Table 1. *Bacteriostatic power of geodin against nineteen Gram-positive and two Gram-negative strains in glucose broth*

Micro-organism	Catalogue No. (National Collection of Type Cultures) *or* London School of Hygiene and Tropical Medicine	Limiting dilution for complete inhibition of growth
Bacterium dysenteriae Shiga	N.C.T.C. 4837	1,000
Vibrio cholerae	N.C.T.C. 1633	2,000 (in plain broth)
Mycobacterium smegmatis	N.C.T.C. 523	2,000
Staphylococcus aureus	N.C.T.C. 4736	4,000
Staph. aureus	N.C.T.C. 3093	8,000
Staph. albus	N.C.T.C. 3256	8,000
Streptococcus viridans	N.C.T.C. 3166	8,000
Myco. smegmatis	N.C.T.C. 523	8,000 (in nutrient agar)
Staph. aureus	N.C.T.C. 4163	16,000
Mycobacterium sp. (saprophytic)	N.C.T.C. 510	16,000
Staph. aureus	N.C.T.C. 3750	32,000
Bacillus subtilis	L.S.H.	32,000
B. anthracis	L.S.H.	32,000
Corynebacterium diphtheriae gravis	L.S.H.	32,000
C. diphtheriae intermedius	L.S.H.	32,000
Staph. aureus	L.S.H.	64,000
Staph. aureus	N.C.T.C. 3761	64,000
Staph. aureus	N.C.T.C. 3095	64,000
C. diphtheriae mitis	L.S.H.	64,000
Staph. citreus	N.C.T.C. 2301	128,000
Strep. pyogenes Griffith type I	N.C.T.C. 2432	128,000
B. anthracis	N.C.T.C. 5444	128,000

Studies on Antibiotics*

2. BACTERIOLOGICAL ACTIVITY AND POSSIBLE MODE OF ACTION OF CERTAIN NON-NITROGENOUS NATURAL AND SYNTHETIC ANTIBIOTICS

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In our search for new antimicrobial agents of natural origin, Dr R. Klein, in our laboratories, found some time ago that the crude culture fluid of *Aspergillus terreus*, strain N.C.T.C. 3911 (Ac 100), possessed a far higher antibiotic activity than could be accounted for by its known content of citrinin (Raistrick & Smith, 1935; 1936). We succeeded in isolating in addition to the latter substance a crystalline, faintly yellow compound which had marked antibiotic activity; this compound was examined chemically by Mr C. H. Hassall and Prof. A. R. Todd of Cambridge University who identified it as geodin, $C_{17}H_{12}O_7Cl_2$, previously isolated from another organism by Raistrick & Smith (1936).

While still engaged on the elucidation of the structure of clavatin or patulin (Bergel, Morrison, Moss & Rinderknecht, 1944; cf. also Raistrick, 1943) we also synthesized a number of compounds possessing some of the structural features of penicillic acid (Birkinshaw, Oxford & Raistrick, 1936) and of the clavatin molecule, which we knew at the time to be an unsaturated keto lactone.

In the following we present the preliminary results of the bacteriological tests and experimental evidence for a possible mode of action of these substances. Numbers following compounds refer to Table 2, where formulae are given.

* Part I, see *J. chem. Soc.* (1944), p. 415.

METHODS AND EXPERIMENTAL

Natural compounds (mycological investigations by R. Klein)

Penicillic acid and clavatin (nos. 1 and 2) were prepared as described by Birkinshaw *et al.* (1936) and Bergel, Morrison, Moss, Klein, Rindernecht & Ward (1943) respectively. For the preparation of geodin (no. 3) from *Aspergillus*

terreus N.C.T.C. 3911 (L.S.H.T.M. Ac 100) cultural conditions were similar to those given by Raistrick & Smith (1935), with the following differences. Stock cultures were kept on Czapek-Dox agar with 1·5% glucose and 2% agar; when these slopes were about 8–14 days old, the spores of one tube were suspended in 10 ml. sterile water. This suspension was used for the inoculation of two 1 l. Erlenmeyer flasks, each containing 50 ml. Czapek-Dox agar with 1·5% glucose and 1% agar. One Erlenmeyer flask culture was used for the inoculation of *c.* fifty 40 oz. square bottles when about 8–14 days old, each bottle containing *c.* 300 ml. Czapek-Dox solution as used by Raistrick & Smith (1935). A spore suspension was made by adding aseptically 250 ml. sterile water to the Erlenmeyer flask and by shaking vigorously for a few minutes. Of this suspension, *c.* 5 ml. were transferred aseptically to each bottle. The bottles were placed on the flat side and kept at 26–27° for 18–20 days. During this time folded mycelial mats with cinnamon spores and yellow transpiration drops were formed on the surfaces of the medium. Samples for activity assays of the culture liquid were taken at regular intervals, and when a satisfactory activity was reached, the liquid was harvested by draining it off the mats.

3 l. of culture fluid were adjusted to pH 6·6 and stirred vigorously with 31 g. of activated charcoal for ½ hr. The charcoal was then filtered off, dried in a desiccator and extracted with three portions of 400 ml. of methanol. The methanol extract was evaporated to a small volume, from which crude geodin crystallized out. Yield: 0·7 g., m.p. 212–213°. It was recrystallized from methanol or aqueous acetone, m.p. 227–230°. (Found: C, 51·2; H, 3·2. Calc. for $C_{17}H_{12}O_7Cl_2$: C, 51·1; H, 3·0%.)

Synthetic compounds

Ethyl Δ^{δ}-α:γ-*diketo*-δ-*methylhexenoate* (no. 4) (prepared by A. R. Moss). Sodium (3·56 g.) was dissolved in 100 ml. ethanol and the excess solvent removed by distillation under reduced pressure. The last traces of ethanol were removed by heating in an oil bath at 180°. Nitrogen was then admitted to the white dry sodium ethoxide and a solution of 22·6 g. of dimethyl oxalate in 130 ml. dry ether was added with ice cooling. Methyl *iso*propenyl ketone (13 g.) was then added over 10 min. and the reaction mixture was allowed to stand overnight. After addition of 100 ml. of dry ether, the yellow sodium derivative of ethyl Δ^{δ}-α:γ-diketo-δ-methylhexenoate was filtered off and kept in a desiccator over P_2O_5. The free ester was obtained by acidification of an aqueous solution of the sodium derivative and extraction with ether. After washing, drying and evaporation of the extract, a mobile oil was obtained which boiled at 71°/0·05 mm. and partly polymerized on distillation. The copper derivative containing 1 molecule of water of crystallization melted at 120–121°, and the anhydrous material at 131–133°, after recrystallization from benzene/light petroleum (b.p. 60–80°). (Found: C, 50·0; H, 4·9. $(C_9H_{11}O_4)_2Cu$ requires C, 50·2; H, 5·1%.)

The sodium enolate of ethyl Δ^{δ}-α:γ-diketo-ε-methylheptenoate (no. 5) (ethyl α-mesityloxido oxalate) was prepared according to Claisen (1896) (cf. also Borsche & Thiele, 1923).

The sodium enolate of ethylacetylpyruvate (no. 6) and that of ethyl α:γ-diketo-δ-dimethylhexanoate (no. 7) were made according to Claisen & Stylos (1887) and Couturier (1910) respectively.

Ethyl benzoylpyruvate (no. 8) and the corresponding p-*chloro compound* (no. 9), m.p. 65° (found: Cl, 13·7. $C_{12}H_{11}O_4Cl$ requires Cl, 13·8%), were prepared according to Beyer & Claisen (1887).

α-Keto-β-benzoyl-γ-phenylbutyrolactone (no. 10) was prepared according to Knoevenagel (1894).

α-*Keto*-β-*benzoyl*-γ-*methylbutyrolactone* (no. 11). A mixture of ethyl benzoylpyruvate (2·2 g.), paraldehyde (0·45 g.), and benzene (1 ml.) was cooled and saturated with HCl. After standing for 2 days at room temperature the mixture, which had partly solidified, was filtered. The crude reaction product was recrystallized from benzene/light petroleum (b.p. 60–80°). It melted at 130–132° and was a colourless substance soluble in sodium carbonate solution. (Found: C, 66·0; H, 4·9. $C_{12}H_{10}O_4$ requires C, 66·1; H, 4·6%.)

The sodium salt of 5-phenylpyrazole-3-carboxylic acid (no. 12), a derivative of benzoylpyruvic acid, was made according to Bülow (1904).

Δ^{δ}-α:γ-*Diketo*-δ-*methylhexenoic acid* (no. 13) (prepared by A. R. Moss). The ester (no. 4) (21·55 g.) was added to a solution of 5·12 g. of sodium in 35 ml. of ethanol and 60 ml. of water cooled in ice. The mixture was then allowed to stand at room temperature for ½ hr. A cold solution of 7·5 ml. conc. H_2SO_4 in 120 ml. of water was then added. The solid which separated was filtered and washed with a small quantity of ice-water. Crude acid thus obtained was dissolved in carbon tetrachloride from which it crystallized on cooling as a pale yellow substance, melting at 104–107°. Sublimation at 60–70°/0·1 mm. of a sample yielded colourless crystals of m.p. 106–108°. (Found: C, 54·0; H, 5·1. $C_7H_8O_4$ requires C, 53·8; H, 5·1%.)

Chloralide of α:γ-*diketo*-δ:δ-*dimethylhexenoic acid* (no. 14). Ethyl α:γ-diketo-δ:δ-dimethylhexenoate (no. 7) (2 g.) and chloral (4·05 g.) were heated in a sealed tube at 120° for 3 hr. The product thus obtained was triturated with water. The residual oil was distilled at 14 mm. pressure. Some unchanged ethyl α:γ-diketo-δ:δ-dimethylhexenoate was recovered. The solid residue in the distillation flask was recrystallized from benzene/light petroleum (60–80°). The colourless material melted at 156–158°. (Found: C, 40·3; H, 4·3; Cl, 35·2. $C_{10}H_{11}O_4Cl_3$ requires C, 40·0; H, 3·7; Cl, 35·2%.)

The chloralide of benzoylpyruvic acid (no. 15) and the corresponding p-*chloro compound* (no. 16), m.p. 185–187°. (Found: Cl, 39·6. $C_{12}H_6O_4Cl_4$ requires Cl, 39·9%) were prepared according to Schiff (1898).

Benzoylacrylic acid (no. 17) was synthesized according to von Pechmann (1882).

p-*Chlorobenzoylacrylic acid* (no. 18) (cf. Kohler & Woodward, 1936). Aluminium chloride (56 g.) was added in portions to a mixture of 19·6 g. of maleic anhydride, 22·4 g. of chlorobenzene and 50 ml. of carbon disulphide. The mixture was gradually heated to the boiling-point and refluxed for 3 hr. The solvent was decanted and the residue decomposed with ice and HCl. The crude *p*-chlorobenzoylacrylic acid thus obtained was recrystallized from benzene. The lemon-yellow crystals had m.p. 157–158°. (Found: Cl, 16·6. $C_{10}H_7O_3Cl$ requires Cl, 16·7%.)

2:4-*Dichlorobenzoylacrylic acid* (no. 19). Powdered aluminium chloride (56 g.) was added in portions to a mixture of 19·6 g. of maleic anhydride, 30 g. of 1:3-dichlorobenzene and 50 ml. of carbon disulphide. After refluxing

for 6 hr., the reaction mixture was decomposed with ice and HCl. The mixture was then extracted with ether. The ether was washed with water, dried and evaporated. The residue was triturated with benzene. 2:4-Dichlorobenzoylacrylic acid crystallized from the benzene extract on concentrating. Recrystallization from chloroform gave a yellow crystalline material of m.p. 191–193°. (Found: Cl, 29·0. $C_{10}H_6O_3Cl_2$ requires Cl, 29·0%.)

The enol lactone of 6:6-dimethyl-3-benzoyltetrahydro-γ-pyrone-2-carboxylic acid (no. 20) was prepared from ethyl α-mesityloxidooxalate according to Meyer (1913). Its constitution will be discussed later.

Anhydro-6-methyl-3-hydroxybenzylidene tetrahydropyrone-2-carboxylic acid (no. 21). (*a*) *Ethyl* Δ^{δ}-α:γ-*diketoheptenoate*. Sodium ethoxide (0·68 g.) was suspended in dry ether (10 ml.). After cooling to −15°, ethyl oxalate (1·48 g.) was added and then ethylidene acetone (0·84 g.) with continuous stirring. After standing for 2 days at room temperature, the sodium derivative was filtered and dried over P_2O_5. The copper salt which was prepared by treating the aqueous solution of the sodium derivative with copper acetate melted at 156–160° (decomp.) after recrystallization from light petroleum (b.p. 100–120°). (Found: C, 50·3; H, 5·3. $(C_9H_{11}O_4)_2Cu$ requires C, 50·2; H, 5·1%.)

(*b*) *No.* 21. The enol lactone of 6-methyl-3-benzoyltetrahydro-γ-pyrone-2-carboxylic acid was obtained by shaking an ethereal solution of its copper derivative with dilute ice-cold H_2SO_4, washing, drying and evaporating the ether. This material (1·3 g.) was mixed with 1·0 g. of benzaldehyde and two drops of piperidine. After standing for several weeks the reaction mixture had solidified. The crystalline material was recrystallized from benzene/light petroleum (b.p. 60–80°) and subsequently from methanol. The colourless product melted at 176–177°. (Found: C, 68·6; H, 5·0. $C_{14}H_{12}O_4$ requires C, 68·8; H, 4·9%.)

Bacteriological examination

Geodin (Table 1) was prepared as a 1/300 ethanolic solution with a trace of dilute ammonia. All other substances were tested in aqueous solutions. Serial dilutions were set up in 2% glucose-broth (pH 6·9). One loop of a 1/100 dilution of a 24 hr. culture was used as inoculum; one loop of undiluted culture was used for *Mycobacterium smegmatis*. Incubation was carried out at 37° and growth was observed after 18 hr. and, in the case of geodin, also after 2 days.

RESULTS AND DISCUSSION

Table 1 gives the results of the preliminary bacteriological investigation on geodin. It is interesting to note that in contrast to clavatin and penicillic acid, geodin possesses relatively little activity against Gram-negative organisms and may thus be classed with some of our synthetic inhibitors. Table 2 contains bacteriological data on our synthetic substances and results of our investigation on the mechanism of action of these and of some natural antibiotics.

In our synthetic approach to this problem we chose penicillic acid (no. 1) as a model and first prepared ethyl Δ^{δ}-α:γ-diketo-δ-methylhexenoate (no. 4) which shows a structural resemblance to the latter and was found to possess bacteriostatic activity when tested as the sodium salt of the enolic form. Replacing the *iso*propenyl radicle in no. 4 by other groups, we found that in order to produce substances with antibiotic activity the δ-carbon atom must not carry a hydrogen atom. Thus the sodium enolates of

Table 1. *Effect of geodin on bacterial growth*

Organism tested	Highest dilution of geodin giving complete inhibition of growth for: 18 hr.	2 days
Staphylococcus aureus N.C.T.C.* 4163	1 : 2,000,000	1 : 1,000,000
Staph. aureus N.C.T.C. 4163 (in 10% serum)	1 : 80,000	1 : 10,000
Bacillus subtilis N.C.T.C. 85	1 : 1,000,000	1 : 1,000,000
Streptococcus pyogenes (Richards) (in 10% blood)	1 : 40,000	—
Pseudomonas fluorescens	1 : 20,000	1 : 10,000
Esch. coli N.C.T.C. 86	1 : 40,000	1 : 20,000
Mycobacterium smegmatis	1 : 20,000	1 : 10,000

* National Collection of Type Cultures.

ethyl Δ^{δ}-α:γ-diketo-ε-methylheptenoate (no. 5); and ethyl α:γ-diketovalerate (ethyl acetylpyruvate; no. 6) were inactive while those of ethyl α:γ-diketo-δ:δ-dimethylhexanoate (no. 7) and ethyl α:γ-diketo-γ-phenylbutyrate (ethyl benzoylpyruvate; no. 8) were slightly active. On the other hand, in compounds such as α-keto-β-benzoyl-γ-phenylbutyrolactone (no. 10) and α-keto-β-benzoyl-γ-methylbutyrolactone (no. 11), where, in contrast to no. 1 and no. 4, both the α- and β-carbon atoms are fully substituted, no activity was found. No. 11 was prepared by condensation of benzoylpyruvic ester with acetaldehyde. Incorporation of the α:β double bond into a heterocyclic ring structure as in no. 12 likewise led to complete loss of activity. The third structural feature which appears to be essential for antibiotic activity is the presence of an α:β double bond as achieved in no. 1 by etherification of the β-enol and in no. 4 by the formation of a sodium derivative of the α-enol, the corresponding free diketo acid (no. 13) being inactive. When the α-enol structure was stabilized in another manner—namely by condensation with chloral as in the formation of β:β:β-trichloroethylidene ether ester of α-hydroxy-β-benzoylacrylic acid (no. 15) and β:β:β-dichloroethylidene ether ester of α-hydroxy-γ-keto-δ:δ-dimethylhexanoic acid (no. 14)—a striking increase in activity was observed amounting in no. 15 to about three times that of clavatin, as tested against *Staph. aureus*.

Table 2. *Compounds tested, and their effects on bacterial growth in the presence and absence of cysteine*

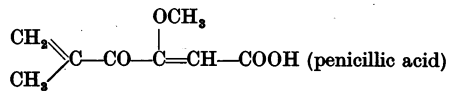
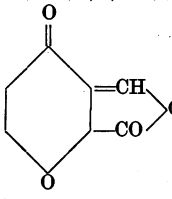
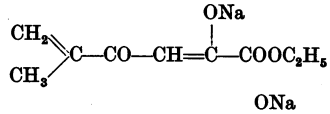
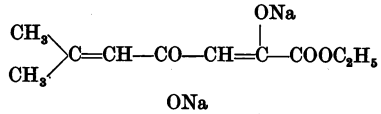
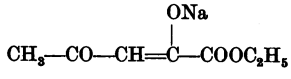
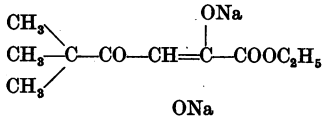
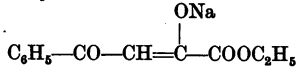
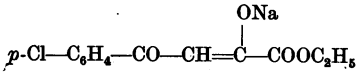
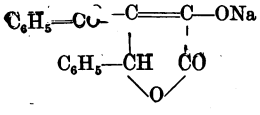
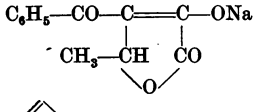
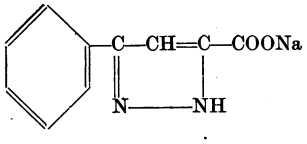
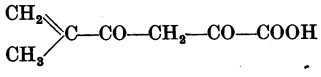
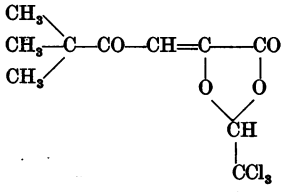
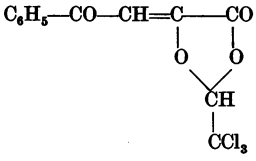
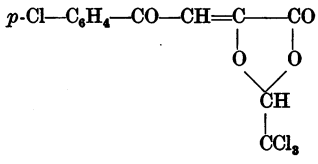
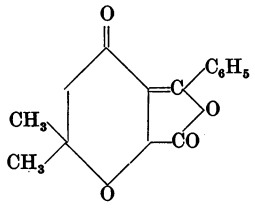
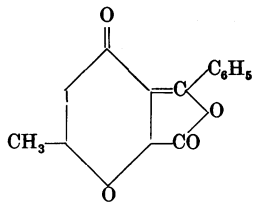
No. (see text)	Compound: Formula	Highest dilution giving complete inhibition of growth: Without cysteine — *Staph. aureus* N.C.T.C. 4163	Without cysteine — *Esch. coli* N.C.T.C. 86	With cysteine (1 : 1000, buffered at pH 6·8) — *Staph. aureus* N.C.T.C. 4163	With cysteine (1 : 1000, buffered at pH 6·8) — *Esch. coli* N.C.T.C. 86
1	 (penicillic acid)	1 : 16,000	1 : 25,000	1 : 1,000	1 : 1,000
2	 (clavatin, patulin)	1 : 100,000	1 : 200,000	<1 : 1,000	1 : 1,000
3	$C_{17}H_{12}O_7Cl_2$ (geodin)*	1 : 2,000,000	1 : 30,000	<1 : 32,000	<1 : 8,000
4		1 : 5,000	—	—	—
5		<1 : 1,000	—	—	—
6		<1 : 1,000	—	—	—
7		1 : 2,000	—	1 : 2,000	—
8		1 : 4,000	—	1 : 4,000	—
9		1 : 2,000	—	1 : 1,000	—
10		⩽1 : 1,000	—	—	—
11		<1 : 1,000	—	—	—
12		<1 : 100	—	—	—
13		1 : 1,000	—	—	—

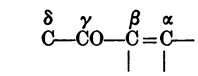
Table 2 (*cont.*)

No. (see text)	Compound: Formula	Highest dilution giving complete inhibition of growth: Without cysteine — *Staph. aureus* N.C.T.C. 4163	Without cysteine — *Esch. coli* N.C.T.C. 86	With cysteine (1 : 1000, buffered at pH 6·8) — *Staph. aureus* N.C.T.C. 4163	With cysteine (1 : 1000, buffered at pH 6·8) — *Esch. coli* N.C.T.C. 86
14		1: 24,000	—	—	—
15		1 : 300,000	<1 : 30,000	<1 : 30,000	<1 : 30,000
16		1 : 300,000	—	—	—
17	C_6H_5—CO—CH=CH—COOH	1 : 25,000	1 : 15,000	<1 : 1,000	<1 : 3,000
18	*p*-Cl—C_6H_4—CO—CH=CH—COOH	1 : 50,000	1 : 5,000	1 : 1,000	<1 : 1,000
19	2 : 4-Cl_2—C_6H_3—CO—CH=CH—COOH	1 : 80,000	1 : 5,000	1 : 1,000	<1 : 1,000
20		<1 : 1,000	<1 : 1,000	—	—
21		1 : 1,000	—	—	—

* On the constitution of geodin see Calam, Clutterbuck, Oxford & Raistrick (1947).

Finally when the enolic double bond in α:β-position was replaced by a simple ethylenic linkage, as in benzoylacrylic acid, substances of relatively high antibacterial activity were obtained (cf. nos. 17, 18, 19). The benzoylacrylic acids were obtained by condensation of maleic anhydride with the appropriate benzenes.

Summarizing, the following structure:



possessing a hydrogen atom either on the α or β carbon atom, may be regarded as being responsible for antibacterial activity. The same features are

present in the clavatin molecule (no. 2) with the exception of a complete substitution of the carbon atom corresponding to the δ-position of the above formula. It is noteworthy that here again (cf. nos. 10 and 11) lack of hydrogen on both carbon atoms of the double bond adjacent to the keto group leads to loss of activity, as observed with compounds nos. 20 and 21 and with *iso*clavatin (Puetzer, Nield & Barry, 1945).

The former substances were prepared by condensation of benzaldehyde with the enolates of ethyl Δ^{δ}-α:γ-diketo-ε-methylheptenoate and ethyl Δ^{δ}-α:γ-diketoheptenoate respectively. No. 20 has been erroneously described by Meyer (1913) as the lactone of γ-hydroxybenzyl-mesityloxidooxalic acid. However, its chemical behaviour which follows that of the cyclic form of β-mesityloxidooxalate very closely and recent work by Puetzer *et al.* (1945), on similar substances, leaves little room for doubt on the structure assigned to it in Table 2.

Effects of cysteine

Some years ago we found independently of Geiger & Conn (1945) that the antibacterial activities of clavatin (no. 2) and benzoylacrylic acid (no. 17) were inhibited by the presence of cysteine. We have subsequently confirmed cysteine-inactivation of penicillic acid and observed the same phenomenon with our synthetic compounds, nos. 15, 18 and 19, but not with the sodium enolates nos. 7, 8 and 9, which are all derivatives of pyruvic acid and have relatively low initial activities. It is noteworthy that geodin as well as our synthetic substances which are inactivated by cysteine possess comparatively low activity against *Esch. coli*, in contrast to the natural antibiotics clavatin and penicillic acid.

Geiger & Conn (1945) suggested that the mechanism of the antibiotic action of clavatin and penicillic acid may be an interaction of their α:β-unsaturated keto group with the sulphydryl group of an enzyme system vital for bacterial metabolism. They associate the order of antibiotic activity of the compounds included in their investigation with their efficacy in adding sulphydryl compounds like cysteine and thioglycollic acid. Our observations on the synthetic compounds, nos. 15, 16, 17, 18 and 19, support this hypothesis for α:β-unsaturated ketones possessing the essential features indicated in a preceding paragraph. It is significant, however, that the increase in antibiotic activity of our compound, no. 17 (benzoylacrylic acid), against Gram-positive organisms which follows the introduction of chlorine into its benzene nucleus is not accompanied by a parallel increase in activity against Gram-negative bacteria. This together with the fact that, in contrast to clavatin and penicillic acid, none of the synthetic substances and geodin possess activity against Gram-negative bacteria of the same order as that against Gram-positive organisms, indicates that some additional structural feature is involved in the antibacterial mechanism of clavatin and penicillic acid which is absent in the synthetic compounds as well as geodin (cf. Calam, Clutterbuck, Oxford & Raistrick, 1947). Cavallito & Haskell (1945) have obtained evidence that this may be the unsaturated lactone structure which is present in clavatin and potentially present in penicillic acid. The results of these authors and those obtained in this paper permit the tentative conclusion that the inhibition of Gram-negative organisms by α:β-unsaturated ketones of the type specified above involves an enzyme system containing SH groups different from that in Gram-positive bacteria, or that the enzyme system in Gram-negative bacteria is less accessible to the action of these compounds than is the case for Gram-positive organisms.

The mode of action of our substances, nos. 4, 7, 8 and 9 which are derivatives of pyruvic acid and are not inhibited by cysteine, seems to be entirely different.

SUMMARY

1. Geodin has been isolated from the culture medium of *Aspergillus terreus* N.C.T.C. 3911 and shown to possess antibiotic activity of a high order against Gram-positive bacteria.

2. The synthesis of certain substances chemically related to penicillic acid and clavatin are described and some features essential for antibiotic activity are pointed out.

3. The inhibitory effect of cysteine on these antibiotics has been investigated and its possible significance in relationship to the mode of action of these substances is discussed.

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The Importance of Folic Acid and Unidentified Members of the Vitamin B Complex in the Nutrition of Certain Insects

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In our work on the nutrition of insects evidence has been accumulating that growth on purified diets containing all the known vitamins of the B complex in pure substance is inferior to that on similar diets which contain yeast or yeast extracts. This was particularly noticeable in the genus *Ephestia* (Lepidoptera) where the caterpillars always grew much more slowly in the absence of yeast or yeast fractions (Fraenkel & Blewett, 1946*a*). From the effect of adding fractions resulting from a charcoal treatment of yeast extract, it seemed probable that the missing factor might be 'folic acid' and this was finally established by tests with synthetic folic acid (Lederle). An even more striking case of the need for folic acid was found in the mealworm, *Tenebrio molitor*, the growth rate of which on certain diets was vastly increased in the presence of folic acid. In our earlier work with another beetle, *Tribolium confusum*, it seemed that a mixture of all the then known vitamins of the B complex would sustain growth equally as well as yeast or a yeast extract (Fraenkel & Blewett, 1943*b*). This result has been difficult to repeat on several occasions but it has now been established that a folic acid deficiency can also be demonstrated in *Tribolium* if the casein in the diet is sufficiently purified.

'Folic acid' is used throughout this paper to signify an unspecified preparation showing folic acid activity, while folic acid designates synthetic folic acid (Lederle) (pteroylglutamate, Angier, Boothe, Hutchings, Movat, Semb, Stokstad, Subbarow, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels & Smith, 1946), which was formerly called *L. casei* factor. Some of the results have been briefly reported elsewhere (Fraenkel & Blewett, 1946*b*).

METHODS

The methods of breeding and of testing and preparing diets have been fully described (Fraenkel & Blewett, 1943*a*, *b*, 1946*a*). All tests were performed at 25° and 70% relative humidity. As a rule, 20 larvae were used in each test. All tests were started with newly hatched larvae and the criterion for the efficiency of a diet was either the length of the larval period (*Ephestia kuehniella*, *E. elutella*, *Tribolium confusum*), or the time taken until emergence of the adult (*Plodia interpunctella*), or growth expressed by the weight increase (*Tenebrio molitor*).

In most experiments the effect of the following three diets was compared:

(1) *Pure vitamins diet.* Casein (Glaxo Laboratories) 20, glucose anhydr. (British Drug Houses Ltd.) 80, McCollum's salt mixture (no. 185) 2, 'insoluble yeast' (yeast exhaustively extracted by boiling water) 2·5, cholesterol 1 and water 10 parts. To this mixture the following vitamins were added (expressed as μg./g. of dry diet): aneurin 25, riboflavin 12, nicotinic acid 25, pyridoxin 12, panthothenic acid 25, choline chloride 500, *i*-inositol 250. In tests with *Tribolium*, the diet consisted of 50 parts of casein and 50 parts of glucose. In tests with *Ephestia* and *Plodia*, 1 part of wheat-germ oil (Vitamins Ltd.) was added.

(2) *Yeast diet.* No. 1 with the addition of 1% dried brewer's yeast (Glaxo Laboratories).

(3) *Folic acid diet.* No. 1 with the addition of synthetic folic acid (Lederle).

The following preparations were also used:

(1) Yeast extract and yeast charcoal filtrate and eluate. Yeast extracts prepared from fresh brewers' yeast according to Chick & Roscoe (1930) or from dried brewers' yeast (Glaxo Laboratories) by a similar method, were added to the diets in quantities corresponding to 5% yeast. The charcoal treatment of yeast was carried out according to Hutchings, Bohonos & Peterson (1941) (adsorption at pH 3 with three successive portions of norite and elution of the norite adsorbates with ammoniacal ethanol).