Cavalieri, L., Patterson, D. B. & Carmack, M. (1945). *J. Amer. chem. Soc.* **67**, 1785.
Croft, P. E. & Richter, D. (1943). *J. Physiol.* **102**, 155.
Feldberg, W. & Vogt, M. (1948). *J. Physiol.* **107**, 372.
Homeyer, A. H., Whitmore, F. C. & Wallingford, V. H. (1933). *J. Amer. chem. Soc.* **55**, 4209.
Kappers, C. U. A., Huber, G. C. & Crosbie, E. C. (1936). *The Comparative Anatomy of the Nervous System of Vertebrates, including Man*, 2 vols. New York: The MacMillan Co.
Little, J. M. (1948*a*). *Amer. J. Physiol.* **153**, 436.
Little, J. M. (1948*b*). *Amer. J. Physiol.* **155**, 60.
Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.
Mounter, L. A. (1951). *Biochem. J.* **50**, 122.
Mounter, L. A. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 525.
Nachmansohn, D. (1939). *Bull. Soc. Chim. biol., Paris*, **21**, 761.
Nystrom, R. F. & Brown, W. G. (1947). *J. Amer. chem. Soc.* **69**, 2548.
Ord, M. G. & Thompson, R. H. S. (1952). *Biochem. J.* **51**, 245.
Paléus, S. (1947). *Arch. Biochem.* **12**, 153.
Pighini, G. (1938). *Biochim. Terap. sper.* **25**, 347.
Richter, D. & Croft, P. G. (1942). *Biochem. J.* **36**, 746.
Sturge, L. M. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 518.
Whittaker, V. P. (1949). *Biochem. J.* **44**, xlvi.
Whittaker, V. P. (1951). *Physiol. Rev.* **31**, 312.
Wideqvist, S. (1949). *Acta. chem. scand.* **3**, 303.
Zeller, E. A. (1949). *Helv. chim. acta*, **32**, 484.

Gladiolic Acid, a Metabolic Product of *Penicillium gladioli*

2. STRUCTURE AND FUNGISTATIC ACTIVITY

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Gladiolic acid, an antifungal metabolic product of *Penicillium gladioli* Machacek has been shown to have the tautomeric structure I (Grove, 1952*a*; Raistrick & Ross, 1952). Ultraviolet and infrared spectroscopic investigation of gladiolic acid and its derivatives (Grove, 1952*b*) showed that whilst gladiolic acid was present in the lactol form I*a* (R = OH) in the solid state, an equilibrium between the lactol and open chain I*b* (R = OH) forms existed in aqueous solution, the pH being the determining factor. Furthermore, the ultraviolet-absorption spectrum of gladiolic acid in alkaline solution showed that the gladiolic acid anion had the hydrated (dihydroxyphthalan) structure II ($R = O^-$) although, as regards chemical activity, this structure was equivalent to I*b* ($R = O^-$); similarly, *o*-phthalaldehyde also existed in aqueous solution in the hydrated form (dihydroxyphthalan). In addition, the esters of gladiolic acid described by Grove (1952*a*) were shown to be pseudo esters of general formula I*a*.

Brian, Curtis & Hemming (1948) found that gladiolic acid inhibited germination of the spores of a number of fungi in Czapek-Dox medium at low pH, and Smith (1952) has presented data on the fungistatic activity of the related cyclopaldic and cyclopolic acids and on dihydrogladiolic acid. In the present paper the results of tests on a comprehensive series of derivatives of gladiolic acid and of simple model compounds chemically related to gladiolic acid are reported, and conclusions are drawn regarding the structural groupings which are responsible for the fungistatic properties of the gladiolic acid molecule. In addition, a number of other factors which influence the fungistatic activity of gladiolic acid are discussed in relation to the specificity shown by the antibiotic.

EXPERIMENTAL

Materials. The preparation and properties of the derivatives of gladiolic acid are described by Grove (1952*a*). Model compounds were prepared by standard methods and purified by distillation or crystallization.

Routine evaluation of fungistatic activity (Tables 1 and 2). Compounds were assayed by the spore-germination test with conidia of *Botrytis allii* Munn. (Brian & Hemming, 1945; Brian *et al.* 1948), but Czapek-Dox was used in place of Weindling medium throughout, since gladiolic acid is unstable in the latter. All solutions were adjusted to pH 3·5 before assay, and dilutions made in ×2 steps. Small discrepancies (within the limits of error arising from such a dilution procedure) between the figures quoted for the fungistatic concentrations of certain compounds in different sections of this paper are due to the dilution of solutions of differing initial concentration. Salicylanilide was used throughout as standard reference substance; all tests were carried out in duplicate and the mean figures quoted in the tables. The highest concentration tested was limited by the water solubility of the particular compound under investigation.

Evaluation of fungistatic activity at different pH *values* (Tables 4 and 5). This was made in the same way as in the routine assay at pH 3·5 (above) by direct comparison with controls at the appropriate pH. Thus the figures quoted for 98–100% inhibition of germination are independent of the variation of germination with pH, which is, in any case, almost negligible for *B. allii* between pH 3 and 8.

Stability of lactol and aldehyde acetates in aqueous solution

In buffer solution. The compounds (50 mg.) in ethanol (2 ml.) were added to McIlvaine buffer (10 ml.), and the solutions allowed to stand at 25° (in some cases the starting material slowly separated) with occasional shaking. After 3 days the acid liberated by hydrolysis was estimated by titration with 0·1 N-NaOH to the first appearance of a pink colour with phenolphthalein (simultaneous titration of a blank).

Compound	Hydrolysis (%) at pH 3·9	Hydrolysis (%) at pH 7·2
Tetraacetyl-*o*-phthalaldehyde dihydrate	0	2·6
Triacetylgladiolic acid hydrate	6·4	5·4
Methyl gladiolate	4·0	6·0

In alkaline medium. The neutral compounds (50 mg.) in 30% (v/v) aqueous ethanol (10 ml.) were titrated with 0·1 N-NaOH to the phenolphthalein end point in CO_2-free air. The red colour produced after the addition of the first drops of alkali rapidly disappeared, in some cases within a few seconds and the titration was continued in this manner, adding alkali two drops at a time, until a pink colour was obtained which remained permanent for 3 hr.

The compounds varied considerably in their behaviour towards hydrolysis under these conditions. Thus, diacetylphenylglyoxal hydrate titrated almost like a dibasic acid and hydrolysis was complete within a few minutes. Acetylgladiolic acid (potential dibasic acid), 3-acetoxyphthalide (dibasic), triacetylgladiolic acid hydrate (tetrabasic) and tetraacetyl-*o*-phthalaldehyde dihydrate (tetrabasic) rapidly consumed alkali at first, but after a time the rate of hydrolysis slowed (titration time to the arbitrary end point (above), 2 hr.; % hydrolysed, 80–90), and the behaviour was more akin to that of a readily opened lactone. Methyl gladiolate was more stable and slowly consumed 0·9 equivalent in 3 hr.

Ultraviolet-absorption spectra of gladiolic acid (Fig. 1). Appropriate quantities of gladiolic acid were dissolved by shaking at room temperature in McIlvaine or Clark and Lubs buffer solutions, the pH values checked on a Cambridge pH meter, and the ultraviolet spectra obtained with a Unicam S.P. 500 spectrophotometer.

It has been shown (Grove, 1952*b*), by comparison with model compounds of known structure, that the ultraviolet-absorption curve *A* (Fig. 1) with maxima at 2670 and 3050 A is given by the lactol form (I*a*, R = OH) of gladiolic acid and is obtained in aqueous solutions of low pH, whereas curve *B* is characteristic of the hydrated open chain form II and is obtained at high pH values. When the ultraviolet absorption of gladiolic acid is examined at pH values between these extremes, a family of curves of intermediate shape is obtained and from the value

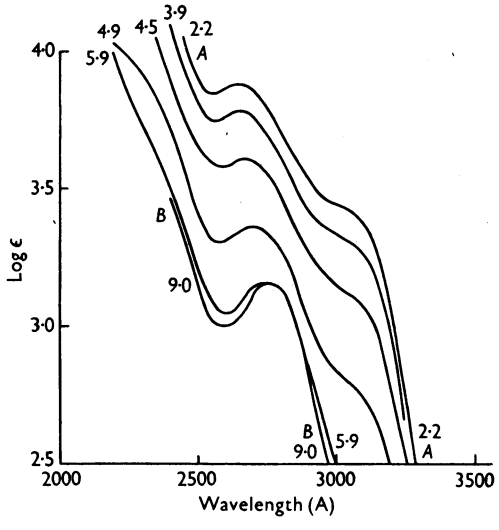


Fig. 1. Ultraviolet absorption spectra of gladiolic acid at different pH values.

of the extinction coefficient at 2670 A it is possible to calculate the percentage of lactol form present. The percentages of open-chain form obtained by subtraction from 100 are compared in Table 3 with the percentages of dissociated molecules calculated from the pK (4·4).

RESULTS

The concentration of gladiolic acid and a number of its derivatives required to produce 98–100% inhibition of germination of the spores of *B. allii* are given in Table 1 and compared with salicylanilide and mercuric chloride. Similar data for the model compounds related to gladiolic acid are collected in Table 2.

The stabilities of acetylgladiolic acid and gladiolic acid at different pH values are compared in Table 4.

Table 5 compares the effect of pH on the fungistatic activity of gladiolic acid and the neutral derivatives acetylgladiolic acid and triacetylgladiolic acid hydrate, with three neutral compounds tetraiodoethylene, *o*-phthalaldehyde and 3-acetoxyphthalide. The logarithms of the equieffective concentrations of these compounds are plotted against pH in Fig. 2.

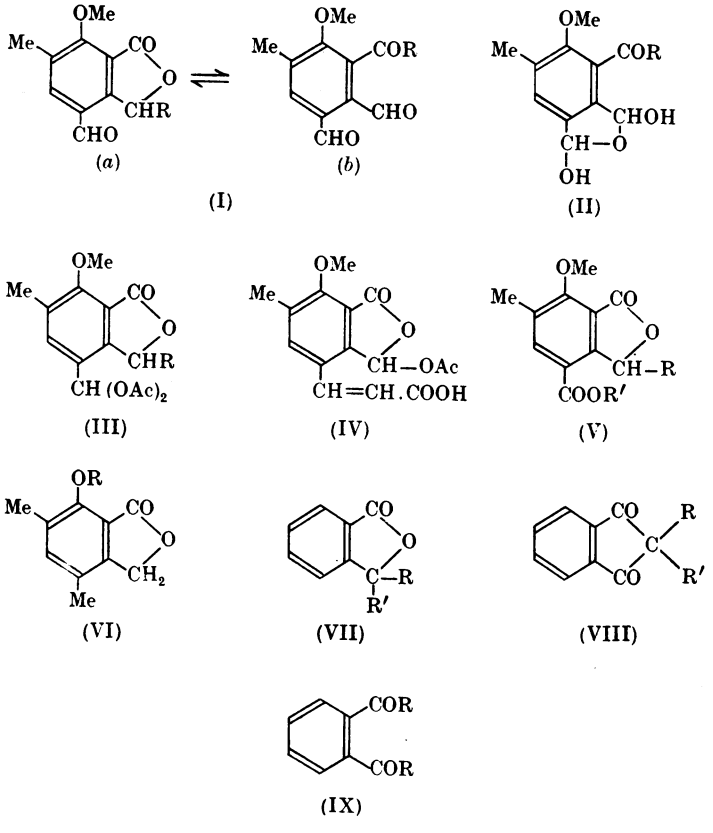
The results of tests with some of these compounds as inhibitors of spore germination for a wide range of fungi are given in Table 6.

Table 1. *Fungistatic activity of gladiolic acid derivatives*

Compound	Concentration inhibiting germination of *Botrytis allii* spores at pH 3·5 (μg./ml.)	($M \times 10^{-5}$)
Gladiolic acid (I, R = OH)	6·25	2·8
Gladiolic acid hemihydrate	6·25	2·7
Acetylgladiolic acid (I*a*, R = OAc)	1·6	0·6
Triacetylgladiolic acid hydrate (III, R = OAc)	6·25	1·7
Methyl gladiolate (I*a*, R = OMe)	12·5	5·3
Diacetyl methyl gladiolate hydrate (III, R = OMe)	25·0	7·4
Ethyl gladiolate (I*a*, R = OEt)	12·5	5·0
Diacetyl ethyl gladiolate hydrate (III, R = OEt)	6·25	1·8
Ethyl gladiolate semicarbazone	>100	>32·3
Acetylgladiolic acid semicarbazone	62·5	19·5
3-Acetoxy-7-methoxy-6-methylphthalidyl-4-β-acrylic acid (IV)	>100	>32·7
7-Methoxy-6-methylphthalide-4-carboxylic acid (V, R = R′ = H)	>100	>45·0
Methyl 7-methoxy-6-methylphthalide-4-carboxylate (V, R′ = Me, R = H)	>100	>42·4
3-Hydroxy-7-methoxy-6-methylphthalide-4-carboxylic acid (V, R = OH, R′ = H)	>100	>42·0
Methyl 3-hydroxy-7-methoxy-6-methylphthalide-4-carboxylate (V, R = OH, R′ = Me)	>100	>39·7
3-Acetoxy-7-methoxy-6-methylphthalide-4-carboxylic acid (V, R = OAc, R′ = H)	>100	>35·7
4-Formyl-7-methoxy-6-methylphthalide (I*a*, R = H)	>100	>48·5
Dihydrogladiolic acid	>100	>44·7
7-Methoxy-4:6-dimethylphthalide (VI, R = Me)	>100	>52·1
7-Hydroxy-4:6-dimethylphthalide (VI, R = H)	>100	>56·2
4-Methoxy-5-methylbenzene-1:2:3-tricarboxylic acid	>100	>39·4
4-Methoxy-5-methylbenzene-1:2:3-tricarboxylic acid trimethyl ester	>100	>33·8
Salicylanilide	62·5	29·3
Mercuric chloride	0·1	0·04

Table 2. *Fungistatic activity of some compounds related to gladiolic acid*

Compound	Concentration inhibiting germination of *Botrytis allii* spores at pH 3·5 (μg./ml.)	($M \times 10^{-5}$)
Benzoic acid	125	102
Methyl benzoate	>1000	>735
Benzaldehyde	100	94·3
Phenyl glyoxal	125	93·3
Diacetylphenyl glyoxal hydrate	>100	>42·4
Dimethyl phthalate	>100	>51·5
Phthalaldehydic acid (VII, R = H, R′ = OH)	>100	>66·7
Phthalide (VII, R = R′ = H)	>100	>74·6
3-Methoxyphthalide (VII, R = H, R′ = OMe)	>100	>61·0
3-Acetoxyphthalide (VII, R = H, R′ = OAc)	50	26·0
o-Acetylbenzoic acid (VII, R = Me, R′ = OH)	>100	>61·0
3-Methoxy-3-methylphthalide (VII, R = Me, R′ = OMe)	>100	>56·2
Benzil-2-carboxylic acid (VII, R = COPh, R′ = OH)	>100	>39·4
3-Acetoxy-3-benzoylphthalide (VII, R = COPh, R′ = OAc)	>100	>33·8
Phthalyl alcohol	>100	>72·5
o-Formylbenzyl alcohol	>100	>73·5
1:3-Diketoindane (VIII, R = R′ = H)	100	68·5
Triketohydrindene hydrate (VIII, R = R′ = OH)	>500	>181
2:2-Diacetoxy-1:3-diketoindane (VIII, R = R′ = OAc)	>100	>38·2
1:2-Diacetylbenzene (IX, R = Me)	>100	>61·8
Terephthalaldehyde	500	373
*iso*Phthalaldehyde	500	373
Tetraacetyl-*o*-phthalaldehyde dihydrate	100	28·8
o-Phthalaldehyde (IX, R = H)	6·5	4·8
Gladiolic acid (4-methoxy-5-methyl-*o*-phthalaldehyde-3-carboxylic acid)	6·25	2·8
Salicylanilide	62·5	29·3



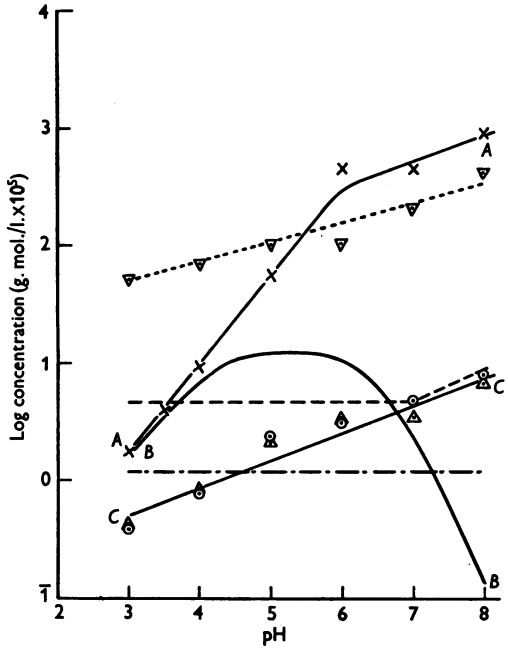


Fig. 2. Equieffective concentrations of fungistatic compounds at different pH values. ▽, 3-Acetoxyphthalide; – – –, *o*-phthalaldehyde; —.—, tetraiodoethylene; ×, *A*, gladiolic acid; *B*, gladiolic acid (undissociated molecules); ⊙, *C*, acetylgladiolic acid; △, triacetylgladiolic acid hydrate.

Table 3. *Effect of* pH *on the tautomeric equilibrium I and on the dissociation of gladiolic acid*

pH	ϵ_{2670} (data from Fig. 1)	Hydrated open-chain (dihydroxyphthalan) form (II) (%)	Dissociated molecules (calc. from pK) (%)
2·2	7600	0	0·6
3·0	7500	1·6	3·8
3·9	6030	24·0	24·0
4·5	3980	55·3	55·7
4·9	2190	82·6	76·0
5·9	1170	98·2	97·0
9·0	1050	100·0	100·0

Table 4. *Fungistatic activity of acetylgladiolic acid and gladiolic acid in Czapek-Dox medium*

		Concentrations inhibiting germination of *Botrytis allii* spores (M × 10^{-5})									
		Acetylgladiolic acid Storage at 25° (days)					Gladiolic acid* Storage at 25° (days)				
	pH of solution	0	1	2	3	16	0	1	2	3	16
(*a*) Assayed at pH 3·5	3·0	0·4	0·4	0·4	0·4	0·8	3·6	4·7	4·7	3·6	4·7
	4·0	0·4	0·4	0·4	0·4	2·4	3·6	4·7	7·2	4·7	3·6
	5·0	0·4	0·4	0·8	0·4	2·4	3·6	7·2	4·7	7·2	4·7
	6·0	0·4	0·8	2·4	0·8	4·7	3·6	4·7	4·7	4·7	4·7
	7·0	0·4	0·8	4·7	2·4	4·7	3·6	7·2	4·7	3·6	4·7
	8·0	0·4	2·4	4·7	4·7	4·7	3·6	7·2	7·2	4·7	4·7
(*b*) Assayed at pH of solution	3·0	0·4	0·4	0·4	0·4	1·2	1·8	3·6	1·8	1·8	1·8
	4·0	0·8	2·4	2·4	2·4	1·2	9·4	9·4	7·2	7·2	9·4
	5·0	2·4	3·2	4·7	9·5	2·4	56·3	56·3	56·3	56·3	56·3
	6·0	3·2	18·9	18·9	37·9	18·9	450	450	450	450	450
	7·0	4·7	37·9	37·9	37·9	37·9	450	450	450	450	450
	8·0	9·5	37·9	37·9	37·9	37·9	900	900	900	450	900

* Calculated from Brian *et al.* (1948).

Table 5. *Fungistatic activity of compounds assayed in Czapek-Dox medium at varying* pH

	Concentration (M × 10^{-5}) causing inhibition of germination of *Botrytis allii* spores					
pH of solution	Gladiolic acid*	Acetyl-gladiolic acid	Triacetyl-gladiolic acid hydrate	*o*-Phthal-aldehyde	Tetraiodo-ethylene	3-Acetoxy-phthalide
3·0	1·8	0·4	0·4	4·7	1·2	52·1
4·0	9·4	0·8	0·85	4·7	1·2	69·5
5·0	56·3	2·4	2·3	4·7	1·2	104
6·0	450	3·2	3·4	4·7	1·2	104
7·0	450	4·7	3·4	4·7	1·2	208
8·0	900	9·5	6·8	9·4	1·2	416

* Calculated from Brian *et al.* (1948).

Table 6. *Fungistatic activity of gladiolic acid, acetylgladiolic acid, methyl gladiolate and* o*-phthalaldehyde*

	Concentrations causing inhibition of germination at pH 3·5 (M × 10^{-5})			
	Gladiolic* acid	Acetylgladiolic acid	Methyl gladiolate	*o*-Phthalaldehyde
Absidia glauca Hagem	0·9	0·6	10·6	0·1
Aspergillus niger van Tiegh.	56·3	2·4	42·4	1·2
Botrytis allii Munn.	3·5	0·6	21·2	4·7
Fusarium coeruleum (Lib.) Sacc.	1·8	0·3	10·6	0·1
F. graminearum Schwabe	0·4	0·1	21·2	0·6
Myrothecium verrucaria (Alb. & Schw.) Ditm. ex Fr.	28·2	9·5	42·4	9·4
Mucor erectus Bain	3·5	0·3	21·2	0·6
Penicillium digitatum Sacc.	7·0	2·4	21·2	1·2
P. expansum Link	1·8	1·2	21·2	0·1
P. gladioli Machacek	3·5	2·4	>42·4	0·3
Stemphylium sp.	28·2	4·8	21·2	2·4
Thamnidium elegans Link	28·2	0·1	42·4	4·8
Trichoderma viride Pers. ex Fr.	113·0	38·0	>42·4	37·3

* Calculated from Brian *et al.* (1948).

DISCUSSION

Effect of structure on fungistatic activity

Most simple aromatic compounds with functional substituents are fungistatic at concentrations of the order of 10^{-3}–10^{-2} M, provided that the pH of the assay medium is so adjusted that ionization of acidic or basic substances is reduced to a minimum. The commercially important fungicidal compound salicylanilide inhibits germination of *B. allii* spores at $2{\cdot}9 \times 10^{-4}$ M at pH 3·5, but at this pH gladiolic acid is fungistatic at much lower concentrations (of the order of 10^{-5} M). For the purposes of the present paper, therefore, the term 'active' is applied to those compounds showing fungistasis in the same concentration range as gladiolic acid; compounds causing no inhibition of germination at concentrations greater than the lowest fungistatic concentration of salicylanilide are designated 'inactive'. It must, nevertheless, be borne in mind that gladiolic acid is only weekly fungistatic compared with mercuric chloride (Table 1). The maximum fungistatic activity of gladiolic acid (pK 4·4) is not observed in the routine assay (see later), nevertheless, it has been found more convenient to carry out the assay at this pH.

Examination of the results obtained with the model compounds (Table 2) shows that benzoic acid, phenylglyoxal and benzaldehyde are all fungistatic at a concentration of approx. 10^{-3} M, but *o*-phthalaldehyde (IX, R = H) is active at 5×10^{-5} M, i.e. in the same concentration range as gladiolic acid. Phthalyl alcohol, *o*-formylbenzyl alcohol, phthalaldehydic acid and dimethyl phthalate, all compounds closely related to *o*-phthalaldehyde but at a different level of oxidation or reduction, are inactive, as are phthalide and all the 3-substituted phthalides tested. The importance of the two formyl substituents is brought out by the fact that not only are compounds of structure (VIII), including 1:3-diketoindane and triketohydrindene hydrate and its diacetyl derivative, inactive, but so also is 1:2-diacetylbenzene (IX, R = Me). That the two formyl substituents must be in the ortho position on the aromatic nucleus is emphasized by the inactivity of *iso*- and tere-phthalaldehydes.

The only active compounds in the series of gladiolic acid derivatives (Table 1), namely gladiolic acid, acetylgladiolic acid (I*a*, R = Ac), triacetylgladiolic acid hydrate (III, R = OAc), the pseudo esters (I*a*, R = OMe and OEt) and the acetyl derivatives of their hydrates, are those in which both formyl groups are either intact or readily available on hydrolysis. The semicarbazones of acetylgladiolic acid and ethyl gladiolate show weak activity of the same order as salicylanilide. But all derivatives in which one or both of the formyl substituents has been oxidized to —COOH (e.g. 3-hydroxy-7-methoxy-6-methylphthalide-4-carboxylic acid (V, R = OH, R′ = H), 4-methoxy-5-methylbenzene-1:2:3-tricarboxylic acid and their neutral esters) or reduced to —CH_2OH (dihydrogladiolic acid, 4-formyl-7-methoxy-6-methylphthalide (I*a*, R = H) or —CH_3 (7-methoxy-4:6-dimethylphthalide (VI, R = Me)) are inactive; *iso*gladiolic acid (7-methoxy-6-methylphthalide-4-carboxylic acid (V, R = R′ = H)), and its neutral methyl ester are also inactive. Thus, of the known metabolic products of *P. gladioli*, namely, gladiolic acid, dihydrogladiolic acid, 4-formyl-7-methoxy-6-methylphthalide and 7-hydroxy-4:6-dimethylphthalide (VI, R = H), only gladiolic acid shows fungistatic activity.

The inactivity of 4-formyl-7-methoxy-6-methylphthalide (I*a*, R = H), taken in conjunction with the results on the model compounds already discussed, suggests that the lactol form (I*a*, R = OH) of the tautomeric system (I) is inactive *per se*. It can be argued that the activity shown by acetylgladiolic acid (I*a*, R = OAc) and the pseudo esters (I*a*, R = OMe and OEt) does not support the conclusion, but this objection is dealt with below. Like gladiolic acid, *o*-phthalaldehyde is rapidly inactivated in Weindling medium at pH 5 or above with the production of dark-green or greenish brown solutions. Moreover, the morphological effects produced by marginal concentrations of *o*-phthalaldehyde on the germ tubes of *B. allii* are identical with those produced by similar concentrations of gladiolic acid. One must conclude, therefore, that gladiolic acid owes its fungistatic activity to the *o*-phthalaldehyde grouping present in the open chain form (I*b*, R = OH), and that this grouping, which undergoes hydration in aqueous solution, probably reacts with some specific cell constituent.

Good agreement (within the limits of experimental error) is obtained when the percentage of dissociated molecules is compared with the percentage of open chain form present at a given pH (Table 3). It follows that in any investigation of the effect of pH on the fungistatic activity of gladiolic acid, the undissociated lactol form (I*a*, R = OH) and the hydrated open chain anion (II, R = O^-) are the most important molecular species to be considered. It is therefore difficult to escape the conclusion that the anion is the principal active form of gladiolic acid. The undissociated open-chain forms (I*b*, R = OH or II, R = OH) in equilibrium with (I*a*, R = OH) and (II, R = O^-) may be present, undetected by the spectroscopic technique employed, but together probably not exceeding 1 % of the total concentration of gladiolic acid, and may contribute to the total activity; but if (I*b*, R = OH) or (II, R = OH) were the sole active species, the effective external fungistatic concentration of active species

would be of the order of 10^{-7} M or less, an entirely different order of concentration from that found with *o*-phthalaldehyde.

Effect of ionization on fungistatic activity

Brian *et al.* (1948) concluded that the effect of the pH of the external medium on the fungistatic activity of gladiolic acid was best explained on the assumption that only undissociated molecules of gladiolic acid penetrated the plasma membrane of the spore. As is shown below, this is not now thought to be wholly correct; nevertheless, the undissociated molecule is the most effective penetrating species. Thus the molecular species which can most readily penetrate the plasma membrane is inactive, while the species which is mainly responsible for fungistatic activity, the hydrated open-chain anion, has greater difficulty in effecting penetration.

Two factors, therefore, penetration and the internal availability at the site of action of the active molecular species, operate in determining the fungistatic power of gladiolic acid, and these factors are determined respectively by the external and internal pH values. Thus, a low external pH favours penetration and a high internal pH favours dissociation to give the active anion.

Unfortunately, nothing is known about the internal pH of *B. allii* spores, and how this might be expected to vary with the external pH. Nevertheless, it is possible to make some predictions of a general nature by analogy with published work on the mycelium of other organisms. Thus if we assume that the (largely aqueous) cytoplasm of the *B. allii* spores is separated from the external medium by a chitinous cell-wall and lipoprotein semipermeable 'membrane', then in Czapek-Dox medium the internal and external concentration of ions will be governed by a Donnan equilibrium. Robbins (1924) found the isoelectric points of pH 5·0 and 5·5 for the intracellular colloids of *Rhizopus nigricans* and *Fusarium lycopersici* respectively. Most workers have found the internal pH of mycelium to fall between 4·8 and 6·5 (Armstrong, 1929; Mahdihassan, 1930), and there is evidence that the internal pH is well buffered against changes in the external pH. Thus Bünning (1936) found the internal pH of mycelium of *A. niger* only altered from 4·4 to 7·0, while the external pH was changed from 2·5 to $>8·0$. The most likely internal pH for the *B. allii* spore would seem therefore to be about 5, being but little affected by changes in external pH of the order studied in the present investigation. Nevertheless, the pH may vary in different parts of the cytoplasm and it is the pH at the site of action which is critical.

Since the pH at which the assay is carried out has such a profound influence on the fungistatic activity of gladiolic acid (Brian *et al.* 1948), it is instructive to examine the effect of varying the pH of assay on the activity of some of the derivatives of gladiolic acid and on *o*-phthalaldehyde (Tables 4 and 5). In the series assayed at pH 3·5 (Table 4*a*), acetylgladiolic acid appears to be less stable than gladiolic acid; however, the concentration of acetylgladiolic acid effective after standing 16 days at pH 8·0 is close to the concentration of gladiolic acid normally effective at the pH of assay (3·5), suggesting that the fall in activity is due to slow hydrolysis of acetylgladiolic acid to gladiolic acid in the external medium. In the series assayed at different pH values (Table 4*b*), acetylgladiolic acid is much less affected by the pH of assay than is gladiolic acid. The concentration of tetraiodoethylene, a stable neutral fungistatic compound (Muirhead, 1949), effective against *B. allii* spores, is independent of the pH of assay (Table 5). *o*-Phthalaldehyde behaves similarly, the slight fall in activity at pH 8 being due to decomposition of the material which occurs readily in the presence of hydroxyl ions (Grove, 1952*b*). It follows either that penetration of a non-electrolyte, and in particular a neutral dialdehyde, to the site of action and the equieffective concentrations at that site are independent of the pH of the external medium, or that any variations in the ability to penetrate at different pH values are exactly balanced by variations with pH of the equieffective concentration at the site of action. The former is more probable and is used in subsequent arguments.

The logarithms of the equieffective concentrations of gladiolic acid plotted against pH (Fig. 2, curve *A*) fall on a line which is initially linear and steeply inclined to the pH axis but flattens out above pH 6. The corresponding equieffective concentrations of undissociated molecules (curve *B*) derived from these values fall away rapidly at pH values greater than 6 and the general picture is typical of that shown by weak acids (Beevers & Simon, 1949; Simon, 1950). Simon has pointed out that since a constant external concentration of undissociated molecules does not produce the same response at each pH level, it cannot he held that the undissociated molecules are solely responsible for toxicity. Indeed, in the case of gladiolic acid, it seems probable from the chemical evidence that toxicity is due to the ion and this may therefore be true for other weak acids which give similar curves. (For summary, see Simon & Blackman, 1949.)

Acetylgladiolic acid and triacetylgladiolic acid hydrate (Fig. 2) give a linear plot of the logarithms of the equieffective concentrations against pH unlike that of the weak acid gladiolic acid or the non-electrolyte tetraiodoethylene. It will be recalled (Table 1) that while acetylgladiolic acid and triacetylgladiolic acid hydrate are more active than gladiolic acid at pH 3·5 (partly due to the absence

of the ionization effect mentioned above), the pseudo esters (I *a*, R = OMe and OEt) and the acetyl derivatives of their hydrates are appreciably less active. These results, and those in Table 4, might be explained on the hypothesis that these neutral gladiolic acid derivatives penetrate the plasma membrane of the spore unchanged but are hydrolysed (by enzyme action) within the cell to gladiolic acid which is the active principle. It is suggested, moreover, that the differences in activity between these

A simplified diagram of the processes involved in the penetration of the acetyl derivatives of gladiolic acid is given in Fig. 3; internal hydrolysis to gladiolic acid is assumed to go almost to completion. If the penetration is a passive process then the equieffective external concentrations [AcA_e] (Fig. 2, curve *C*) will be proportional to the equieffective total internal concentrations of gladiolic acid ([HA_i] + [A_i^-]) resulting from the external application of these substances. In Table 7 the variation

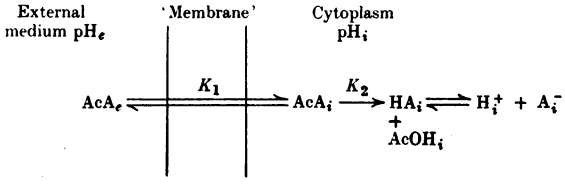


Fig. 3. Penetration of acetylgladiolic acid.

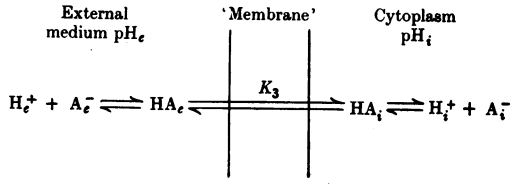


Fig. 4. Penetration of gladiolic acid.

AcA represents the molecule of acetylgladiolic acid, HA the undissociated molecule of gladiolic acid and A^- the gladiolic acid anion. The subscripts *e* and *i* denote external and internal, respectively. K_1, K_2 and K_3 are constants.

compounds are due to their differing susceptibilities to enzymic hydrolysis, paralleled by their behaviour towards acid hydrolysis *in vitro*. Lactol acetates, aldehyde acetates and the pseudo esters of gladiolic acid are all rapidly hydrolysed in alkaline solution at room temperature but are relatively stable in acid solution, tetraacetyl-*o*-phthalaldehyde dihydrate being the most and triacetylgladiolic acid hydrate the least stable. The inactivity of tetraacetyl-*o*-phthalaldehyde dihydrate can thus be explained on the above hypotheses. The logarithms of the equieffective concentrations of 3-acetoxyphthalide plotted against pH lie on a line of similar slope to the acetyl derivative of gladiolic acid (though at 100 times the concentration), and it is clear that this is an effect characteristic of this type of compound which is readily hydrolysed to a weak electrolyte.

It has been shown that the susceptibility of triacetylgladiolic acid hydrate towards hydrolysis does not alter significantly between pH 3·9 and 7·2. One may conclude that it is unlikely that the increase in the equieffective concentration of this substance between these pH limits is due to increasing hydrolysis in the external medium.

of internal with external pH found by Bünning (1936) has been assumed to hold for the *B. allii* spores and internal concentrations corresponding to the equieffective external concentrations calculated in order to see whether a constant internal concentration of anion is obtained (cf. *o*-phthalaldehyde, above). This constant concentration of anion is not found, and indeed is only obtained in the unlikely event of the internal pH being low (approx. 3) when the external pH lies between 8 and 5 and then rises (to 4·5) as the external pH falls from 5 to 3. There is, of course, no valid reason for supposing that what appeared to hold true for a non-electrolyte would also hold true for the anion, but since small variations in internal pH are involved, a constant internal concentration of anion might be expected. K_1 is probably close to unity and independent of pH, but K_2 would be expected to be pH dependent; in addition, the pH at the (unknown) site of action may be very different from the bulk internal pH, and these factors may contribute to the observed deviations from expectation.

However, rather better agreement with expectation is obtained in the case of gladiolic acid. The

Table 7. *Equieffective internal concentrations of gladiolic acid from data on the acetyl derivatives* (M × 10^{-5})

External pH (pH_e)	Internal pH (pH_i) (assumed, after Bünning, 1936)	$K_1[HA_i]$	$K_1[A_i^-]$	$K_1([HA_i]+[A_i^-])$ (from Fig. 2, curve *C*)
3·0	4·5	0·2	0·2	0·4
4·0	4·5	0·4	0·4	0·8
5·0	4·5	0·7	0·9	1·6
6·0	5·0	0·5	2·0	2·5
7·0	5·5	0·3	3·7	4·0
8·0	7·0	0·03	8·97	9·0

Table 8. *Equieffective internal concentrations of gladiolic acid* (M × 10^{-5})

External pH (pH_e)	Internal pH (pH_e) (assumed, after Bünning, 1936)	$K_3[HA_i]$ (from Fig. 2, curve *B*)	$K_3[A_i^-]$	$K_3([HA_i]+[A_i^-])$
3·0	4·5	3·8	4·8	8·6
4·0	4·5	9·2	11·6	20·8
5·0	4·5	12·5	15·7	28·2
6·0	5·0	10·0	30·0	40·0
7·0	5·5	2·3	28·8	31·1
8·0	7·0	0·14	55·9	56·0

equilibria involved are represented diagrammatically in Fig. 4. The internal concentrations of undissociated molecules ($[HA_i]$) will be proportional to the corresponding external concentration ($[HA_e]$) but local potential barriers due to local differences in charge on the protein layers of the 'membrane', combined with the differing internal and external pH values would be expected to produce a large concentration gradient across the membrane of the undissociated molecules of a weak electrolyte.

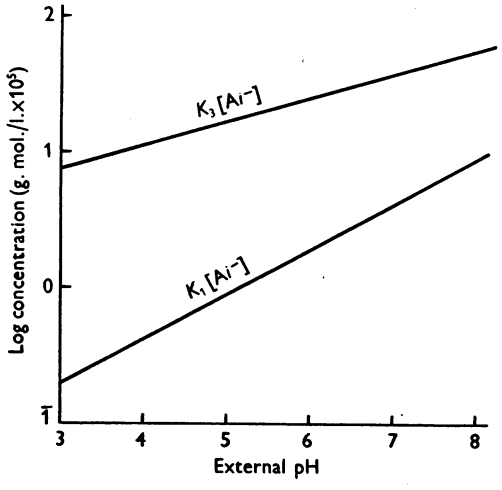


Fig. 5. Internal anion concentrations corresponding to the equieffective external concentrations of gladiolic acid and acetylgladiolic acid.

In Table 8 values of $K_3[A_i^-]$ corresponding to values of $K_3[HA_i]$ taken from Fig. 2, curve *B*, have been calculated for the same variation of internal with external pH as was assumed in Table 7. The logarithms of these values are plotted in Fig. 5 (together with the corresponding data for $K_1[A_i^-]$ from Table 7) and lie on a line only slightly inclined to the pH axis. The decrease in equieffective concentration below pH 4 (which is also apparent in Fig. 2, curve B) probably reflects the increasing contribution due to the penetration of gladiolic acid anions on the acid side of the isoelectric point.

To sum up. The effects of pH on the fungistatic activity of gladiolic acid can be broadly explained by the theory that the anion is the active molecular species but only the undissociated molecules penetrate the plasma membrane of the spore, and assuming a variation of internal and external pH that seems probable from the results of earlier work. Agreement is not perfect but is somewhat better for gladiolic acid than for acetylgladiolic acid which is assumed to penetrate unchanged but is then hydrolysed internally to gladiolic acid.

A great deal of the foregoing is of necessity speculative owing to the deficiencies in our knowledge of the system being studied, and the final elucidation of these problems must await further work, particularly on the biological side.

Specificity of fungistatic activity

Brian *et al.* (1948) tested gladiolic acid for inhibition of spore germination against a number of fungi and described a wide range of susceptibility among the fungi tested. The fungistatic concentrations at pH 3·5 of the neutral derivatives of gladiolic acid, acetylgladiolic acid and methyl gladiolate, and *o*-phthalaldehyde, fall into much narrower concentration bands when the substances are tested against the same fungi (Table 6), and although the variations from species to species are still quite large, the deviations from the means are significantly smaller than for gladiolic acid.

The fungistatic concentration of *o*-phthalaldehyde is of the order of 10^{-5} M for all the fungi tested, and is independent of the external pH between 3 and 7; gladiolic acid shows more marked specificity, and fungistatic concentrations are highly dependent on external pH. It is reasonable to assume slightly different membrane potentials and internal pH values for each species of fungus at a given external pH; and near the pK of gladiolic acid, small changes in pH produce relatively large changes in the ratio of undissociated molecules to ions. It is difficult to escape the conclusion, therefore, that the enhanced specificity of action of gladiolic acid arises from the presence of the carboxyl substituent, which not only gives the molecule the penetration specificity characteristic of any weak acid, but also, being situated next to one of the formyl groups and taking part in the tautomeric system (I), limits the proportion of fungistatic molecules and makes it pH dependent.

SUMMARY

1. Gladiolic acid (4-methoxy-5-methyl-*o*-phthalaldehyde-3-carboxylic acid in the open-chain form) is the only fungistatic compound among the known metabolic products of *Penicillium gladioli* Machacek.

2. *o*-Phthalaldehyde shows fungistatic activity in the same concentration range as gladiolic acid and tests on a large number of derivatives of gladiolic acid and on simple model substances indicate that activity is connected with the presence of two formyl substituents in the ortho position on the aromatic nucleus. The lactol form of gladiolic acid (4-formyl-3-hydroxy-7-methoxy-6-methylphthalide) is believed to be inactive.

3. The percentage of dissociated molecules of gladiolic acid present at any given pH is in good agreement with the percentage of hydrated open-chain (dihydroxyphthalan) form calculated from the ultraviolet absorption. The undissociated lactol and hydrated open-chain anion make up 99% of the total gladiolic acid present at any given pH.

4. An attempt to explain the variation of the equieffective concentration of gladiolic acid with the external pH, on the basis that whereas the hydrated open-chain anion is the active species, only the undissociated lactol form penetrates into the spore, is not unsuccessful.

5. The variation with pH of the activity of acetylgladiolic acid and certain closely related neutral derivatives is not quite so satisfactorily explained on the hypothesis that the molecules penetrate unchanged but undergo internal enzymic hydrolysis to gladiolic acid.

6. The enhanced specificity of the fungistatic action of gladiolic acid compared with that of *o*-phthalaldehyde arises from the presence of the carboxyl substituent adjacent to the *o*-diformyl grouping.

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REFERENCES

Armstrong, J. I. (1929). *Protoplasma*, **8**, 222.
Beevers, H. & Simon, E. W. (1949). *Nature, Lond.*, **163**, 408.
Brian, P. W. & Hemming, H. G. (1945). *Ann. appl. Biol.* **32**, 214.
Brian, P. W., Curtis, P. J. & Hemming, H. G. (1948). *J. gen. Microbiol.* **2**, 341.
Bünning, E. (1936). *Flora, Jena*, **131**, 87.
Grove, J. F. (1952*a*). *Biochem. J.* **50**, 648.
Grove, J. F. (1952*b*). *J. chem. Soc.* p. 3345.
Mahdihassan, S. (1930). *Biochem. Z.* **226**, 203.
Muirhead, I. (1949). *Ann. appl. Biol.* **36**, 250.
Raistrick, H. & Ross, D. J. (1952). *Biochem. J.* **50**, 635.
Robbins, W. J. (1924). *J. gen. Physiol.* **6**, 259.
Simon, E. W. (1950). *Nature, Lond.*, **166**, 343.
Simon, E. W. & Blackman, G. E. (1949). *3rd Symp. Soc. exp. Biol.* p. 253.
Smith, G. (1952). *Biochem. J.* **50**, 629.

New Zealand Fish Oils

6. SEASONAL VARIATIONS IN THE COMPOSITION OF NEW ZEALAND GROPER (*POLYPRION OXYGENEIOS*) LIVER OIL

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The groper (*Polyprion oxygeneios*) is one of the larger and more important New Zealand edible fish. The fatty-acid composition of the head oil and of the liver oil has been described by Shorland (1938), while the vitamin A and vitamin D contents of the liver oils have been determined respectively by Shorland (1937, 1948, 1950), Cunningham (1935), Cunningham & Scott (1944) and Weeber (1945).

This paper describes the seasonal changes in size of liver, yield of oil, vitamin A, cholesterol, total unsaponifiable content, phospholipid and iodine value.

EXPERIMENTAL

Thirty-nine samples, each containing from 8 to 53 (average 24) livers, representative of a day's catch from one boat, were collected in the period 1938–43, at approximately equal intervals of time to minimize the possible differences between years. During the months of September and October, however, when there was invariably a marked increase in vitamin A potency, eleven samples were taken as