This method could therefore be used as a simple and rapid method of insulin assay, measurement of the area under the peak giving the insulin content of the sample used. In addition to speed and simplicity this method has an advantage over biological assay in that it appears to be as satisfactory for crude as for pure insulin. It would be equally possible to use this technique for preparative work, as a single step appears to be sufficient to obtain pure insulin from crude material. The maximum weight used with these columns so far has been 150 mg. crude insulin on a 50 g. column, but there is no evidence that this loading may not be raised.

SUMMARY

1. The preparation is described of liquid two-phase systems using a variety of glycol ethers, water and organic and inorganic solutes. These systems have been found to be suitable for partitioning proteins.

2. Three such systems containing water, ethyl and butyl cellosolves and sodium or potassium phosphate have been used for the partition chromatography of insulin.

3. The insulin thus prepared from crude or crystalline material was of uniform activity of about 24 i.u./mg. The recovery from the columns appeared to be near 100%.

4. Throughout these chromatographic studies the insulin behaved as a single component.

5. The inherent difficulties of the method and the range of applicability to other proteins are discussed.

I wish to thank Dr W. Dickinson of Boots Pure Drug Co. Ltd., Nottingham, for the generous supply of all the insulin preparations used, Dr G. A. Stewart of Burroughs Wellcome Ltd. for the accurate assay of a sample of insulin from the column, and Dr A. J. P. Martin for many valuable discussions during the course of this work.

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The Isolation and some Chemical Properties of Viridicatin, a Metabolic Product of *Penicillium viridicatum* Westling

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Penicillium viridicatum was first described by Westling (1911); it was classified by Raper & Thom (1949) as a member of the *P. viridicatum* series, one of the features of which is the production of a strong, penetrating, mouldy or earthy odour. Smith (1942) states that it is often impossible to decide definitely whether a given strain should be called *P. cyclopium* or *P. viridicatum*, and suggests that 'the best way of dealing with this group is to lump together all the blue-green and yellow-green strains with similar morphology as the *P. cyclopium-viridicatum* series'. The principal strain used in this work, our catalogue number F 22 and of unknown origin, corresponded closely in its characteristics with those described by Raper & Thom (1949) for *P. viridicatum* Westling. Members of the *P. viridicatum* series are of frequent occurrence upon decaying vegetation in contact with the soil. There are a number of reports of their isolation from stored grains. Koehler (1938) reported *P. viridicatum* capable of growing upon shelled maize at a moisture level of 17·6%. Semeniuk & Barre (1944) found *P. viridicatum* to be the chief mould present on maize stored in steel bins in Iowa. Marchionatto (1942), working in Buenos

Aires, isolated *P. viridicatum* from maize infected with 'mildew'. The strain was identified by Thom. The infected grain was reported to the Argentine Ministry of Agriculture as the cause of poisoning pigs and horses. Carbon balances of three strains of *P. viridicatum* Westling were reported by Birkinshaw, Charles, Hetherington & Raistrick (1931), who found large amounts of 'carbon unaccounted for' in cultures in Czapek-Dox medium. No other biochemical studies on *P. viridicatum* have been reported.

The mycelium of the mould grown in Czapek-Dox medium yielded on extraction with chloroform a crystalline compound for which the name viridicatin is proposed. Further extraction of the mycelium with ethanol gave a second product, mannitol, the yield being 3–4% by weight of the dry mycelium. Mannitol has been reported as a metabolic product of species of *Aspergillus*, *Penicillium*, *Helminthosporium* and *Clasterosporum*, and as the principal metabolic product of *Byssochlamys fulva* (Raistrick & Smith, 1933). The yield of viridicatin, although high in the initial experiments, later fell to a negligible amount, which was not improved by variations in the medium. Two other strains of *P. viridicatum* failed to yield viridicatin on several media. Viridicatin, m.p. 269°, is a colourless, crystalline compound, $C_{15}H_{11}O_2N$, which does not contain carboxyl, methoxyl, carbon-methyl or nitrogen-methyl groups. No optical rotation could be detected in glacial acetic acid solutions of the pure compound. The infrared absorption spectrum of a Nujol suspension of viridicatin (Fig. 1) exhibits an intense band at 6·08 μ., a wavelength characteristic of the carbonyl group of an amide, but high for a ketonic carbonyl function. Additional bands in the regions 3·0 and 6·40 μ. support a substituted amide structure, but these bands are of low intensity (Randall, Fowler, Fuson & Dangl, 1949). Bands at 6·16 and 6·68 μ. suggest a phenyl nucleus, while the band at 6·34 μ. suggests further conjugation. No absorption clearly due to a hydroxyl group could be detected. Attempts to prepare a carbonyl derivative of viridicatin failed. On the other hand, the compound reacted readily with benzoyl chloride in pyridine to yield a monobenzoate. Since no picrate, methiodide or hydrochloride of viridicatin could be isolated it was concluded that this acylation involved a hydroxyl rather than an amino group. Furthermore, treatment of viridicatin with dilute or concentrated aqueous sodium hydroxide solution yielded a monosodium salt, which was reconverted to viridicatin by the action of mineral acid or carbon dioxide. An aqueous solution of the salt rapidly deposited crystalline viridicatin on exposure to the atmosphere. The sodium salt also reacted readily with *p*-phenylphenacyl bromide to yield the corresponding derivative, and these indications of a phenolic function in solutions of viridicatin were supported by the fact that a deep-red colour was produced when the sodium salt of viridicatin was allowed to react with diazotized sulphanilic acid, whilst a dilute ethanolic solution of viridicatin gave an intense, permanent, green colour with neutral aqueous, ferric chloride solution. The sodium salt of viridicatin is strongly alkaline and the potentiometric titration of an aqueous solution of the salt with dilute hydrochloric acid gave the pH of half-neutralization of the salt as 9·95. Viridicatin is therefore a very weak acid.

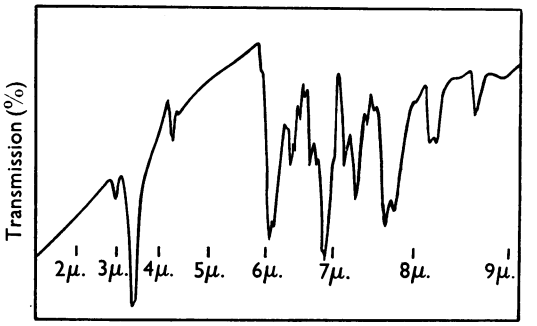

Fig. 1. Infrared absorption spectrum of viridicatin; mull in Nujol.

Although some of the properties of viridicatin are very similar to those of a betaine-like structure, such as that attributed to 5-hydroxyacridine (Lehmstedt, 1935), the evidence, other than that of the infrared spectrum, suggests the presence of a normal phenolic hydroxyl group. That this inference is correct was established when the sodium salt of viridicatin was found to react with methyl iodide to yield methylviridicatin, in which the methyl group is attached to the nucleus by an oxygen atom and not by a nitrogen atom as it is in the corresponding derivative of a structure of the 5-hydroxyacridine type. The same product was also isolated from the reaction of the silver salt of viridicatin with methyl iodide. The ultraviolet absorption spectra of viridicatin and methylviridicatin (Fig. 2) exhibit an intensification of absorption at a region (3200 A.) frequently associated with phenolic or phenyl ether absorption (Friedel & Orchin, 1951), although the bathochromic effect of the maximum of the methyl derivative in this region is associated more with *N*-alkylation. The absorption of viridicatin in 0·1N-sodium hydroxide solution, pH 11 (sodium viridicatin), is markedly altered and the intensity and wavelength range of absorption of these three compounds suggest a highly conjugated nucleus.

Viridicatin contains two active hydrogen atoms, one of which is accounted for by the hydroxyl group. The presence of a cyclic amide group, inferred from the infrared absorption spectrum, may account for the other, and also for the high melting point and

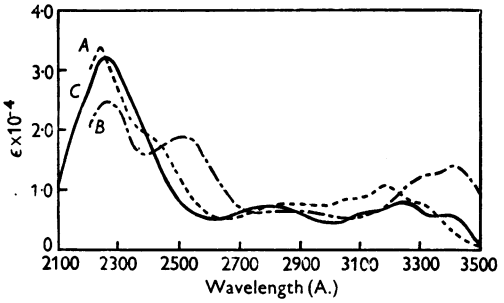

Fig. 2. Ultraviolet absorption spectra of viridicatin in ethanol (*A*); in aqueous sodium hydroxide (*B*); and methylviridicatin in ethanol (*C*).

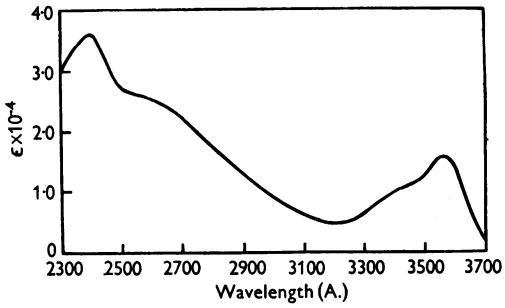

Fig. 3. Ultraviolet absorption spectrum of chloroviridicatin in ethanol-chloroform.

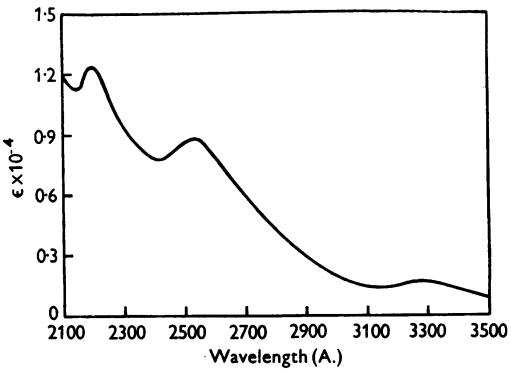

Fig. 4. Ultraviolet absorption spectrum of oxyviridicatin in ethanol.

stability of viridicatin. The substance is not attacked by a boiling solution of hydriodic acid in glacial acetic acid. It is not reduced by sodium in boiling ethanol, acidification of the solution yielding unchanged starting material; nor does its glacial acetic acid solution react with hydrogen in the presence of reduced Adams's catalyst at atmospheric temperature and pressure. Viridicatin is stable to phosphorus oxychloride at 140°, but at 180° decomposition occurs. With thionyl chloride, however, an oxygen and hydrogen atom are replaced by halogen. The ultraviolet light absorption of the compound is shown in Fig. 3. The product isolated from the addition of bromine to a glacial acetic acid solution of viridicatin is a dibromo compound in which one hydrogen atom and one hydroxyl group appear to have been replaced by halogen. The metabolic product is readily oxidized by dilute nitric acid to yield oxyviridicatin (Fig. 4) as a yellow crystalline product which contains three active hydrogen atoms.

Viridicatin shows no antibiotic activity against *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* (*Micrococcus pyogenes*, var. *aureus*). Our colleague, Dr C. T. Calam, has shown that the substance is active *in vitro* against *Mycobacterium tuberculosis* at a dilution of 1:15 000 but has no activity against *Entamoeba histolytica*.

EXPERIMENTAL

All melting points are corrected. Analyses are by Weiler and Strauss, Oxford, and the Microanalytical Section, Imperial Chemical Industries Ltd., Nobel Division.

Isolation of viridicatin. 'Glaxo' type culture flasks containing Czapek-Dox medium (5% glucose; 800 ml./flask) were inoculated in batches of fifty flasks with a spore suspension of *P. viridicatum* and the cultures incubated at 25°. After 3–4 days the surface of the medium was covered by an unrestricted, heavily sporing felt and by the end of the second week tufts of white, non-sporing mycelium appeared on the surface of the mould. By the twenty-first day the average sugar concentration of the medium (as determined by a modification of the method of Munsen & Walker (1906, 1912) in which Cu_2O was estimated by the permanganate method of Bertrand (1906)) was less than 1 g./l., and the mycelium was collected, washed several times with water, air-dried (40°) and ground (150 g.). The mycelium was continuously extracted with $CHCl_3$ in a Soxhlet type apparatus and *viridicatin* slowly separated from the hot $CHCl_3$ solution. The crude product (6·15 g., 4·1%), m.p. 240–260°, was crystallized twice from $CHCl_3$, from which it separated as rods, m.p. 262–265°, contaminated with a small quantity of soft, dull plates. Repeated recrystallization from ethanol yielded viridicatin as lustrous needles, m.p. 269°. The compound is sparingly soluble in cold organic solvents and in dilute mineral acids, but is much more soluble in boiling, conc. HCl, from which it separates unchanged on cooling. It crystallizes from acetone, glacial acetic acid and ethylene glycol monomethyl ether as prisms. The compound is optically inactive, $[\alpha]_D^{18°}$ 0° ± 2° in $CHCl_3$ (*c*, 1·0). A sample was dried at 80°/10^{-2} mm. for 2 hr. over P_2O_5. (Found: C, 75·9, 75·9, 76·0; H, 4·6, 4·7, 4·7; N, 5·6, 5·8, 6·3; mol.wt. 209, 274. $C_{15}H_{11}O_2N$ requires C, 75·9; H, 4·7; N, 5·9%; mol.wt. 237.)

Viridicatin was first isolated in 1948 from freshly harvested, dried mycelium and high yields of the crude product were obtained. When chemical work on the substance was

resumed in 1951, freshly harvested batches of mycelium from the same strain (F22) gave only 0·1% of material, identical with that previously isolated. Enrichment of Czapek-Dox medium with thiamine hydrochloride (0·1 p.p.m.), calcium D-pantothenate (0·1 p.p.m.), inositol (0·1 p.p.m.) and biotin (0·0004 p.p.m.) using 400 ml. or 800 ml./flask, gave a vigorous growth of the mould with no increase in the yield of viridicatin. The same results were obtained with beer-wort medium, and a range of media containing 2–5% glucose and 0·25–1·0% of Pronutrin (an enzymic casein hydrolysate). Furthermore, a culture of Raper & Thom's reference strain (N.R.R.L. 963) of *P. viridicatum* Westling obtained from the Commonwealth Mycological Institute and another strain of *P. viridicatum* from the same source (I.M.I. 49162) both failed to yield viridicatin on various media.

Viridicatin benzoate. Benzoylation of viridicatin (0·1 g.) in pyridine solution yielded an oil which was washed with water and dissolved in warm ethanol. *Viridicatin benzoate* separated slowly from the cooled solution as lustrous plates, and recrystallization from ethanol yielded large colourless plates, m.p. 180°. A specimen was dried at 80°/10^{-2} mm. for 1 hr. (Found: C, 77·4, 77·5; H, 4·2, 4·2. $C_{22}H_{15}O_3N$ requires C, 77·4; H, 4·4%.)

Sodium salt. Viridicatin (0·18 g.) was suspended in aqueous NaOH (3 ml. of 7·5 N) and the mixture was warmed on a steam bath for 15 min. The opaque prismatic needles which separated on cooling were collected and dried *in vacuo* (0·19 g.), m.p. 240–260° (decomp.). Recrystallization of the *sodium salt* raised the melting point to 260–265° (decomp.), and this was depressed by 30° on admixture with viridicatin. A sample was dried at 80°/10^{-2} mm. for 3 hr. (Found: Na, 8·9, 9·2. $C_{15}H_{10}O_2NNa$ requires Na, 8·9%.)

Viridicatin p-phenylphenacyl ether. A mixture of the sodium salt of viridicatin (0·19 g.), m.p. 255–265° (decomp.) and *p*-phenylphenacyl bromide (0·1 g.), was suspended in water (1·5 ml.) and ethanol (2·5 ml.) was added. The mixture was heated under reflux for 1 hr. and was cooled to 0° overnight. The solid, which separated as slightly yellow microcrystalline prisms (0·092 g.), m.p. 218–224°, was recrystallized from ethyl acetate-ethanol, from which *viridicatin p-phenylphenacyl ether* separated as colourless prisms, m.p. 225–230°, unaltered on repeated recrystallization. A sample was dried at 80°/10^{-2} mm. for 1 hr. over P_2O_5. (Found: C, 80·6, 80·8; H, 4·9, 5·0; N, 3·5. $C_{29}H_{21}O_3N$ requires C, 80·7; H, 4·9; N, 3·2%.)

Viridicatin methyl ether. A suspension of viridicatin (0·3 g.) in 5 ml. 3 N-NaOH was warmed on a steam bath for 5 min. The sodium salt was collected from the cooled solution and was washed with ice-cold ethanol (5 ml.) and dried (0·3 g.). The salt was dissolved in water (10 ml.) and excess of aqueous $AgNO_3$ was added. The yellow silver salt which separated was collected, washed with water and dried. It was then suspended in a solution of CH_3I (1 ml.) in ethanol (15 ml.) and the mixture was refluxed for 30 min. The reaction mixture was filtered free from AgI and excess CH_3I and ethanol were removed *in vacuo* to yield the crude *methyl ether* (0·2 g.), m.p. 130–230°, as an almost colourless amorphous solid. The product was crystallized from methanol-water from which it separated as rods (0·05 g.), m.p. 225–235°, and recrystallization from acetone yielded rods, m.p. 239°. A sample was dried at 80°/10^{-2} mm. for 2 hr. over P_2O_5. (Found: C, 77·0, 76·6; H, 5·2, 5·1; OMe, 11·5. $C_{15}H_{10}ON.OCH_3$ requires C, 76·5; H, 5·2; OMe, 12·3%.)

Dibromoviridicatin. Viridicatin (0·05 g.) was dissolved in glacial acetic acid (5 ml.) and excess Br_2 (about 0·2 g.) was added. The solution was left overnight at room temperature and was then warmed on a steam bath and concentrated (2 ml.). The microcrystalline material which separated from the cooled solution was collected (0·03 g.; m.p. 300–320°), and recrystallized from aqueous ethanol from which the *dibromo compound* separated as needles, m.p. 325–330°. A sample was dried at 80°/10^{-2} mm. for 3 hr. over P_2O_5. (Found: C, 48·2; H, 2·6; N, 3·9; Br, 40·5. $C_{15}H_9ONBr_2$ requires C, 47·5; H, 2·4; N, 3·7; Br, 42·2%.)

Chloroviridicatin. A solution of viridicatin (0·15 g.) in acid-free $SOCl_2$ (3 ml.) was heated under reflux on a water bath for 5 hr. Removal of $SOCl_2$ under reduced pressure yielded a yellow gum which solidified on washing with ethanol. The product was suspended in ethanol (4 ml.) and warmed under reflux. The material which did not dissolve was separated and crystallized from glacial acetic acid to yield *chloroviridicatin* as colourless needles (0·02 g.), m.p. 308–312°. This material was sublimed at 200°/10^{-2} mm. to yield a crystalline product, m.p. 310–312°. (Found: C, 70·9; H, 4·2. $C_{15}H_{10}ONCl$ requires C, 70·5; H, 3·9%.)

Oxyviridicatin. A suspension of viridicatin (0·15 g.) in 2 ml. 2 N-HNO_3 was warmed on a water bath at 70° for 10 min. The temperature was then raised to 85° over a period of 30 min. and the solution became yellow as the viridicatin dissolved with the deposition of an amorphous yellow solid. The solution was cooled and the product was collected (0·13 g.; m.p. 140–180° (decomp.)), and dissolved in acetone leaving a residue (0·02 g.) of viridicatin. Evaporation of the acetone and recrystallization from aqueous ethanol yielded *oxyviridicatin* as small clustered yellow prisms, m.p. 178–179° (decomp.). The product was dried at 80°/10^{-2} mm. for 1 hr. (Found: C, 70·7; H, 4·4; N, 5·5; active H, 1·4, 1·5; $C_{15}H_{11}O_3N$ requires C, 71·1; H, 4·4; N, 5·5; 3 active H, 1·2%.)

SUMMARY

1. The air-dried mycelium of *Penicillium viridicatum* (strain F. 22 of the Departmental collection) has been shown to contain a hitherto undescribed compound (viridicatin) and mannitol.

2. Subsequent experiments after some years with the same strain of *P. viridicatum* gave very poor yields of viridicatin. Two other strains failed to give viridicatin in a variety of media.

3. Viridicatin has been shown to have the molecular formula $C_{15}H_{11}O_2N$, in which one oxygen atom is present as a phenolic hydroxyl group, whilst the other may be present in a cyclic amide. The molecule is of a polycyclic aromatic nature.

4. Seven derivatives of viridicatin are described, but insufficient material was available for degradation work.

5. Viridicatin showed slight antibiotic activity against *Mycobacterium tuberculosis in vitro*.

The authors wish to express their thanks to Mr H. B. Colquhoun for his assistance in the preparation of viridicatin.

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Separation of Tea Polyphenols on Paper Chromatograms

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Previous work on the chromatography of tea polyphenols (Roberts & Wood, 1951*a*) was carried out with phenol and *n*-butanol-acetic acid as the solvents. It has now been shown that water is also a useful solvent for the separation of polyphenols on a paper chromatogram. In particular, results have been obtained which are most simply interpreted by assuming that the water effects a separation of optical antipodes of catechins and gallocatechins, thus enabling a provisional decision to be made as to which of the various optically active forms of these substances occur naturally in the tea leaf. The use of water as a solvent in paper chromatography also differentiates between aglycones and glycosides in the flavonol group.

A preliminary account of this work has already been presented (Roberts & Wood, 1951*b*).

METHODS AND MATERIALS

Chromatographic technique. This is as described by Roberts & Wood (1951*a*). Two-way chromatograms are carried out using water as the first solvent, and the *n*-butanol-acetic acid mixture, previously described, as the second solvent. Such a chromatogram will be referred to as a two-way water chromatogram. Ferric alum (0·2%, w/v) and ammoniacal $AgNO_3$ (equal volumes of 0·1 N-$AgNO_3$ and 5 N-NH_3 aq.) are used as sprays for the detection of polyphenols. Chlorogenic acid is detected by spraying first with 1% (w/v) $NaNO_2$ dissolved in 10% acetic acid (v/v), and then with N-NaOH (Roberts & Wood, 1951*c*). Exposure of the dry paper, before spraying, to NH_3 vapour shows up anthoxanthins and chlorogenic acid as yellow patches.

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Polyphenols. (±)-Gallocatechin, (−)-epigallocatechin and (−)-epicatechin were samples obtained from Dr A. E. Bradfield. Quercitrin was obtained from Prof. W. Baker, rutin from Dr J. F. Crouch and myricetin from Prof. T. R. Seshadri.

The (+)-catechin was Merck's commercial product. Its aqueous solution was rather dark in colour which made polarimetry difficult, but $[\alpha]_D^{26°}$ was found to be +16·1° in acetone-water (50%, v/v), which leaves little doubt that it is (+)-catechin.

Quercetin was prepared from rutin by hydrolysis with 0·1% (v/v) HCl for 4 hr., and butin from the blooms of *Butea frondosa* by the method of Rupe & Schaerer (1932).

A dilute solution of (+)-gallocatechin was obtained from tea-leaf juice by the following procedure. A close row of spots of tea-leaf juice was applied along the starting line of a chromatogram. After development with *n*-butanol-acetic acid, the (+)-gallocatechin band was located by spraying guide strips. Transverse strips of the paper, containing the (+)-gallocatechin, were then eluted with ethyl acetate saturated with water. The solvent was removed *in vacuo* and the residue taken up in water.

A dilute solution containing gallocatechin-*a* gallate and catechin-*a* gallate was obtained by a similar procedure from reconstituted juice autoclaved at 130° for 1 hr. (Roberts & Wood, 1951*a*). The solvent used for development in this case was water.

Tannase. This was prepared from *Aspergillus niger* according to Freudenberg (1933). In later experiments a culture of *A. niger* M, obtained from Long Ashton Research Station, was used.

RESULTS

A typical two-way water chromatogram is illustrated in Fig. 1, which shows the pattern of spots for polyphenols from clone 14/3/32. This source is chosen as it contains naturally occurring gallocatechin-*a* gallate. Catechin-*a* gallate, if present,