespie, Engelman & Graff (1954, 1956) have synthesized a series of benzimidazole derivatives which inhibit the growth of certain protozoa and frog embryos, and Tamm (1954, 1955, 1956) has studied the inhibition of the growth of influenza virus by halogen-substituted benzimidazoles. Tamm found that the inhibitory action of benzimidazole increased with increasing substitution by chlorine in positions 4, 5 and 6, while the action of the trichloro derivatives was increased some 15 times by substitution with a β -D-ribofuranosyl group in position 1.

ORGANISM AND METHODS

The organism, *S. aureus* Duncan, its growth, harvesting, disintegration, preparation of the disrupted-cell fraction and its depletion of nucleic acid have been described in previous papers (Gale & Folkes, 1955*a, b*). Incubation was carried out under condition 1 of those papers, each tube containing approximately 1 mg. (dry wt.) of disrupted-cell preparation and 0.02 μ C of [¹⁴C]glycine (generally labelled, specific activity 4 mc/m-mole)/3.0 ml.; other reagents were as previously described. Procedures for preparing the 'trichloroacetic acid (TCA) precipitate' and 'protein fraction' of the cells, drying, and counting their radioactivities were also described in those papers. In a number of tests it was found that the reduction in counts/min. on removing nucleic acid from the TCA precipitate was of the order of 10-13% and that such removal did not significantly alter the inhibitions studied. Consequently, this step was

* Part 23: Rowlands, Gale, Folkes & Marrian (1957).

usually omitted and, except where it is stated that counts were determined on protein fractions, results are expressed as counts/min. of the TCA precipitate obtained from 1.0 mg. (dry wt.) of cell preparation. The experiments recorded in this paper were carried out over several months and the activity of the disrupted-cell preparations varied from time to time with alterations in the efficiency of the supersonic disintegrator; in experiments with forty-seven different preparations the mean value for incorporation of glycine after 90 min. incubation under condition 1 was 2977 counts/min./TCA precipitate from 1.0 mg. (dry wt.) preparation, standard deviation 1086, range 1445–4423. The counting equipment used was calibrated against the [^{14}C]glycine used; 1.0 μm -mole gave 362 counts/min.

Incorporation of glycine under condition 1 is not linear; the rate decreases with time and incorporation ceases after 90–120 min. at 37° (see Fig. 5). In preliminary surveys the radioactivity of the preparation was measured after 90 min. incubation in the presence and absence of inhibitor; in key instances inhibition was checked by repetition of the experiment, samples being taken after 30, 60 and 90 min. incubation. Inhibition in all cases is expressed as the percentage decrease in the rate of incorporation measured in the absence of inhibitor. There is some variation in the degree of

inhibition obtained with any particular inhibitor in different experiments; for 5:6-dimethylbenzimidazole at 0.001M concentration, the mean inhibition of incorporation of glycine was 62%, n 25, standard deviation 6, range 30–86. All other inhibitors have been compared with 5:6-dimethylbenzimidazole as standard.

Inhibitors. We are indebted to the following for the substances indicated by numbers in brackets in the tables: Dr D. W. Woolley (3, 5, 7, 23, 31, 36, 53–56); Professor Sir Alexander Todd and Dr V. M. Clark (4, 18, 28, 32, 37–40, 46); Dr C. H. Schunk (21–23, 25, 27, 29, 33, 34); Dr M. Engelman (19, 20, 24, 26, 30, 41–45, 47–49); Dr R. E. F. Matthews (6, 8, 9, 12–16, 50, 51); Dr D. B. Dunn (2); Dr D. Raacke (17); Dr B. A. Newton for 57 and the substances shown in Fig. 7b; Mr M. Richmond for 59, 1:2-dimethyl-4-(*p*-nitrobenzenesulphonamido)benzene and *p*-nitrobenzenesulphonamide.

RESULTS

Table 1 shows the inhibition of incorporation of glycine produced by a number of common metabolic inhibitors. The incorporation is an energy-requiring reaction and is consequently affected by substances which inhibit energy production or 'coupling' under the experimental conditions used (Gale & Folkes, 1955*a*). The object of the present survey was to find substances whose inhibitory action could be related to the mechanism of incorporation itself. Table 1 shows that reagents which combine with aldehydes or ketones have little inhibitory action, being much less effective than they are in pyridoxal-activated systems such as the amino acid decarboxylases (Taylor & Gale, 1945). Of the chelating agents tested, ethylenediaminetetra-acetic acid (EDTA) and α -dipyridyl were without significant effect, whereas oxine was a potent inhibitor.

Oxine

Fig. 1 shows the effect of oxine concentration on the inhibition of incorporation of glycine; it is unusual in that high concentrations, of the order of 0.001M-oxine, are less effective than concentrations two or three orders lower. The optimum concentration for inhibition in the experimental conditions used is 10^{-5}M . Fig. 2 shows the action of oxine on the incorporation of glycine in a disrupted-cell preparation which has been depleted of nucleic acid and then tested in the presence and absence of added staphylococcal deoxyribonucleic acid (DNA). The diphasic effect of oxine concentration is much less marked on the nucleic acid-depleted preparation, and it can be seen that 10^{-5}M -oxine completely abolishes the activation of incorporation by DNA. Higher concentrations of oxine are again less effective. The same general result is obtained if incorporation is stimulated by DNA, RNA or a purified preparation of the glycine-incorporation factor (Gale, 1957).

Effect of metal ions. The inhibitory action of oxine might be due to chelation of essential multi-

Table 1. *Action of some inhibitors on the incorporation of glycine in disrupted staphylococcal cells under condition 1*

Disrupted-cell preparation was incubated under condition 1: 3.0 ml. of incubation mixture containing 4.0 mg. of adenosine triphosphate, 0.3 ml. of hexose diphosphate (=30 mg. of Ba salt), 0.02 μC of [^{14}C]glycine, buffered saline (pH 6.2), with and without inhibitor as below. Incorporation into protein fraction was estimated after 90 min. incubation at 37°. Inhibition is expressed as percentage rate of incorporation in the absence of added inhibitor. Results are collected from six experiments. Mean value of controls without inhibitors = 2865 counts/min./mg. of preparation (estimations on protein fraction); range 2020–3026.

	Concn. (mm)	Inhibition (%)
Hydroxylamine	1.0	56
	0.1	0
Hydrazine	1.0	23
Semicarbazide	1.0	15
Potassium cyanide	10.0	75
	1.0	47
	0.1	9
Sodium azide	1.0	40
	0.1	9
α -Dipyridyl	1.0	0
EDTA	30.0	10
	10.0	0
	1.0	0
Oxine	0.01	75
	0.001	23
Aminopterin	3.0	34
	1.0	10
Sulphamezathine	1.0	0
<i>p</i> -Nitrobenzenesulphonamide	1.0	11

valent metal ions. It has, for example, been shown that inhibition by oxine of the accumulation of glutamic acid in intact *S. aureus* can be antagonized and abolished by manganese or magnesium (Gale, 1949). Consequently the action of oxine was tested in the presence of equivalent concentrations of various multivalent metal ions; Table 2 shows that, with the possible exception of Co^{2+} ions, no significant antagonism was found. Cobalt forms stable complexes with oxine and has been found to exert a protective action in other cases of oxine toxicity (Albert *et al.* 1953); it may act in these cases by displacing other metals from chelate complexes to form less toxic complexes, or by reducing the effective concentration of oxine itself. No evidence could be obtained that the action of oxine on the incorporation of glycine could be overcome by provision of excess of any of the metals listed in Table 2.

Albert *et al.* (1953) found that oxine was non-toxic to bacteria suspended in distilled water but became toxic in the presence of heavy-metal ions such as Cu^{2+} or Fe^{2+} . To test whether the same is true in the present investigations, a batch of incubation medium, containing all the ingredients other than $[^{14}\text{C}]$ glycine and disrupted-cell prepara-

tion, was stripped of heavy metals by the method of Waring & Werkman (1942). The procedure involves shaking the medium with oxine and then removing excess of oxine and its metal complexes by shaking with chloroform; the efficiency of removal of metal

Table 2. *Effect of metal ions on the inhibition of incorporation of glycine by oxine*

Incorporation of glycine was studied in disrupted-cell preparation under condition 1 as in Table 1; oxine was added at concentrations shown below, with and without an equivalent concentration of metal ion added as the chloride. Estimations were made on the TCA precipitate; control without inhibitor = 2823 counts/min./mg. of preparation.

Metal ion added	Percentage inhibition of incorporation of glycine	
	0.01 mM-Oxine	1.0 mM-Oxine
None	65	50
Mo^{3+}	70	—
Co^{2+}	54	32
Cu^{2+}	91	99
Fe^{2+}	59	94
Zn^{2+}	68	74
Mn^{2+}	61	41
Mg^{2+}	65	45
Ca^{2+}	65	48

Fig. 1. Inhibition by oxine of incorporation of glycine in disrupted staphylococcal cell preparation. The preparation was incubated under condition 1:3.0 ml. of incubation mixture containing 4.0 mg. of adenosine triphosphate, 0.3 ml. of hexose diphosphate (=30 mg. of Ba salt), 0.02 μ of $[^{14}\text{C}]$ glycine, buffered saline (pH 6.2) and approximately 1 mg. (dry wt.) of disrupted-cell preparation. Incorporation in the TCA precipitate was estimated after 90 min. incubation at 37°. Results are expressed as counts/min./TCA precipitate from 1.0 mg. (dry wt.) of preparation.

Fig. 2. Effect of oxine on the nucleic acid stimulation of the incorporation of glycine in nucleic acid-depleted disrupted staphylococcal cell preparation. The disrupted-cell preparation was incubated for 1 hr. at 37° with 30 μ g. of deoxyribonuclease/ml. and centrifuged down before incubation with glycine under condition 1 as in Fig. 1. Curve A: depleted preparation. Curve B: as A with addition of 0.2 mg. of staphylococcal deoxyribonucleic acid/3.0 ml. Incorporation was estimated after 90 min. incubation and expressed as counts/min./TCA precipitate from 1.0 mg. (dry wt.) of preparation.

ions depends upon the number of times the treatment is repeated and on the absence of metal contamination from glassware, etc., used in the experimental procedure. In our experience the stripping procedure improves in successive experiments, probably owing to progressive removal of metals from glassware, and Fig. 3 shows the effect of oxine on incorporation experiments carried out in normal medium and in three successive batches of stripped medium. It is clear that, as heavy metals are removed from the experimental system, so the incorporation reaction becomes less sensitive to oxine.

Attempts were made to discover what metals were responsible for oxine toxicity by adding known metal ions to the stripped medium. It was not possible to demonstrate significant effects by the addition of Fe^{2+} or Fe^{3+} ions, but results somewhat similar to those obtained in normal media were found after the addition of Cu^{2+} ions. At this stage it was found that incorporation of glycine is highly sensitive to Cu^{2+} ions, and Fig. 4 shows that Cu^{2+} ions are markedly more inhibitory in media stripped of other multivalent ions than in untreated incubation medium. It can be seen also that oxine has little effect on the toxicity of Cu^{2+} ions. The results in Fig. 4 suggest that the incorporation of glycine might be protected against Cu^{2+} ions by the presence of other heavy metals normally present in the incubation medium. Ferric and Co^{2+} ions were found to antagonize the toxicity of Cu^{2+} ions to some extent; 10^{-6}M-Cu^{2+} gave rise to 89, 50 and 56% inhibition when tested in stripped medium containing no added metal, 10^{-7}M-Fe^{3+} and 10^{-6}M-Co^{2+} respectively. It was not possible to abolish the toxicity of Cu^{2+} ions by addition of these or other metals.

Analogues of purines and pyrimidines

Table 3 shows the effect of a variety of analogues of purines and pyrimidines on the incorporation of glycine. 5-Nitro- and 5-bromo-uracil are the only pyrimidine analogues tested which have significant inhibitory activity; a number of purine analogues have some activity at concentrations of 0.003M but 5:6-dimethylbenzimidazole is the only substance listed in Table 3 that can be regarded as an effective inhibitor. Webster & Johnson (1955) obtained somewhat similar results for the incorporation of glutamic acid by a particulate preparation from pea seedlings; of the substances tested by these authors, aminopterin proved to be the most effective inhibitor, 0.001M -aminopterin giving 80% inhibition. The glycine-incorporation system studied here is less sensitive to aminopterin (Table 1), but displays approximately the same sensitivity to benzimidazole.

Fig. 3. Effect of removal of heavy metals from incubation mixture on inhibition of incorporation of glycine by oxine. Curve 1: incubation for 90 min. under condition 1 as for Fig. 1. Control = 1863 counts/min./mg. of preparation (TCA precipitate). Curve 2: as for curve 1 but with previous treatment of incubation mixture with oxine to remove heavy metals, as in text. Control = 1291 counts/min./mg. of preparation (TCA precipitate). Curves 3, 4: successive batches of incubation mixture stripped of heavy metals as for curve 2. Control = 1252 (3), 960 (4) counts/min./mg. of preparation (TCA precipitate).

Fig. 4. Inhibition of incorporation of glycine by copper. Curve A: effect of Cu^{2+} ions when incubation takes place for 90 min. as in Fig. 1 in normal medium. Control = 2800 counts/min./mg. of preparation (TCA precipitate). Curve B: effect of Cu^{2+} ions when incubation takes place in medium stripped of heavy metals. Control = 1815 counts/min./mg. of preparation (TCA precipitate). Curve C: as for curve B with 1 equiv. of oxine added in each case. Curve D: as for curve B with 2 equiv. of oxine added in each case.

Table 3. *Action of analogues of purines and pyrimidines on the incorporation of glycine in disrupted staphylococcal cells*

Conditions were as for Table 1. Estimations were made on the TCA precipitate; control without inhibitor = 3445 counts/min./mg. of preparation.

	Percentage inhibition	
	0.003 M	0.001 M
(1) 5:6-Dimethylbenzimidazole	92	66
(2) 5-Bromouracil	35	10
(3) 8-Azaxanthine	26	9
(4) Benzimidazole	22	5
(5) 2-Azahypoxanthine	21	—
(6) 5-Nitrouracil	20	4
(7) 2-Aza-adenine	10	0
(8) Isocytosine	0	—
(9) Thiouracil	0	—
(10) Theobromine (3:7-dimethylxanthine)	—	0
(11) Caffeine (1:3:7-trimethylxanthine)	—	0
(12) 2-Aminopyrimidine	—	0
(13) 2:4:6-Triaminopyrimidine	—	0
(14) 2-Amino-4-hydroxypyrimidine	—	0
(15) 2:4-Amino-6-hydroxypyrimidine	—	0
(16) 2:4:5-Amino-6-hydroxypyrimidine	—	0
(17) 2-Hydroxy-6-mercaptopurine	—	0

Benzimidazole derivatives

Inhibitors. Table 4 shows the degree of inhibition of incorporation of glycine produced by a series of derivatives of benzimidazole. Benzimidazole itself is a weak inhibitor whose activity is markedly increased by substitution with EtO-, MeO-, NO₂-, CH₃- or Cl- in positions 4(7), 5(6) or 6(5). In general the greater the degree of substitution, the more inhibitory is the derivative. Thus the inhibitory activities of the mono-(5)-, di-(5:6)- and tri-(4:5:6)-chloro derivatives relative to benzimidazole are approximately 5, 19 and 25 respectively. Further substitution in the 1-position may modify markedly the inhibitory activity of derivatives containing CH₃- or Cl- in positions 5 and 6. For 5:6-dimethylbenzimidazole, substitution in position 1 with an allyl group produces the most potent inhibitor of this series yet tested, whereas substitution in the same position with 2:3-dihydroxypropyl, β -D-glucopyranosyl or β -D-ribofuranosyl groups greatly decreases or abolishes the inhibitory activity. The natural nucleotide 5:6-dimethyl-1-(α -D-ribofuranosyl)benzimidazole 3'-phosphate has an inhibitory activity of the same order as that of the free base. Substitution in position 1 with β -D-ribofuranosyl greatly decreases the inhibitory activity of 5:6-dichlorobenzimidazole, but slightly enhances the activity of 4:5:6-trichlorobenzimidazole; in the latter case no significant difference is produced by substitution with 1- β -D- or 1- α -D-ribofuranosyl groups.

Substitution with HO-, CH₃- or C₂H₅- in position

2 appears to have little effect, although Table 5 shows that there are significant differences in the activities of certain benzimidazole as opposed to corresponding benzotriazole derivatives. The difference is not consistent, as in three cases the triazole derivative is more effective and in three cases less effective than the corresponding imidazole compound.

Stimulatory substances. Substitution with an amino group in position 6 of benzimidazole gives rise to substances which stimulate the incorporation of glycine. Table 6 shows the action of some stimulatory substances of this type; the most effective so far tested is 6-amino-4-hydroxybenzotriazole. The degree of stimulation is variable; in the majority of experiments the incorporation of glycine is increased by 40–80 % in the presence of 0.001 M-6-amino-4-hydroxybenzotriazole, but in occasional experiments the stimulation amounts to 100–150 % and on one occasion rose to 250 % (mean 55; *n* 27; standard deviation 45; range 8–250). The concentration giving optimum stimulation is also somewhat variable, normally lying between 0.0003 and 0.001 M, higher concentrations frequently being inhibitory. The reason for the variable nature of the effect has not been traced, although it may lie in variable amounts of certain trace metals in the incubation system. It is probable that many of these substances act as chelating agents, but it has not been possible to alter their activity significantly by carrying out tests in the presence of EDTA, in media stripped of heavy metals as described above, or by the addition of 10⁻⁶ M-Cu²⁺, -Co²⁺ or -Fe³⁺ ions to the incubation medium.

Antagonism of inhibition by benzimidazole derivatives. Woolley (1944) found that the growth-inhibitory action of benzimidazole could be antagonized by purines, guanine being a more effective antagonist than adenine. Table 6 shows that the purines or their ribosides are not markedly effective as antagonists of the inhibition of the incorporation of glycine by 5:6-dimethylbenzimidazole. The stimulatory derivatives, however, do act as antagonists, and complete antagonism is given by an equimolar concentration of 6-amino-4-hydroxybenzotriazole. Fig. 5 shows the time course of the incorporation of glycine in the presence of 5:6-dimethylbenzimidazole and 6-amino-4-hydroxybenzotriazole separately and together. 6-Amino-4-hydroxybenzotriazole (0.001 M) also gives complete antagonism of inhibition by 0.0001 M-1-allyl-5:6-dimethylbenzimidazole and partial antagonism of inhibition by 4-methoxy-6-nitrobenzotriazole (see Table 8).

Analogues of dimethyldiaminobenzene

Woolley & Pringle (1952) showed that a number of substituted benzenes are potent inhibitors of the growth of *S. aureus* and suggested that they act as

Table 4. *Inhibition of incorporation of glycine in disrupted staphylococcal cells by benzimidazole derivatives*

Conditions as for Table 1. Results were collected from fifteen experiments, 5:6-dimethylbenzimidazole being included in all experiments (see text). Mean value of controls = 3189 counts/min./mg. of preparation (estimations on TCA precipitates); range 2277–4134.

	Concn. (mm) giving 50 % inhibition	Percentage inhibition given by 0.001 M
(18) 1-Allyl-5:6-dimethylbenzimidazole	0.044	—
(19) 6-Methoxy-4-nitrobenzimidazole	0.09	—
(20) 4-Methoxy-6-nitrobenzotriazole	0.15	—
(21) 4:5:6-Trichloro-1-β-D-ribofuranosylbenzimidazole	0.21	—
(22) 4:5:6-Trichloro-1-α-D-ribofuranosylbenzimidazole	0.21	—
(23) 2:3:6-Trimethylbenzoxazole	0.25	—
(24) 5-Methoxy-7-nitroquinoxaline	0.28	—
(25) 4:5:6-Trichlorobenzimidazole	0.40	—
(26) 4-Hydroxy-2-methyl-6-nitrobenzimidazole	0.50	—
(27) 5:6-Dichlorobenzimidazole	0.52	—
(1) 5:6-Dimethylbenzimidazole	0.7	—
(28) 5:6-Dimethyl-1-α-D-ribofuranosylbenzimidazole 3'-phosphate	0.85	—
(29) 2-Ethyl-5-methylbenzimidazole	1.3	—
(30) 5-Nitrobenzimidazole	1.8	—
(31) 4-Nitrobenzimidazole	3.0	43
(32) N-D-Glucosyl-4:5-dimethyl-N'-thioformyl o-phenylenediamine	—	43
(33) 5:6-Dimethyl-1-β-D-ribofuranosylbenzimidazole	—	28
(34) 5:6-Dichloro-1-β-D-ribofuranosylbenzimidazole	—	27
(35) Benzimidazole	—	22
(36) 2-Hydroxybenzimidazole	—	13
(37) Benzotriazole	—	9
(38) 1-(2:3-Dihydroxypropyl)-5:6-dimethylbenzimidazole	—	7
(39) 5:6-Dimethylbenzotriazole	—	4
(40) 1-β-D-Glucopyranosyl-5:6-dimethylbenzimidazole	—	0

Table 5. *Comparison of benzimidazole and benzotriazole derivatives as inhibitors of incorporation of glycine*

Conditions were as for Table 1. Estimations were made on the TCA precipitate; control without inhibitor = 3097 counts/min./mg. of preparation.

	Substituent in position			Concn. (mm)	Percentage inhibition by	
	4	5	6		Benzimidazole derivative	Benzotriazole derivative
(41, 42) NO ₂	MeO	—	—	0.1	38	0
(43, 44) NO ₂	—	—	EtO	0.1	25	65
(19, 45) NO ₂	—	—	MeO	0.3	5	32
(30, 31) —	—	NO ₂	—	0.3	26	67
(1, 46) —	—	CH ₃	CH ₃	1.0	65	10
(35, 37) —	—	—	—	1.0	15	7

Table 6. *Stimulation of incorporation of glycine by benzimidazole derivatives and their antagonistic action against 5:6-dimethylbenzimidazole*

Conditions were as for Table 1; all derivatives were tested at a final concentration of 0.001 M. Control without derivative = 2631 counts/min./TCA precipitate from 1.0 mg. of preparation.

	Percentage effect on incorporation of glycine	Percentage antagonism of inhibition by 0.001 M-5:6-di- methylbenz- imidazole
5:6-Dimethylbenzimidazole	- 70	—
(47) 6-Amino-4-hydroxybenzotriazole	+ 55	100
(48) 6-Amino-4-hydroxybenzimidazole	+ 20	42
(49) 6-Aminobenzimidazole	+ 15	34
Guanine	+ 13	23
(50) 8-Azaguanine	+ 10	20
(51) Guanosine	+ 10	0
Guanlyic acid	0	0
Adenine	0	0
Adenosine	0	0

inhibitors of the metabolism of 4:5-diamino-1:2-dimethylbenzene, a precursor of benzimidazole derivatives such as vitamin B₁₂ and riboflavin. They found that the inhibitory nature of the derivatives increased with progressive substitution in positions 1, 2, 4 and 5 with active groups such as Cl⁻, NO₂⁻ and HO⁻. A somewhat similar situation has been encountered in the present studies. Substances which have no significant inhibitory effect on the incorporation of glycine include *o*-phenylenediamine, *o*-nitroaniline, 3:4-dichloroaniline and xylylene; Fig. 6 shows the degree of inhibition obtained in the presence of other analogues of dimethyldiaminobenzene. 4:5-Diamino-1:2-dichlorobenzene has approximately the same inhibitory action as 5:6-dichlorobenzimidazole, while 2-amino-4:5-dimethylphenol is as active as 1-allyl-5:6-dimethylbenzimidazole. The most potent inhibitor is 1:2-dichloro-5-nitro-4-(*p*-nitrobenzenesulphonamido)-benzene (DCDNS); omission of the 5-nitro group decreases the activity by 10 times, while the corresponding 1:2-dimethyl-4-(*p*-nitrobenzenesulphonamido)benzene gives less than 30% inhibition at 0.3 mM. Woolley (1952*a*) found that DCDNS was the most effective growth inhibitor of this series. *p*-Nitrobenzenesulphonamide has no effect on the incorporation of glycine when tested at concentrations of 1 mM or less. No significant antagonism of

DCDNS has been obtained with 6-amino-4-hydroxybenzimidazole, the corresponding benzotriazole, benzimidazole, *p*-aminobenzoic acid, guanine, guanosine, adenine or azaguanine.

Antibiotics and antimicrobial drugs

Fig. 7 shows the inhibitory action of a variety of antibiotics and antimicrobial drugs. The actions of penicillin and chloramphenicol have been reported previously (Gale & Folkes, 1955*a*) and are included for purposes of comparison; together with puromycin they differ from other inhibitors in that inhibition reaches a plateau value which is much less than 100%. Of the substances tested penicillin is the most potent inhibitor at low concentrations, but even so this antibiotic is less effective than DCDNS. Ethidium bromide (2:7-diamino-10-ethyl-9-phenylphenanthridinium bromide), an antiprotozoal drug (Watkins & Woolfe, 1952), is an inhibitor of the same order of activity as Terramycin. Substances which had no significant effect at concentrations of 0.001 M or less included streptomycin, suramin and isoniazid.

Inhibition of incorporation of glycine in the presence of other amino acids (condition 2)

In Table 7 are compared the degrees of inhibition produced by a variety of inhibitors tested on the incorporation of glycine under condition 1 (glycine

Fig. 5. Effect of 5:6-dimethylbenzimidazole and 6-amino-4-hydroxybenzotriazole on the incorporation of glycine by disrupted staphylococcal cells incubated under condition 1. Conditions were as for Fig. 1 with additions as follows: curve 1, no addition; curve 2, mM-5:6-dimethylbenzimidazole; curve 3, mM-6-amino-4-hydroxybenzotriazole; curve 4, mM-5:6-dimethylbenzimidazole + mM-6-amino-4-hydroxybenzotriazole.

Fig. 6. Inhibition of glycine incorporation by analogues of benzimidazole and diaminodimethylbenzene. Conditions were as for Fig. 1; incorporation was estimated after 90 min. incubation in presence and absence of inhibitor. Radioactivity was estimated on TCA precipitate and inhibition expressed as percentage rate of incorporation in absence of inhibitor; the results show two experiments: control for nos. 18, 27, 56, 59 = 2963 counts/min./mg. of preparation, for nos. 27, 52, 53, 54, 55 = 2054 counts/min./mg. of preparation. Inhibitors: 52, *o*-aminophenol; 27, 5:6-dichlorobenzimidazole; 53, 4:5-diamino-1:2-dichlorobenzene; 54, 2-amino-4:5-dimethylphenol; 18, 1-allyl-5:6-dimethylbenzimidazole; 59, 1:2-dichloro-4-(*p*-nitrobenzenesulphonamido)benzene; 55, 2-bromo-4:5-dichlorophenol; 56, DCDNS.

being the only amino acid present in the incubation medium) and condition 2 in the presence of a complete mixture of amino acids (Gale & Folkes, 1955*a*). In general there is little difference in the degree of inhibition produced under the two conditions, although 5:6-dimethylbenzimidazole and its 1-allyl derivative are less effective under condition 2 than under condition 1. Oxine is less effective at 10^{-5} M than at 10^{-3} M under condition 2 whereas, as shown above, the lower concentration is the more

effective under condition 1; this is compatible with the suggestion that the toxic substance is a metal complex, as the amount of free metal ion in the medium would be reduced by the presence of amino acids under condition 2.

Antagonism of inhibitors by 6-amino-4-hydroxybenzotriazole

Since the inhibitory benzimidazole derivatives were found to be antagonized by 6-amino-4-hydroxybenzotriazole (see Table 6), the action of the latter substance has been tested in the presence of some of the other substances found to be inhibitors of the incorporation of glycine. Table 8 lists the results and shows that 6-amino-4-hydroxybenzotriazole antagonizes the action of 2-amino-4:5-dimethylphenol but not that of 4:5-diamino-1:2-dichlorobenzene or 2-bromo-4:5-dichlorophenol. The interesting antagonism is that of oxine, whose inhibitory action at concentrations of 10^{-5} M (giving maximal inhibition, see Fig. 1) or less is largely abolished by 0.001 M 6-amino-4-hydroxybenzotriazole. There may be some antagonism of ethidium bromide at low concentrations but no significant antagonism of the antibiotics tested was obtained.

Action of inhibitors on the incorporation of other amino acids

Table 9 summarizes the action of some of the inhibitors on the incorporation of other amino acids each tested under condition 1. The general picture is the same in all cases tested, although there are variations in the degree of sensitivity. It

Fig. 7. Inhibition of incorporation of glycine by antibiotics (a) and other antimicrobial substances (b). Conditions were as for Fig. 1; incubation was for 90 min.; estimations were on the TCA precipitate. Results are expressed as percentage inhibition of rate of control without inhibitor. Value of control = 3379 (a), 2859 (b) counts/min./mg. of preparation. (a) ▲, Puromycin; △, chloramphenicol; ●—●, penicillin; ○, Terramycin; ×, aureomycin; ····, DCDNS. (b) ⊙, Antrycide; △, proflavine; ○, propamidine; ▲, acriflavine; ×, pentamidine; ●, ethidium bromide.

Table 7. *Action of inhibitors on the incorporation of glycine by disrupted staphylococcal cells incubated under conditions 1 and 2*

Conditions were as for Table 1: condition 1 was with glycine as sole amino acid present; condition 2 was in the presence of a complete mixture of naturally occurring amino acids, each component at a concentration of 0.4 mg. of L-isomer/3.0 ml., only glycine being labelled with ^{14}C . Estimations were made on the TCA precipitate; controls without inhibitor = 3123 (condition 1), 989 (condition 2) counts/min./mg. of preparation.

Inhibitor	Concn. (mM)	Percentage inhibition	
		Condition 1	Condition 2
5:6-Dimethylbenzimidazole	3.0	90	52
	1.0	65	11
1-Allyl-5:6-dimethylbenzimidazole	0.05	50	8
5:6-Dimethyl-1-β-D-ribofuranosylbenzimidazole	1.0	0	0
4:5:6-Trichlorobenzimidazole	0.3	49	44
4:5:6-Trichloro-1-β-D-ribofuranosylbenzimidazole	0.3	53	23
4:5:6-Trichloro-1-α-D-ribofuranosylbenzimidazole	0.3	48	65
4-Methoxy-6-nitrobenzotriazole	0.1	52	36
Ethidium bromide	1.0	97	98
	0.1	43	71
Oxine	1.0	70	63
	0.01	95	0
DCDNS	0.001	66	68

Table 8. *Antagonism of inhibitors by 6-amino-4-hydroxybenzotriazole*

Conditions were as for Table 1. Results were collected from seven experiments. Mean value of controls = 3190 counts/min./mg. of preparation (estimations on TCA precipitates); range 2631-3277.

Inhibitor	Concn. (mm)	Percentage inhibition in presence of 6-amino-4-hydroxybenzotriazole at final concn.				Percentage antagonism (1.0 mm)
		0	0.1 mm	0.3 mm	1.0 mm	
(18) 1-Allyl-5:6-dimethylbenzimidazole	0.1	57	23	17	0	100
(1) 5:6-Dimethylbenzimidazole	1.0	76	61	12	4	95
(54) 2-Amino-4:5-dimethylphenol	0.1	56	25	15	14	75
(20) 4-Methoxy-6-nitrobenzotriazole	0.1	42	32	31	27	45
(55) 2-Bromo-4:5-dichlorophenol	0.01	86	80	80	76	11
(53) 4:5-Diamino-1:2-dichlorobenzene	1.0	35	40	40	45	0
Oxine	0.1	82	81	81	80	0
	0.01	86	75	65	31	64
	0.003	74	—	18	9	88
(57) Ethidium bromide	0.3	89	90	91	91	0
	0.1	30	28	24	23	23
Aureomycin	0.03	67	71	66	60	10
Penicillin	0.01	48	47	44	43	10
Chloramphenicol	0.1	40	39	39	43	0
(58) Aminopterin	3.0	38	—	42	45	0
(56) DCDNS	0.001	36	—	44	55	0

Table 9. *Inhibition of incorporation of various amino acids tested under condition 1*

Conditions were as for Table 1 except that glycine was substituted by one other amino acid as below. Estimations were made on the TCA precipitate; results are expressed as percentage inhibition of control without inhibitor, shown in final column.

Amino acid incorporated	Inhibitor					Incorporation in control (counts/min./mg. of prep.)
	5:6-Dimethyl- benzimidazole 1.0 mm	1-Allyl- 5:6-dimethyl- benzimidazole 0.1 mm	4-Methoxy- 6-nitrobenzo- triazole 0.1 mm	DCDNS μ M	Oxine 0.01 mm	
Glycine	70	70	57	67	70	2862
Aspartic acid	22	49	15	—	73	1867
Glutamic acid	40	48	72	65	76	3832
Lysine	38	56	27	—	—	1562
Leucine	44	28	10	36	40	1956
Arginine	25	44	40	—	48	750
Phenylalanine	33	46	41	—	—	1045
Isoleucine	0	0	24	41	—	1362
Valine	0	6	0	25	—	501
	3.3 mm	1.0 mm	0.2 mm	0.01 mm	—	
Valine	54	84	21	85	—	501

has been shown before (Gale & Folkes, 1955a) that the sensitivity of incorporation reactions to penicillin and chloramphenicol varies widely with the amino acid studied; there is no evidence of similar variations with the inhibitors listed in Table 9. The incorporation of valine and isoleucine is less sensitive than that of the other amino acids tested, but the bottom line of Table 9 shows that, at least for valine, this is again a question of degree rather than an absolute difference in sensitivity. 6-Amino-4-hydroxybenzotriazole at a concentration of 0.3 mm stimulated the incorporation of all the amino acids tested, the degree of stimulation varying between 10 and 60 % of the control.

DISCUSSION

Benzimidazole appears to act as an inhibitor of growth by competitive inhibition of purine metabolism (Woolley, 1944); Tamm (1954, 1955, 1956) found that the derivatives most effective in inhibiting the growth of influenza virus are those related to the purine nucleosides. Thus 1- β -ribofuranosyl-4:5:6-trichlorobenzimidazole is some 15 times as inhibitory as trichlorobenzimidazole itself, whereas the 1- α -ribofuranosyl derivative is only 3 times as effective as the base. These results differ from those in the present studies in that the ribofuranosyl derivatives are only slightly more

effective than the trichloro derivative in inhibiting the incorporation of glycine. A further difference appears in the 5:6-dichloro derivatives: 1- β -ribofuranosyl-5:6-dichlorobenzimidazole is approximately 7 times as effective as the base in inhibiting the multiplication of influenza virus but has only a small fraction of the activity of the base in inhibiting the incorporation of glycine. It would appear from these differences that the benzimidazole derivatives are affecting different mechanisms in the two systems; certainly the purines are not effective antagonists of the inhibition of the incorporation of glycine by benzimidazole (see Table 6). It may be that the component of the incorporation mechanism which is inhibited by benzimidazole derivatives is not a purine or purine nucleoside. If we take into account the general principles that have been evolved as a result of studies of antimetabolites (Woolley, 1952*b*; Kodicek 1949) we can deduce that the active component must bear a structural relationship to benzimidazole and that positions 4, 5 and 6 are important in determining its activity. Position 1 is clearly important also since substitution with α -ribofuranosyl, glucopyranosyl or dihydroxypropyl in that position abolishes, whereas substitution with allyl increases, the inhibitory activity of 5:6-dimethylbenzimidazole.

It is of interest that 6-amino-4-hydroxy derivatives of either benzimidazole or benzotriazole stimulate incorporation and antagonize the inhibitory action of other benzimidazole derivatives and related structures. The same structural unit is found in oxine, which is a potent inhibitor in media containing heavy metals. It seems improbable that the action of oxine is due simply to its action as a chelating agent since, on the one hand, it is antagonized by 6-amino-4-hydroxybenzotriazole whereas, on the other, EDTA is without inhibitory effect. It may be that the structure

present in many of the active substances described here, represents an active centre in the incorporation mechanism. Such structures form chelate complexes, and the potency of oxine chelates may be due to their structural resemblance to, and consequent competition with, the natural component of the system. The necessity for a metal component is consistent, again, with the demonstration by Saz & Slie (1954*a, b*) and Saz & Martinez (1956) that the inhibitory action of aureomycin can be ascribed to inactivation of metal.

SUMMARY

1. The incorporation of glycine by disrupted staphylococcal cells, incubated with a source of energy and [14 C]glycine only, is inhibited by 8-hydroxyquinoline (oxine) but not by ethylenediaminetetra-acetic acid. The maximal inhibitory concentration of oxine is 10^{-5} M, higher concentrations being less inhibitory. This concentration abolishes the stimulation of incorporation produced by the addition of nucleic acid to the nucleic acid-depleted preparation.

2. Oxine is not inhibitory in media from which heavy metals have been removed.

3. Benzimidazole is a feeble inhibitor whose activity is markedly increased by substitution with CH_3^- or Cl^- in positions 4, 5 and 6. The inhibitory activity of 5:6-dimethylbenzimidazole is increased by substitution with an allyl group in position 1, but similar substitution with β -D-ribofuranosyl, β -D-glucopyranosyl or 2:3-dihydroxypropyl groups abolishes inhibitory activity.

4. 6-Amino-4-hydroxybenzimidazole and 6-amino-4-hydroxybenzotriazole stimulate the incorporation of glycine and antagonize the inhibition by 5:6-dimethylbenzimidazole, 1-allyl-5:6-dimethylbenzimidazole and oxine.

5. Analogues of 4:5-diamino-1:2-dimethylbenzene are inhibitors of the incorporation of glycine. The most potent inhibitor found is 1:2-dichloro-5-nitro-4-(*p*-nitrobenzenesulphonamido)benzene.

6. Aureomycin, Terramycin, penicillin, ethidium bromide, pentamidine and acriflavin inhibit the incorporation of glycine; streptomycin, suramin and isoaniazid are among inactive substances tested.

REFERENCES

- Albert, A., Gibson, M. I. & Rubbo, S. D. (1953). *Brit. J. exp. Path.* **34**, 119.
 Gale, E. F. (1949). *J. gen. Microbiol.* **3**, 369.
 Gale, E. F. (1957). *Harvey Lect.* **51**, 25.
 Gale, E. F. & Folkes, J. P. (1955*a*). *Biochem. J.* **59**, 661.
 Gale, E. F. & Folkes, J. P. (1955*b*). *Biochem. J.* **59**, 675.
 Gillespie, H. B., Engelman, M. & Graff, S. (1954). *J. Amer. chem. Soc.* **76**, 3531.
 Gillespie, H. B., Engelman, M. & Graff, S. (1956). *J. Amer. chem. Soc.* **78**, 1651, 2445.
 Kodicek, E. (1949). *Brit. J. Nutr.* **2**, 373.
 Rowlands, D. A., Gale, E. F., Folkes, J. P. & Marrian, D. H. (1957). *Biochem. J.* **65**, 519.
 Saz, A. K. & Martinez, L. M. (1956). *J. biol. Chem.* **223**, 285.
 Saz, A. K. & Slie, R. B. (1954*a*). *J. biol. Chem.* **210**, 407.
 Saz, A. K. & Slie, R. B. (1954*b*). *Arch. Biochem. Biophys.* **51**, 5.
 Tamm, I. (1954). *Science*, **120**, 847.
 Tamm, I. (1955). *Bull. N.Y. Acad. Med.* **31**, 537.

- Tamm, I. (1956). *J. Bact.* **72**, 43.
Taylor, E. S. & Gale, E. F. (1945). *Biochem. J.* **39**, 52.
Waring, W. S. & Werkman, C. H. (1942). *Arch. Biochem.* **1**, 303.
Watkins, T. I. & Woolfe, G. (1952). *Nature, Lond.*, **169**, 506.
Webster, G. C. & Johnson, M. P. (1955). *J. biol. Chem.* **217**, 641.
Woolley, D. W. (1944). *J. biol. Chem.* **152**, 225.
Woolley, D. W. (1950). *Proc. Soc. exp. Biol., N.Y.*, **75**, 745.
Woolley, D. W. (1951). *J. exp. Med.* **93**, 13.
Woolley, D. W. (1952a). *J. Amer. chem. Soc.* **74**, 5450.
Woolley, D. W. (1952b). *A Study of Antimetabolites*. New York: John Wiley and Sons.
Woolley, D. W. & Pringle, A. (1952). *J. biol. Chem.* **194**, 729.
Woolley, D. W. & Schaffner, G. (1954). *Cancer Res.* **14**, 802.

The Solubility and Fractionation of β_1 -Lactoglobulin

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β -Lactoglobulin is an easily accessible crystalline protein and has been used in many experiments, often with the assumption that it is homogeneous. Since Li (1946) demonstrated electrophoretic heterogeneity, it has been firmly established that it is a mixture, and attempts at fractionation have been made (Polis, Schmukler, Custer & McMeekin, 1950; Preaux, Hulsmans & Lontie, 1954; Smithies, 1954). More recently it has been found that it is a member of the rapidly increasing number of proteins known to be under direct genetic control. Aschaffenburg & Drewry (1955) showed that some cows produced two lactoglobulins, β_1 - and β_2 -, some only β_1 - and some only β_2 -. β_1 -Lactoglobulin was the faster in paper electrophoresis at pH 8.6. This made all work carried out on mixed milk preparations (the great majority) of uncertain validity. Ogston & Tombs (1957) studied the separate proteins, obtained from appropriate cows, and found that the titration curves and ultraviolet spectra were different, though the molecular weights were very similar. Green, North & Aschaffenburg (1956) had reached the latter conclusion from X-ray studies. However, the electrophoretic boundaries obtained with both separate proteins at pH 4.66 were complex, the feature which initially led to doubts on the homogeneity of mixed preparations, and leads equally to doubts of the homogeneity of the individual proteins.

Among previous workers only Ogston & Tilley (1955) made efforts to ensure that milk from single cows was used for their preparations, and they were able to distinguish 'normal' and 'abnormal' types of lactoglobulin. The 'normal' type (identified as β_1 -lactoglobulin by Ogston & Tombs, 1957) gave a boundary pattern indicating two components on U-tube electrophoresis at pH 4.66, while the proportion of slow component varied with protein concentration down to a limit of 20% at high concentrations. To account for this they supposed that this

material consists of two proteins: one, about 80% of the whole, polymerizes with increasing concentration; the other, about 20% of the whole, migrates slowly and does not polymerize. The heterogeneity of β_1 -lactoglobulin has now been investigated further by means of solubility tests.

EXPERIMENTAL

Typing of cows and preparation of β_1 -lactoglobulin. This was by the method of Aschaffenburg & Drewry (1957), slightly modified as described by Ogston & Tombs (1957), and the samples of β_1 -lactoglobulin, two in all, were the same as used by them.

Concentration. Concentrations (g./100 ml. of solution) were found with a refractometer (Cecil & Ogston, 1951). The specific refractive increment used was 1.8×10^{-3} in all cases.

U-tube electrophoresis. A Hilger Tiselius apparatus was used as described by Ogston & Tilley (1955). The 3.5 ml. cell was used with a bath temperature of 1°. The current was always 10 ma at about 400 v. Boundaries were divided to estimate 'fast' and 'slow' components according to the method of Ogston & Tilley (1955).

Ultraviolet absorption. This was determined in a Hilger Uvispek spectrophotometer with matched 1 cm. cells of 3 ml. capacity.

pH measurements. These were made at room temperature with a Cambridge Instrument Co. glass electrode and valve voltmeter calibrated against potassium hydrogen phthalate, pH 4.0.

Solubility tests

Variable-solvent solubility curves. The standard solvent system was similar to that used by Smithies (1954). The solutions were made from A.R. reagents and glass-distilled water. The acetate buffer, pH 4.95, contained 1.6 M-sodium acetate-1.06 M-acetic acid. Ammonium sulphate solutions in the range 2.53-3.2 M were made up by diluting a 3.2 M-standard solution (422.8 g./l.). Wet protein crystals (which had been dialysed against water) were separated by centrifuging and dissolved in 1.6 M-sodium chloride. The solution was then dialysed against 1.6 M-sodium chloride for at least 24 hr. Protein concentrations of about 1% (w/v) were suitable for all these experiments. Acetate buffer, pH 4.95,