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ISOLATION OF VIVOMYCIN AND BORRELIDIN, TWO ANTIBIOTICS WITH ANTI-VIRAL ACTIVITY, FROM A SPECIES OF *Streptomyces* (*C*2989)

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In a screening programme based on the examination of streptomyces isolated from soils, a culture designated *Streptomyces C*2989 was found which produced two anti-viral antibiotics. The first solid obtained possessed only *in vitro* activity and was afterwards identified as borrelidin by Anderton and Rickards[1], while the second, a polysaccharide of high molecular weight, was active only *in vivo* and has been named 'vivomycin'. The anti-viral activity of these two antibiotics was detected by Dickinson *et al.*[2], who have also carried out all the antiviral tests involved in this investigation.

The culture, *Streptomyces C*2989, was isolated from a sample of soil obtained from a caravan site at Mablethorpe in Lincolnshire. The organism grew well on a number of solid media and produced abundant spores which were borne in short flexuous chains; no spirals were observed. The organism was non-chromogenous, and it reduced nitrates to nitrites. From these observations and from consideration of the colour of the aerial mycelium on various solid media, the organism was considered to be closely related to *Streptomyces griseus*, and this was confirmed by direct morphological comparisons of *Streptomyces C*2989 with strains of *S. griseus* obtained from Prof. S. A. Waksman and the Northern Regional Research Laboratory at Peoria.

Differences in sporophore morphology, in the colour of the vegetative mycelium on solid media, and in carbon utilization were noted between *Streptomyces C*2989 and *S. rochei*, the organism producing borrelidin and described by Berger *et al.*[3].

Tests by Spooner[4] indicated that *C*2989 brews were inactive against most bacteria and fungi, but were slightly active against some species of *Bacillus* and very active against members of the genus *Corynebacterium*. The hypothesis that this antibacterial activity was due to the substance responsible for the anti-viral activity was supported by the fact that bioautograms obtained from paper electrophoresis strips placed on plates of agar seeded with *C. xerosis* indicated the same movement of the active component (slowly towards the anode at *p*H 7, none at *p*H 5) as had been found for the anti-viral component.

On the basis of this a cup plate diffusion assay was developed using *C. xerosis*. Arbitrary units were used defined as 1 unit $\equiv$ 10 μg of the provisional standard solid (batch LAB1) which was later found to be *c.* 15 per cent pure. The highest potency solid obtained assayed at 660 units/mg. The assay gave a straight line response over a wide range. Solutions containing 0·2–4 units/ml. were normally used.

At an early stage in this investigation, *C*2989 liquors were found to show good activity against encephalomyocarditis (EMC) virus in mice. It was tentatively assumed that this activity was due to the substance responsible for the activity *in vitro*, but discrepancies were soon observed between the activity *in vitro* and *in vivo* of various samples.

However, because of the difficulty of assaying in mice, it was decided to concentrate on the isolation of the substance active *in vitro*, and then determine whether it was active against EMC virus in mice.

A suitable medium for the growth of the organism *C*2989 and the production of the *in vitro* activity in both shaken and stirred aerated forms of submerged culture was one containing 1·5 per cent corn starch, 0·5 per cent glucose, 0·1 per cent v/v corn steep liquor, 1·0 per cent soya flour, 0·2 per cent calcium carbonate and 0·5 per cent sodium chloride made to volume with tap water and adjusted to *p*H 6·5–6·7 before sterilization. Activity was detected after 48 h fermentation, but did not usually reach a peak until 96 h, by which time the liquors usually contained between 70 and 90 units/ml.

*C*2989 brews were usually collected at 96 h and they were filtered at *p*H 5·5–6. The filtrate was extracted with about a quarter of its volume of butanol or, better, methyl isobutyl ketone, and the extract was concentrated at reduced pressure in a climbing film evaporator. The concentrate was stirred with methanol and the solution was decanted from the insoluble fat and concentrated at reduced pressure. Addition of light petroleum precipitated the crude antibiotic, usually 10–20 per cent pure, in a yield of *c.* 0·5 g/l. of brew.

Solids produced by this method had high activity against *C. xerosis* and viruses *in vitro*, but none was active against EMC virus in mice.

Thin-layer chromatography of the crude solids using chloroform–acetic acid (10 : 1) and carbon tetrachloride–chloroform–dioxane–toluene–acetic acid–water (5 : 3 : 3 : 1 : 2 : 3) (lower phase) on thin layers of 'Kieselgel G' showed a number of spots with different-coloured fluorescence in ultra-violet light after spraying with 15 per cent phosphoric acid in ethanol and heating for 15 min at 105° C. Bioautograms using *C. xerosis* showed that the activity was associated with a yellow fluorescing spot with R_F *c.* 0·5 in both solvent systems.

Bioautograms of descending chromatograms using benzene–ethyl acetate (10 : 1) on Whatman No. 1 paper indicated the presence of two active zones, R_F 0 and 0·2, but when material from either of these was rechromatographed it gave the same two zones again and it was concluded that the two zones represented different forms of one component.

The crude solid described here had a characteristic strong absorption maximum at 258 mμ. It was purified by countercurrent extraction in the system water–methanol–methyl isobutyl ketone–ligroin (5 : 5 : 2 : 8), the water being replaced by McIlvain's buffer at *p*H 3 in the tubes to which the sample was added. After 200 transfers the component with the ultra-violet absorption at 258 mμ, the activity against *C. xerosis* and the characteristic behaviour on thin-layer chromatography, was located in the middle tubes. Concentration and precipitation with light petroleum gave a colourless solid, m.p. 98°–105° C, containing 640 microbiological units/mg. Crystallization from chloroform + carbon tetrachloride gave colourless hexagonal plates of solvate.

At this stage it became necessary for us to discontinue work on this substance because we had become sensitized to it, and handling it, even with stringent precautions, resulted in severe skin irritation. It was identified by Anderton and Rickards as borrelidin, an antibiotic described by Berger *et al.*[3] in 1949, but not previously reported to be active against viruses.

When crude and purified borrelidin samples were found active against viruses *in vitro* but not *in vivo*, while the waste aqueous liquors were active *in vivo* but not *in vitro*, it became clear that a second component was involved, and this was named 'vivomycin'. The separation of vivomycin and borrelidin was complicated by the fact that very low levels of borrelidin potentiated the action of vivomycin against EMC virus in mice so that at certain stages an apparent loss of activity occurred on separation.

Further investigations of the fermentation brews of *C*2989 from shaken and stirred aerated vessels were undertaken, and it was found that the same fermentation conditions could be used for producing vivomycin as for borrelidin but that the former was produced earlier with a peak activity at 48–60 h.

By acidifying *C*2989 brews with hydrochloric acid and filtering at *p*H 2 the amount of borrelidin in the liquor could be reduced to less than 10 units/ml. (that is, about 15 mg/l.). This residual amount could be removed by washing with butanol or methyl isobutyl ketone; freeze-drying the neutralized aqueous solution then gave very crude, but borrelidin-free, samples of vivomycin. Samples free from most of the inorganic electrolyte were obtained by adsorption of the antibiotic on charcoal ('Norit *SX*' with about 5 g/l. of brew filtrate), eluting with 50 per cent aqueous acetone at 50°, concentrating and freeze-drying. The solvent wash was found to be unnecessary because the borrelidin remaining in the filtrate was adsorbed on the carbon and not eluted. Crude batch *FA*1, which was active against EMC virus in mice at 100–400 mg/kg, was typical of the crude vivomycin obtained by the foregoing process.

The crude vivomycin solid could be separated into a number of components by circular thin-layer chromatography on plates of 'Kieselgel *G*' made up with 0·2 M boric acid. The solvent system was butanol–acetic acid–water (12 : 3 : 5), and after development the plates were sprayed with anisaldehyde–sulphuric acid–acetic acid (1 : 2 : 97) and heated for 15 min at 110°. Partial separation of these components by various forms of column chromatography showed that the antibiotic was the fraction giving a green zone at the origin in the thin-layer chromatography. This could be separated further by thin-layer chromatography using *tert*-butanol–acetic acid–water (8 : 2 : 5). Two of the components separated in this system were shown to be active against EMC virus in mice, the main one which remained at the origin, the subject of all further work, and a less active one with R_F *c.* 0·4.

The crude solid was dissolved in water saturated with chloroform, heated at 60° for 20 min to pasteurize it, cooled and poured on to a column of *G*25 'Sephadex'. It was eluted with water saturated with chloroform and cuts were examined by thin-layer chromatography. Freeze-drying the fractions containing the origin component gave an almost colourless solid, designated *P*1, active against EMC virus in mice at 25 mg/kg. This was further purified on a column of *G*75 'Sephadex', the separation being followed by means of the ultra-violet absorption at 215 mμ. Absorption was plotted against volume of eluate and the material corresponding to the first peak was freeze-dried to give a fibrous white solid, *P*2, active against EMC virus in mice at 0·4–1·5 mg/kg.

Paper chromatography of acid hydrolysates (N-sulphuric acid at 100° C for 16 h) of crude vivomycin solids indicated the presence of at least five amino-acids and three carbohydrates identified as arabinose, galactose, and glucose. Hydrolysis of fractions from *G*25 and *G*75 'Sephadex' columns indicated that, as the activity was increased by taking higher molecular weight cuts, the proportion of amino-acids fell almost to zero, while the proportion of arabinose increased. The arabinose content was determined by spraying the paper chromatograms with alkaline triphenyltetrazolium chloride solution, eluting the red spots with pyridine–conc. hydrochloric acid (9 : 1), and comparing the colour of the eluate with that of a standard. Analysis of the hydrolysate of *P*2 material by this method indicated an arabinose content of 8 per cent, but a control experiment with arabinose showed that more than 50 per cent was lost or destroyed in this method.

Lowe and Dickinson[5] subjected vivomycin solids to further separation on *G*100 and *G*200 'Sephadex' columns, following the course of elution by continuous monitoring by ultra-violet absorption in a flow-cell. They

showed that the most active material had a molecular weight of more than 100,000, but obtained no evidence of a single active component of definite molecular weight. Crampton and Henry[6] subjected fractions from *P*2 on a *G*200 'Sephadex' column to methanolysis, trimethylsilylation and gas chromatography, but failed to show any correlation between arabinose : glactose : glucose ratios and molecular weight or activity. The fraction with the highest molecular weight (*P*2/*D*1), active against EMC virus in mice at 0·4 mg/kg, yielded 48 per cent of arabinose, 21 per cent of galactose and 31 per cent of glucose. Schrager[7] analysed the same fraction by hydrolysis, trimethylsilylation and gas chromatography, and obtained the results: arabinose, 55·5 per cent; galactose, 16·8 per cent; glucose, 22·5 per cent; 2 per cent of amino-sugars were found. A colour test for sialic acid on *P*2 material indicated a mucoprotein content of *c.* 5 per cent.

Vivomycin solids were subjected to electrophoresis at voltages up to 800 in a *p*H 10 borate buffer (0·005 M) on a 40-cm column of ethanolized cellulose. Samples of eluate from this column were examined by electrophoresis on thin layers of 'Kieselgel *G*' and by ultra-violet absorption at 215 mμ.

Solid *P*1 was separated into two anionic fractions, a fast one moving at *c.* 5 cm/h, giving a blue colour with the anisaldehyde reagent, and a slower one giving a green colour. Samples of these, freed from buffer on a small column of *G*25 'Sephadex', were both active against EMC virus in mice at 25 mg/kg. In solid *P*2 only the slow component was found and was not separated further by more prolonged electrophoresis. In the eluates from a column in which *P*1 had been fractionated by chromatography on *G*75 'Sephadex' the fast component was found in increasing amounts after the *P*2 fraction had been collected.

In recent years there have been various reports of anti-viral, anti-tumour, or non-specific anti-infectious activity in substances of high molecular weight produced by micro-organisms. However, the properties of vivomycin appear to distinguish it from all those described previously. The anti-viral agent, helenine[8], from *Penicillium funiculosum*, was believed to be a ribonucleoprotein. Statolon[9], from *Penicillium stoloniferum*, active against viruses and tumours, was described as a polyanionic polysaccharide composed of galacturonic acid, galactose, galactosamine, glucose, arabinose, xylose and rhamnose. Actinogan[10,11], an anti-tumour agent of high molecular weight from a species of the genus *Streptomyces*, was reported to be a glycopeptide. On hydrolysis it yielded glucose, rhamnose, hexosamine, and several amino-acids. Extracts of the organism producing actinogan (ATCC 13748) were inactive against EMC virus in mice[13]. Antiviral activity in a bacterial polysaccharide was reported by Horsfall and McCarty[12]. Other authors have observed similar activity in tissue culture, in eggs and in mice.

The action of vivomycin in stimulating host defence mechanisms is similar to that of the endotoxins from the cell walls of Gram-negative bacteria which have been described by many authors. Vivomycin is distinguished from these by being produced by a species of *Streptomyces*, by being found in the culture liquor, by its chemical composition, and by being less toxic to mice.

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ANTI-VIRAL ACTIVITY OF TWO ANTIBIOTICS ISOLATED FROM A SPECIES OF *Streptomyces*

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CULTURE liquors from micro-organisms isolated from soil samples may be tested for anti-viral activity by means of a plaque inhibition test, based on Dulbecco's method[1] for titrating cytopathic viruses. This report describes the behaviour of two anti-viral factors isolated from culture liquors of a member of the genus *Streptomyces*, *C*2989, which was selected by this test. The organism, and the isolation and properties of the two antibiotics, are described by Lumb *et al.*[2].

One of these factors was responsible for the inhibition of virus plaques and is highly active against many viruses in tissue culture but also very toxic to growing cells. It has been identified[3] as borrelidin, a toxic antibiotic described in 1949 by Berger *et al.*[4], who found it active against experimental *Borrelia* infections, but no reports of its anti-viral action have been published. The organism described by these workers, *Streptomyces rochei*, is not the same as *C*2989 which produces the antibiotics described here. The other anti-viral factor, which we have called 'vivomycin', is not significantly active in any tissue culture systems, but is of particular interest because it is active against encephalomyocarditis virus (EMC) in mice, with a therapeutic index of at least 100, and has some action on influenza virus infections in mice. Vivomycin seems to be a new antibiotic and the evidence suggests that it stimulates the host defence mechanisms of the mice.

Selection of organism and preliminary tests on culture liquors. For the detection of effects against cytopathic viruses, 80–100 liquors were spotted on to 9 in. × 9 in. squares of filter paper, which were then sterilized by ethylene oxide or ^{60}Co irradiation and placed on an agar medium overlying virus-infected cells. After a suitable time (2–3 days) the cells were stained by neutral red; virus plaques remained unstained. Zones of anti-viral activity appeared as red areas of live cells whereas toxic zones were usually clear and unstained. In the work on *C*2989 liquors, anti-viral activity was shown against influenza virus (WSNF strain), Newcastle disease virus (NDV) and vaccinia virus (Salaman strain), all of which were grown on monolayers of chick embryo cells. Large zones were also obtained against EMC virus growing on suspensions of Krebs ascites tumour cells in agar. In each case the medium consisted of Earle's solution with Difco agar, plus 2·5 per cent calf serum when monolayers were used as host cells.

The liquors were inactive *in vitro* against a wide range of bacteria, except some species of *Corynebacteria*, such as *C. xerosis*. The activity in the plaque test paralleled