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.

We thank the technical staff of the Antibiotics and Fermentation Division for their help.

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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

activity against *C. xerosis* which was later used to assay borrelidin.

One active liquor was tested against EMC virus in mice. The virus was given intraperitoneally (i.p.) and the liquor was given either by the same route or subcutaneously (s.c.), starting the day before infection. Only 2/10 untreated mice survived after 7 days whereas 9/10 and 8/10 treated (i.p. and s.c. respectively) still survived at 14 days, although treatment stopped after 6 days.

In view of these results the work was continued on a larger scale and it was decided to isolate the plaque-inhibitory factor first and to see whether it was responsible for the activity *in vivo*. There were, of course, other possibilities. The activity *in vivo* might be due to non-specific toxic effects of the liquor, even though the mice appeared healthy, or the drug might act on the host defence mechanisms (specifically anti-viral or general). Another possibility was the conversion *in vivo* into a specific anti-viral agent which might be shown to act in tissue culture. All these possibilities were investigated.

Plaque-inhibitory factor (borrelidin). Pure borrelidin was used for the work described here. It was prepared as a 1 per cent solution in acetone and diluted into distilled water when required, in order to obtain stable suspensions; these were kept at +4° C. Borrelidin was highly active in the plaque-inhibition tests against NDV, influenza, vaccinia and EMC viruses, and gave active zones at levels as low as 1–10 μg/ml., using fish spine beads to apply the solutions. It was, however, inactive against EMC virus mice at doses up to the maximum tolerated daily dose (i.p.) of 1 mg/kg. It was also inactive against influenza virus and ectromelia virus infections in mice.

Borrelidin is highly toxic to growing cells and prevents cell division at concentrations of about 1 μg/ml., but suspended cells withstand much larger amounts for 24 h or more. Even 20 μg/ml. is not toxic to suspended uninfected cells; chick embryo cells grow and Krebs cells produce tumours in mice when the drug is removed. The growth of, for example, WSNF strain of influenza and EMC viruses in suspensions of chick embryo cells and Krebs ascites cells respectively is completely inhibited at concentrations above about 1 μg/ml. and usually at 0·1 μg/ml. for as long as the drug is present. The activity in the plaque-inhibition test probably depends on the fact that the cells in monolayers under agar are not infected until after the cells have formed the layer and that when the borrelidin is applied the cells are not actually growing under the agar medium. 5-Iododeoxyuridine prevents plaque formation by DNA viruses in a similar test and it is also highly toxic to growing cells.

The anti-viral action of borrelidin in suspended cells will be described elsewhere. It does not affect virus adsorption but prolongs the latent period by roughly the time for which it is present, provided it is not added later than about half-way through the normal latent period. If added later it may reduce the yield of virus. In these experiments with suspended cell cultures in Hanks's solution, single cycles of infections were used for WSNF strain of influenza virus on chick embryo cells (latent period about 4 h) and EMC on Krebs ascites cells (latent period about 7 h). Borrelidin was used at concentrations of 0·16 μg/ml. and 0·8 μg/ml. respectively (see Figs. 1 and 2).

It should be noted that borrelidin is a most potent sensitizer to the human skin and even if it had been active *in vivo* its toxicity would limit its use.

In vivo factor (vivomycin). Since culture liquors were active *in vivo* whereas borrelidin was not, obviously some other factor was responsible for the activity *in vivo*. The isolation of this factor (vivomycin) depended on animal assays, well known to be inaccurate and slow. Since the active liquors had very little anti-bacterial activity, contamination of samples after collecting the liquors was a problem. Steaming for 15–30 min was shown to have no adverse effect on the activity *in vivo*

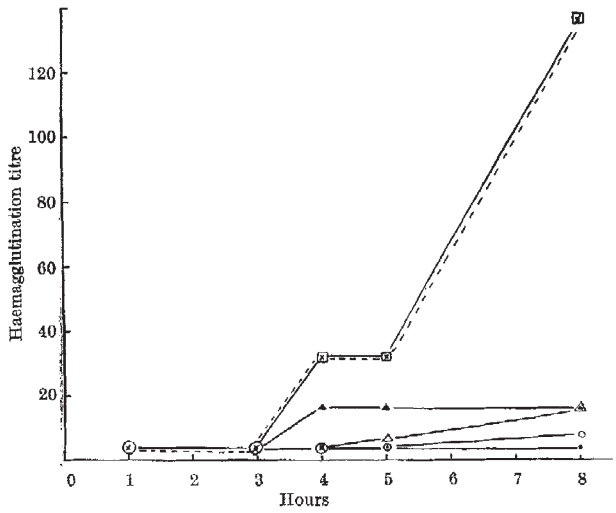

Fig. 1. Action of borrelidin (0·16 μg/ml.) on influenza virus (WSNF) in suspended chick embryo cells. Single cycle growth experiment in Hanks's solution. Growth assessed by haemagglutination, expressed as reciprocal. × - - - ×, control; ●, drug present 0–8 h; ○, drug present 1–4 h; △, drug present 1–2 h; ▲, drug present 3–5 h; □, drug present 5–8 h

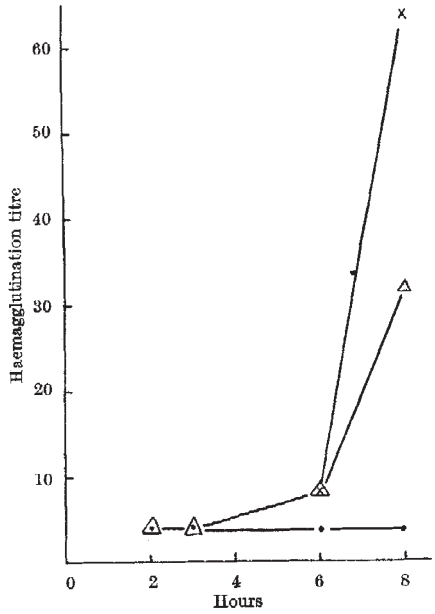

Fig. 2. Action of borrelidin (0·8 μg/ml.) on EMC virus in suspended Krebs ascites cells. Single-cycle growth experiment in Hanks's solution. Growth assessed by haemagglutination, expressed as reciprocal. ●, Present from 0, 2 or 4 h; △, present from 6 h; ×, added at 8 h (control)

and it also eliminated local toxicity, which sometimes occurred when liquors were injected s.c. This method of 'partial sterilization' was used until purified material was available.

The assays were standardized as far as possible and the mice or cages were randomized; male mice, weighing 18–20 g, were used. The EMC virus was kept at −65° as stock suspensions of mouse brain and normally 0·2 ml. of a 10^{-4} dilution was used in the tests. 0·2 ml. of a 10^{-5} dilution given i.p. usually killed all or most of a group of 10–20 mice and a 10^{-6} dilution usually killed some mice. Treatment (0·2 ml. i.p. in most tests) started the day before infection and was continued daily. In later assays the drug was given twice on day 0, that is, 3 h before and 3 h after infection, but equally good protection was obtained by the s.c. route and even when treatment was started after infection. The tests were usually terminated when all the control mice were dead, or at 6–7 days, but

in some cases mice were left to see whether the disease flared up when treatment ceased. Results were calculated statistically on the numbers of survivors (using tables for four-fold contingency tests[5]) and average survival times. For the latter, the ranking method of White[6] was used. Except where supplies of material were limited, several four-fold dilutions of samples were tested. This enabled the therapeutic ratio of samples to be followed. At later stages of the work a standard of known activity was included in the tests.

The work described here relates to a crude solid, *FA*1, which was available in large amounts from a 3,000-gallon fermentation, and to a purified preparation *P*2, derived from *FA*1. *FA*1 was active against EMC in mice at 100–400 mg/kg and sometimes at lower doses; it contained less than 0·0025 per cent borrelidin. *P*2 was active at 0·1–1·5 mg/kg and contained no detectable borrelidin. Details of the preparation and properties of these solids are described by Lumb *et al.*[2].

The most active material obtained (*P*2) was a white amorphous solid, very soluble in water. Although chromatographic changes could be demonstrated on heating there was no detectable loss of activity within the limits of the assays (four-fold dilutions) after steaming or autoclaving at 10-lb. pressure for up to 30 min. Possibly there was some loss at higher temperatures (see Table 1). The solutions could be sterilized by filtration through membrane filters, although this was difficult with concentrated *P*2 solutions. Solids could be sterilized by ^{60}Co radiation and in several tests there was no detectable differences in activity after these various procedures.

Table 1. ACTION OF VIVOMYCIN ON EMC VIRUS IN MICE I.P. INFECTION BY 10–100 LD_{50}

Treatment from day before infection and then daily to end of test except where stated

Sample	Daily dose mg/kg	Route	Survivors		Test read (days)
*FA*1 (filtered)	400	i.p. to day 3	10/10†		4
	100		6/10*		
	25		4/10‡		
	6		2/10		
Control	0		0/10		
*FA*1 (steamed)	400	i.p.	11/20†		6
	400	s.c.	13/20†		
	400	Oral	1/20		
	400	i.p. starting +3 h	10/20†		
Control	0		0/20		
*P*2 (comparison of filtered solution with one autoclaved at 15 lb./20 min	25	i.p. to day 4	Filtered 8/10†	Autoclaved 7/10*	6
	6		8/10†	8/10†	
	1·5		10/10‡	6/10	
	0·37		8/10†	5/10	
	0·01		6/10‡	4/10	
Control	0		1/10		
Significance	*$P < 0.05$		†$P < 0.01$		

‡ Significant only when calculated on survival times[6].

Experimental results on vivomycin. Table 1 gives typical examples taken from many experiments carried out with *FA*1 and *P*2, all giving similar results. It shows that vivomycin was active both i.p. and s.c., but not orally, against i.p. infection by EMC. No toxic symptoms were observed with *FA*1. The therapeutic index for *P*2 was at least 100, toxic symptoms sometimes being observed at 100 mg/kg although this dose was not lethal. The table also illustrates that rather flat dose response curves were obtained, using either numbers of survivors or average survival times. In some tests there was a definite plateau effect and generally results were expressed as the lowest dose (mg/kg) which gave significant protection. Even when significant numbers were not totally protected, the average survival time was often significantly lengthened. A single dose of drug given 1 or 2 days before infection sometimes protected mice, but results varied. Treatment after infection was only effective if given before the virus entered the brain and this could occur within a few hours after infection.

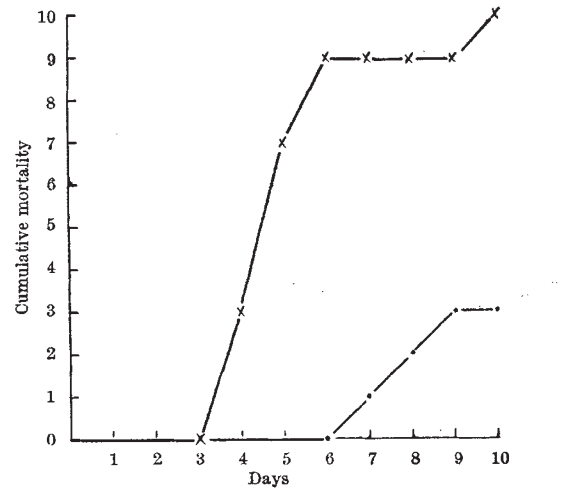

Fig. 3. Effect of vivomycin on EMC virus in mice (1·5 mg/kg daily i.p. from day −1 to day +4). ×, Controls; ●, treated

In some treated mice virus was apparently eliminated from the body and the mice survived for many weeks. If challenged, they were susceptible to EMC virus. In other animals, the disease flared up when treatment ceased. These results suggested that the disease process was slowed down by the drug, but if sufficient virus remained it could later multiply. Fig. 3 illustrates a typical result for *P*2. Relapses can be expected with any virostatic drug (even with the effective tetracyclines against members of the psittacosis virus group) and antibody production is important to prevent this.

Vivomycin was inactive when the infection was by the intracerebral route, suggesting it could not pass the blood–brain barrier, or that it was active only against peripheral multiplication of the virus. Details of the effect of vivomycin on the pathogenesis of experimental EMC infections will be published elsewhere, but the evidence suggests that it acts by stimulating the host defence mechanisms, with consequently more efficient removal of infecting organisms (virus or bacterium). EMC virus and *Pseudomonas aeruginosa* were cleared more rapidly from the peritoneal cavity of treated mice. Organs of treated mice had a much lower virus content than those of control mice, proving that vivomycin did not act by preventing entry into the brain. No evidence was obtained for conversion *in vivo* to any substance which would prevent the growth of EMC virus in tissue culture, or that borrelidin was in any way involved in its activity.

Vivomycin was tested against several viruses in tissue culture, eggs and animals. Except at high doses of drug, low doses of virus and with pretreatment of cells in some tests, it was inactive in all the tissue-culture systems tested. These included plaque-inhibition tests with EMC, herpes, vaccinia, NDV and influenza strain WSNF, and dilution tests with monolayer or suspension cultures with the same viruses. It was not significantly active against influenza virus (strains *PR*8, WSNF and Asian), Rous sarcoma, sheep abortion or vaccinia viruses in eggs. It was inactive against ectromelia in mice and Rous sarcoma virus in chicks but was significantly active in some tests against influenza (mouse-adapted *PR*8) in mice. In these tests groups of 20 randomized mice were infected with about 10^6 EID_{50} virus intranasally under anaesthesia. This dose was sufficient to infect all the mice and to kill some within 7–10 days. Treatment was either i.p., as in the EMC tests, or intranasally (one dose before infection). Results were assessed statistically on numbers of survivors[5] and average lung score[6] at 8–10 days (0–4 for pulmonary consolidation, 5 for a death). Some results are given in Table 2, but high doses of drug were necessary for activity. Influenza tests are not very satisfactory because

Table 2. Action of Vivomycin on Influenza Virus in Mice

Treatment	Survivors at 8 days	Average lung score	No. lungs with −ve scores
*P*2: 1 dose 0·05 ml. 4% solution i.n. at −1 day	20/20	0·9†	8†
As above and daily i.p. at 25 mg/kg	18/20	1·55*	4
100 mg/kg i.p.	20/20	1/37*	5*
25 mg/kg i.p.	19/20	1/77	3
Nil	17/20	2·3	0
*FA*1: dose 4% i.n. at −1 day	19/20†	1·68†	4
400 mg/kg i.p.	14/20	2·8	2
100 mg/kg i.p.	12/20	3·2	0
Nil	10/20	3·5	1

Significance * = $P < 0.05$. † = $P < 0.01$.

of the great variation in lung scores of control mice, but vivomycin was significantly active in several tests, although not in all. The fact that activity was found even when the drug was given intranasally is not necessarily inconsistent with an action on host defence mechanisms because the drug (like the virus) reaches the lungs of mice and could therefore be absorbed.

Both the EMC and influenza tests in mice are very artificial models of actual clinical infections. The same objection applies to almost any virus–host system used in experimental chemotherapy, but influenza and various Picorna viruses, particularly Coxsackie viruses, could be considered as most relevant for clinical trials. Unfortunately, suitable tests using adult mice are not available for Coxsackie viruses, and suckling mice must be used. For these tests animals were kept in their litters and litter mates were used as controls. Allocation of treatment to individual mice was randomized and the lowest dose of virus which would kill all control mice was used; mice were infected at ages of 2–5 days. *P*2 was inactive against EMC in suckling mice at the maximum tolerated dose of 25 mg/kg and it was also inactive against Coxsackie *A*2 and *A*21. These results were not surprising as presumably the host defence mechanisms of suckling mice have not developed.

Although two antibiotics are present in culture liquors of *C*2989 there is no evidence to support the attractive hypothesis that the two are related structurally or biogenetically, and their appearance in the same liquors may be fortuitous. However, it is interesting that when small amounts of borrelidin (less than the maximum tolerated dose of 1 mg/kg) are added to sub-optimal doses of vivomycin, synergism occurs. Table 3 illustrates this for EMC using material similar to, but less active than, *P*2. Synergism probably explains the good results which were sometimes obtained with liquors; these contained varying amounts of borrelidin and vivomycin. Synergism also occurred in tests against Krebs ascites tumour in mice (Table 4). Vivomycin itself was inactive against ascites tumour in mice, but borrelidin sometimes showed activity as might be expected from a potent cytostatic agent. The two together were markedly active in suppressing tumour growth, but the tumour grew when treatment ceased. Similar synergism was shown against influenza (*PR*8) in eggs, in which test system vivomycin is quite inactive. Borrelidin showed some action against low doses of virus (10 EID_{50}) only, but the two together showed actitivy against 1,000 EID_{50} virus. Synergism could not be demonstrated in tissue culture. Previously, borrelidin had been found to enhance the anti-syphilitic action of penicillin G *in vivo*[7,8].

Vivomycin stimulates the host defence mechanisms in a similar manner to the endotoxins. Although it is

Table 3. Effect of Borrelidin on the Activity of Vivomycin against EMC Virus in Mice

I.p. infection by 10–100 LD_{50}; treatment daily i.p. from the day before infection. Results expressed as number of survivors/total

Borrelidin (mg/kg)	Vivomycin/*CA*10 (mg/kg) 25	6	0
0·25	32/39*	34/40†	5/40
0	22/40	11/40	1/40

Significance * $P < 0.05$. † $P < 0.01$.

The results of two experiments, each giving similar results, have been pooled in this table. The significance refers to the increased protection in the presence of 0·25 mg/kg borrelidin at given dose of vivomycin.

Table 4. Effect on Mixtures of Vivomycin/*FA*1 and Borrelidin against Krebs Ascites Tumour in Mice

Mice were implanted i.p. with 5×10^6 cells and randomized. Daily i.p. treatment for 8 days. Mice weighed daily. Drug controls, treated similarly, but not implanted, showed weight gains of 2·0 to 2·75 g compared with 3·75 for controls

Drug	Dose (mg/kg)	Average wt. gain or loss	Remarks
*FA*1	400	+7·2	
	50	+8·2	
Borrelidin	0·1	+11·9	
	0·5	+8·5	
*FA*1 + Borrelidin	400 0·01	+3·9	1/10 died
*FA*1 + Borrelidin	100 0·1	+1·1	Tumours grew after treatment stopped
*FA*1 + Borrelidin	50 0·1	+5·6	,, ,,
*FA*1 + Borrelidin	50 0·5	+1·3	,, ,, (1/10 died)
Nil		+12·8	2/10 died

much less toxic to mice than known endotoxins it resembles them in many ways. Of course, it is not a typical endotoxin, a term usually reserved for the lipopolysaccharide component of the cell wall of Gram-negative bacteria, since vivomycin is found in the culture medium, not in cell wall preparations. Mr. A. B. Spicer supplied cell wall preparations of *C*2989 and of *Escherichia coli*. Those from *C*2989 were inactive but one from *E. coli* was active against EMC. Although about 100 times more toxic than vivomycin the therapeutic ratio was high. Like vivomycin, it increased the clearance rates of EMC virus and *Ps. aeruginosa* from the peritoneum.

The antibacterial action of endotoxins has been known for many years, but the mechanism is not clear. They are active only if given before infection of the mice. Dr. D. F. Spooner and Mr. L. J. Hale (personal communication) found that vivomycin protected mice and rabbits against certain bacterial infections if given before infection but was inactive *in vitro*.

There have been few reports on the anti-viral activity of endotoxins and variable results have been reported. Either increased or decreased resistance to virus infections may occur[9–11]. Vivomycin has been consistently active against EMC in mice, but its possible usefulness in other species can only be decided when further trials, and detailed toxicity work, have been completed. Preliminary results indicate that vivomycin, like endotoxin, is much less toxic to mice than to other species, particularly dogs and rabbits. Dr. B. Lessel (personal communication) has shown that *P*2 is emetic in dogs at doses of 10 μg/kg i.v. and has a pyrogenic action in rabbits at similar doses.

The question of how substances which act on the host defence system of mice exert their anti-viral action is of general interest, and several mechanisms, apart from effects on clearance rates, may be involved.

We thank Mrs. P. Bestwick, Mrs. W. Ward, Mr. G. Cooling and Mr. H. Robinson for their help.

Dr. J. S. Porterfield, of the National Institute for Medical Research, has found that borrelidin is also active against several arboviruses. In a diffusion test carried out in chick embryo monolayer cultures, 8 μg/ml. of borrelidin gave large zones (3–5 cm) of plaque inhibition against Semliki Forest, Sindbis, West Nile, and Bunyamwera viruses.

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