PROPERTIES OF AN IRIDESCENT VIRUS FROM THE BEETLE, *SERICESTHIS PRUINOSA*

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Summary

The recently described iridescent virus from *Sericesthis* (SIV) is closely related to *Tipula* iridescent virus (TIV). TIV and SIV are morphologically similar and have similar sedimentation coefficients ($S_{20,w} = 2200$ S). SIV differs from TIV serologically and in DNA content. Large yields of purified SIV, of the order of 2 mg virus protein per pupa, maybe obtained from the wax moth, *Galleria mellonella*.

SIV is thermolabile, ether- and chloroform-resistant, and its infectivity is retained after exposure to either pH 3·0 or 10·5 for 3 hr. SIV shares these properties with the adenovirus group.

Particle counts of SIV in the electron microscope have been related to infectivity, to total protein, and to absorbance at 260 mμ. 1 absorbance unit represents 0·056 mg SIV protein per millilitre, and this is equivalent to $1{\cdot}8 \times 10^9$ particles. The total particle/infective particle ratio is approximately 1 : 1.

SIV developed in *Galleria* larvae kept at 22°C, but not at 28°C.

I. Introduction

In July 1962 a diseased larva of the scarabaeid, *Sericesthis pruinosa* (Dalman), was found by Dr. R. J. Roberts in the vicinity of Tia, northern New South Wales. This larva (one of three found in 1962) was shown by Steinhaus and Leutenegger (1963) to be infected by a virus similar to the *Tipula* iridescent virus (TIV) described by Smith and his collaborators (see Smith 1963).

We wished to determine whether the Australian virus (designated as SIV) was closely related to TIV and this paper describes an examination of the properties of SIV. The two viruses are almost identical morphologically, but it was soon found that SIV differed from the published description of TIV in several respects. These findings suggest the desirability of re-investigating some of the properties of TIV.

II. Materials and Methods

The SIV was obtained from Professor E. A. Steinhaus who had passaged the original virus in *Galleria mellonella* (L.). This first-passage virus was sent to the Division of Entomology, CSIRO, in Canberra and was established in both *G. mellonella* and *Sericesthis pruinosa*. A small sample of TIV, obtained by Professor F. J. Fenner, Australian National University, from Mr. C. Rivers, Virus Research Unit of the Agricultural Research Council, Cambridge, was used in gel diffusion tests. Mr. Rivers also kindly supplied antiserum to TIV prepared in rabbits by Dr. R. H. Stobbart, University of Durham.

The protein content of the virus samples was estimated by the Folin–Ciocalteu method (Lowry *et al.* 1951), bovine serum albumin being used as a standard. Total

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nitrogen was estimated by the Kjeldahl method, and DNA was estimated by the method of Dische, as outlined by Schneider (1957), with calf thymus DNA as a standard.

Measurements of ultraviolet absorption were routinely performed in a Beckman DU spectrophotometer. Continuous spectra were recorded by a Unicam model SP700 spectrophotometer.

Electron-microscopic examinations were made on a Siemens Elmiskop I electron microscope. Particle counts were made by the method of Williams and Backus (1949), in which latex spheres 3650 Å in diameter (Dow Chemical Co. Batch No. LS–061A) were used. It was unnecessary to use metal shadowing or negative staining, since both virus and latex were readily countable at a magnification of 2000.

Infectivity assays were performed by inoculating 10-fold serial dilutions of virus into the prolegs of last instar *G. mellonella*, which were then kept unfed in petri dishes for 14 days at about 22°C. Approximately 0·02 μl of liquid was inoculated into each larva, this volume being estimated by inoculating a similar sample into a 1-μl Microcap disposable micropipette (Drummond Scientific Co.). The larvae usually pupated and evidence of infection could readily be seen on the ventral side of the pupae; the characteristic iridescent bluish fat-body could also be seen through the cuticle of larvae. If infection developed under suboptimal conditions, it could not always be determined by superficial inspection. However, a single low-speed centrifugation (2000 r.p.m., 5 min, Servall SS 34 rotor) followed by high-speed centrifugation (15,000 r.p.m., 20 min, Servall SS 34 rotor) of a larval extract was sufficient to obtain an iridescent pellet if the larvae were infected.

Antisera to highly purified SIV were prepared in rabbits by intramuscular inoculation of approximately 1·0 mg of virus in Freund's incomplete adjuvant, followed 30 days later by an intravenous inoculation of the same amount of virus without adjuvant. Serum was obtained 7 days later.

III. Experimental and Results

(*a*) *Preparation of Purified Virus*

Infected *Galleria* pupae were at first ground in distilled water and the virus purified by the method described by Smith (1963). Repeated differential centrifugations failed to improve the uniformity of these preparations and electron-microscopic examination revealed that numbers of virus particles appeared to be damaged (Plate 2, Fig. 1). Sedimentation of SIV from sodium chloride solutions of from 1·25 to 0·05M gave iridescent pellets of the blue colour characteristic of infected fat-body. In a solution of 0·005M NaCl the pellet was greenish. Sedimentation from solutions of sucrose gave pellets resembling those from distilled water. The inclusion of salts of heavy metals (iron, mercury, lead, zinc) in buffers resulted in the precipitation of the virus, whereas iridescent pellets were obtained from solutions of salts of lithium, caesium, magnesium, sodium, potassium, and calcium. When phosphate buffers were used the virus was precipitated, but citrate and borate buffers yielded virus pellets of high infectivity. Borate at pH 7·5 containing 0·1M NaCl gave clean pellets from which contaminants could be readily removed and the resulting virus showed few damaged membranes (Plate 1, Fig. 1).

A striking difference between these preparations and those made from virus prepared in distilled water was in the colour of the virus pellets resulting from centrifugation. In a preparation made in distilled water the pellets resembled a white opal, whereas in one made in buffer the pellets were uniformly iridescent blue.

The purity of virus preparations was not improved by treatments with Genetron or with ether, and attempts at isoelectric precipitation were unsuccessful.

As a result of these experiences, the following method was routinely adopted for the preparation of purified SIV. Infected *Galleria* pupae were macerated in a Teflon grinder in a standard buffer consisting of 0·01M borate–HCl at pH 7·5 containing 0·1M NaCl. For the preparation of large quantities of virus 0·08% sodium azide was added to the solution to inhibit bacterial growth. These suspensions were clarified at 1000 r.p.m. for 5 min (Servall SS 34 rotor). The supernatant was strained through muslin and then centrifuged at 15,000 r.p.m. for 20 min. A layer of cell debris generally covered the virus pellet, but this could be removed readily by gently washing with buffer. The virus pellet was resuspended by allowing to stand in buffer overnight at 4°C; the solution was clarified by low-speed centrifugation and sedimented again. After resuspending in buffer, each 1 ml virus was added to 22 ml of a sucrose density gradient (40%–5% in 0·01M borate buffer) and centrifuged for 20 min at 15,000 r.p.m. (Spinco SW 25 rotor). The visible virus zone from each tube was removed, mixed with those from other tubes, diluted in buffer, and the virus sedimented at 15,000 r.p.m. for 20 min (Spinco No. 30 rotor). Pellets were resuspended in borate–NaCl buffer, and the virus solution dialysed against buffer overnight at 4°C.

(*b*) *Evidence for Purity of the Preparation*

Virus suspensions prepared by the above method were found to contain very little non-viral material when examined in electron micrographs. Sections of pellets showed regular packing with little evidence of contaminants (Plate 1, Figs. 1 and 2). The suspensions gave single clear bands after centrifuging in density gradients. Sedimentation in a model E ultracentrifuge, and electrophoresis in a Perkin–Elmer model 38A apparatus (for details, see below) gave no indication of impurities.

(*c*) *Infectivity of the Virus Preparation*

Three questions could now be asked: (1) Is inoculation into *G. mellonella* larvae a sensitive assay for SIV? (2) What is the ratio of the number of total particles to the number of infectious particles? (3) Is the infectivity of virus prepared in distilled water different from virus prepared in buffer solutions? Virus was prepared from two similar infected pupae of *G. mellonella*. One preparation was made in 1 ml distilled water and the other in 1 ml buffer. Tenfold serial dilutions of each preparation were inoculated into *Galleria* larvae, and infections were found 14 days later in all larvae inoculated with either preparation at a dilution of 10^7. There was no difference in the infectivity of the two preparations. The volume inoculated was $1{\cdot}7\times10^{-5}$ ml. Therefore, one infectious unit was contained in a volume of $1{\cdot}7\times10^{-12}$ ml and each pupa contained $1/(1{\cdot}7\times10^{-12})$ or 6×10^{11} infectious units. Absorbance measurements (see below) showed that each pupa contained 2×10^{12} particles. Thus,

one infectious unit corresponded to 3·3 particles. This point was subsequently examined in more detail (see below). Furthermore, it appears that *G. mellonella* is very sensitive to infection. There were considerable errors associated with both the infectivity measurements and the particle counts, but the evidence shows that the total particle/infective particle ratio is not greater than 10 : 1.

(*d*) *Morphology of SIV Virus*

The appearance of SIV particles was studied in the electron microscope in sections of virus pellets and of cells of infected larvae [stained by uranyl acetate, lead hydroxide, and phosphotungstic acid], by negative and positive staining, and by shadowing of the isolated particles. These established that SIV and TIV are very similar in morphology. The SIV particles were icosohedral in shape (as shown by the shadows cast in metal-shadowed preparations) and measured 1300 Å in smallest diameter in sections (Plate 1, Fig. 2). The diameter was increased in phosphotungstic acid preparations (Plate 1, Fig. 1). Particles with disorganized membranes were common in most unfixed phosphotungstic acid preparations (Plate 2, Fig. 1), suggesting that SIV was more sensitive to damage than TIV, as described by Smith (1963). In spite of many attempts with negative staining we failed to obtain evidence of the 812 subunits described for TIV by Smith and Hills (1962).

(*e*) *Chemical and Physical Properties of SIV*

A standard sample of SIV was prepared and determinations made of particle numbers and infectivity, ultraviolet absorption, sedimentation rates, and electrophoretic mobility of the preparation.

(i) *Particle Numbers and Infectivity.*—Counts of virions demonstrated that the preparation had $6{\cdot}01\times10^{11}$ (standard error $0{\cdot}33\times10^{11}$) particles per millilitre. Tenfold serial dilutions of this preparation resulted in 7 out of 10 infections at a dilution of 10^7. There were thus 5×10^{11} infectious particles per millilitre, when measured by inoculation in last instar *Galleria* larvae, indicating a total particle/infectious particle ratio of approximately unity. The preparation contained 14·8 mg protein per millilitre (Lowry method). Kjeldahl estimation of total nitrogen gave 2·65 mg/ml. The sample contained 2·86 mg/ml DNA, i.e. 17·6% of the total weight. This value for DNA was not reduced by treatment of the virus with deoxyribonuclease (50 μg/ml, 0·1% $MgCl_2$) at room temperature for 1 hr. The preparation contained 264 absorbance units per millilitre, so that one absorbance unit at 260 mμ is equivalent to 0·056 mg protein per millilitre. Portion of this preparation (3 ml) was exhaustively dialysed against distilled water. After drying overnight over P_2O_5 the sample weighed 16·23 mg. The weight was not further reduced by heating to 80°C *in vacuo* for 3 hr. Thus, 1 virion weighs $0{\cdot}01623/(6{\cdot}01\times10^{11})$ g, or $2{\cdot}7\times10^{-15}$ g, a value of the same order of magnitude of the weight of the TIV particle ($2{\cdot}3\times10^{-15}$ g) reported by Thomas (1961).

From these data it may be calculated that there are $2{\cdot}46\times10^{-11}$ mg protein per virion, and that there are $2{\cdot}3\times10^{9}$ virions per absorbance unit. Similar measurements revealed that each infected *Galleria* pupa (weighing approximately 90 mg)

contained not less than 2·0 mg virus, equivalent to $7{\cdot}4\times10^{10}$ virions per pupa. This amount of virus represents 2% of the wet weight of the host insect. Approximately 0·05 mg SIV could be detected as an iridescent pellet after centrifugation.

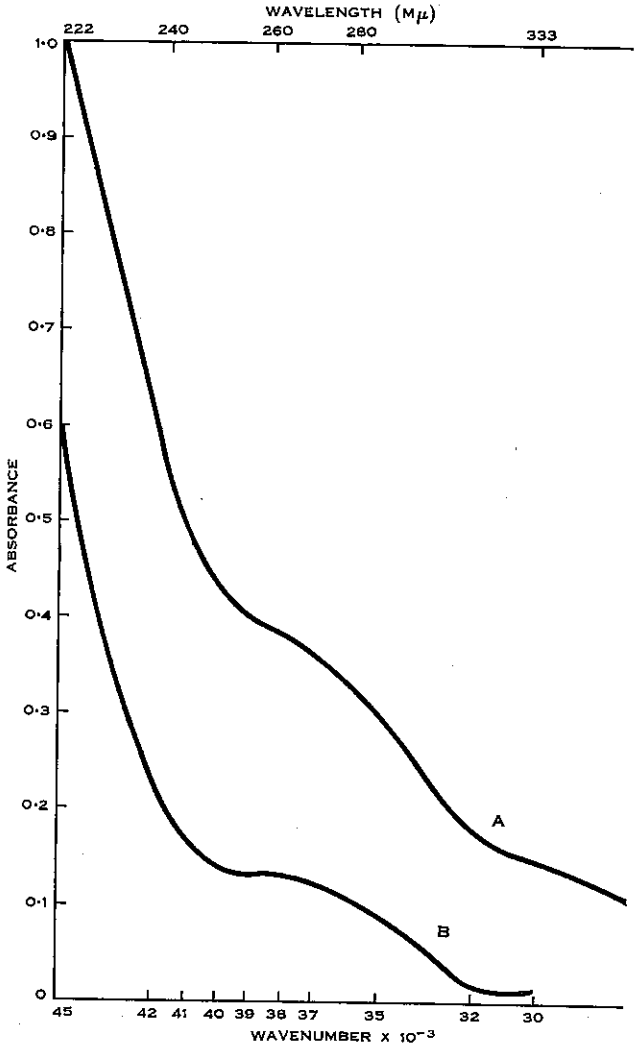

Fig. 1.—Ultraviolet spectrum of SIV in aqueous suspension. *A*, uncorrected curve; *B*, curve corrected for light scattering.

This represents approximately $1{\cdot}8\times10^{9}$ virions. The following tabulation compares data on the composition of SIV with comparable data given by Green (1963) for adenovirus type 2:

	Dry Weight (mg/ml)	Protein (mg/ml)	Phosphorus (mg/ml)	DNA Content (mg/ml)	DNA Content (%)	DNA Content (μg/mg protein)
Adenovirus 2:	7·90	7·19	0·110	1·02	12·9	143
SIV:	16·23	14·80	0·243	2·86	17·6	193

(ii) *Ultraviolet Absorption.*—The ultraviolet absorption spectrum of SIV is illustrated in Figure 1,*A*. Approximately 66% of absorption at 260 mμ is due to light scattering. When a correction for light scattering by the method of Bonhoeffer and Schachman (1960) is used the absorption spectrum is that shown in Figure 1,*B*. The ratio of absorbance at 260 mμ to that at 280 mμ is 1·23.

(iii) *Sedimentation Coefficient.*—In some sucrose density gradient preparations some virus was occasionally found in pellets, as well as a substantial part in light-scattering zones. The $S_{20,\,w}$ of both fractions was very close to 2200 S, the value reported by Weber, Kupke, and Beams (1963) for TIV. It is surprising that the sedimentation coefficients of SIV and TIV are so close, considering that the former contains more DNA than TIV. Details of the sedimentation characteristics of SIV will be published in more detail in a later paper.

TABLE 1

INACTIVATION OF SIV BY HEAT

Numbers of *Galleria* larvae infected out of 5 inoculated

Temperature (°C)	Time (min): 5	10	30	60
70	0	0	0	0
60	0	0	0	0
50	5	4	0	0
50*	0	0	0	0
Room temp.	5	5	5	5

* In the presence of 1M $MgCl_2$.

(iv) *Electrophoretic Mobility.*—Electrophoresis of the purified virus was performed in a pH 7·4 buffer consisting of 0·01M Na_2HPO_4, 0·0033M NaH_2PO_4, and 0·167M NaCl. A potential drop of 1·45 V/cm during 370 min gave a single apparently homogeneous boundary with a mobility of $0{\cdot}6\times10^{-5}$ cm^2 V^{-1} sec^{-1}. This low value could mean that pH 7·4 was near the isoelectric point of the virus, but there is other evidence that the particles carry a large sheath of water molecules (Klug, Franklin, and Humphreys-Owen 1959), and this could be the explanation of the low mobility observed.

(*f*) *Biological Properties of SIV*

(i) In vitro *Temperature Sensitivity.*—Although it has been stated (Smith 1963) that TIV is a resistant virus, no details have been reported. Table 1 shows that the infectivity of a dilute SIV solution is destroyed completely at 60°C for 5 min and by 30 min at 50°C, but not by 10 min at 50°C. The addition of magnesium chloride to a final concentration of 1M in the solution greatly increased the thermal instability of SIV. In this respect SIV is similar to the adenovirus and pox virus groups (Wallis and Melnick 1962). SIV is therefore not particularly thermostable

and the results suggest the desirability of maintaining low temperatures during preparative manipulations to retain optimum infectivity.

(ii) In vivo *Temperature Sensitivity.*—The effect of environmental temperature on the development of infection in the intact *S. pruinosa* was observed by placing larvae at 11, 16, 22, and 28°C following inoculation with a standard infecting dose. At 16 and 22°C the infection developed fully within 14 days, at which time much of the larval fat-body was characteristically iridescent. Very slight signs of infection were evident in larvae kept at 11°C after 14 days. At 28°C no signs of infection appeared at all, and the larvae died of desiccation after 21 days without exhibiting any evidence of infection. This experiment was repeated with *Galleria* which is better adapted to high temperatures than is *Sericesthis*. Evidence of virus produced at various temperatures given in Table 2 suggests that virus multiplication takes place only at the lower temperature.

TABLE 2

AMOUNT OF SIV PRODUCED BY GALLERIA LARVAE AT VARIOUS TEMPERATURES

First Treatment		Subsequent Treatment		Virus Concentration*	Antigens Detected in Gel Diffusion Tests
Time (days)	Temp. (°C)	Time (days)	Temp. (°C)		
14	22			0·700	++
4	22	10	28	0·040	−
8	22	6	28	0·295	+
12	22	2	28	0·750	±
1	28	13	22	0·745	+
2	28	12	22	0·700	++
4	28	10	22	0·330	+
8	28	6	22	0·060	±

* Absorbancy measurements at 260 mμ on purified virus preparation from one larva diluted 10 times (average of 10 readings).

(iii) *Sensitivity to Organic Solvents.*—The property of ether or chloroform sensitivity is an indication of the presence of lipid essential for infectivity of viruses and has been used as a criterion of relationship in several schemes of viral classification. The sensitivity of SIV to ether was tested according to the procedure of Andrewes and Horstmann (1949), and to chloroform by following the method of Feldman and Wang (1961). No decrease in infectivity occurred, showing that SIV is completely resistant to both ether and chloroform. This was so whether the treated virus had been prepared in buffer or in distilled water. Thus, although the presence of lipid-containing substances in TIV has been reported by Thomas (1961), a lipid is apparently not essential for infectivity in SIV. The appearance of SIV in the electron microscope after treatment with ether is not significantly different from the appearance of untreated virus.

(iv) *pH Lability.*—A criterion of viral relationship used by Kelter, Hamparian, and Hilleman (1962) and by Hamparian, Hilleman, and Kelter (1963) is the stability of virus to pH 3·0 for 3 hr at room temperature. An aliquot of the standard SIV preparation was taken to pH 3·0 with 0·1M HCl and another to pH 10·5 with 0·1M NaOH. After 3 hr these samples were neutralized and 10-fold dilutions inoculated into *Galleria* larvae. Both samples were infectious after dilution to 10^{-7}. It was unexpected to find infectivity retained after treatment with alkali because it is known that pH 10·5 results in the appearance of "soluble" viral antigens in a virus preparation [see Section III(*f*)(v)]. It is apparent that sufficient infectious particles remained to infect even after considerable dilution. This may be taken as evidence that the viral "membrane" is not necessary for infectivity as measured by inoculation into *Galleria* larvae.

(v) *Serological Properties.*—Antisera produced in rabbits against SIV gave several very pronounced lines in Ouchterlony tests against macerated infected (but not uninfected) *Galleria* larvae. An aqueous extract from a single larva showed a precipitation zone in agar only if not diluted to more than 10 ml. The antiserum had a titre of 10^{-2} in similar tests.

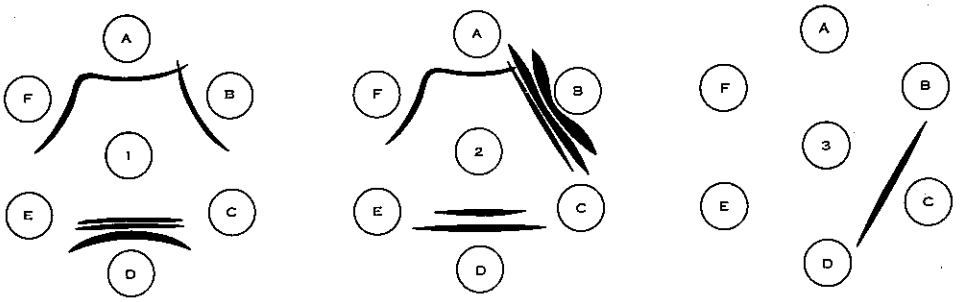

Fig. 2.—Diagrams of gel diffusion plates. Antisera in centre wells: *1*, SIV antiserum; *2*, TIV antiserum; *3*, adenovirus 5 antiserum. Peripheral wells: *A*, SIV soluble antigen; *B*, TIV, pH 10·5; *C*, adenovirus 5; *D*, SIV, pH 10·5; *E*, extract of normal *Galleria* larva; *F*, extract of *Galleria* larva infected by SIV.

This antiserum did not react against intact SIV particles prepared either in buffer or in distilled water. If, however, the pH of the SIV solution was raised to 10·5, then antigens were detectable, showing identity with those of the infected larvae (Plate 2, Fig. 2). No such antigen appeared after treatment of the virus at pH 3·0. Aqueous extracts of infected larvae centrifuged at 39,000 r.p.m. (Spinco 40 rotor) for 1 hr contained a soluble antigen, which is serologically identical with an antigen obtained from the purified virus either by treatment at pH 10·5 or by sonic disintegration produced by a Mullard ultrasonic disintegrator (20 kc/s). This antigen is destroyed by ethanol and by acetone but not by heating to 75°C for 5 min. An attempt was made to estimate the molecular weight of this soluble antigen by the method of Allison and Humphrey (1960). A strong line was present making an angle of 52° with the antigen, suggesting a molecular weight of the order of 70,000.

A comparison between the serological responses of SIV and TIV showed unequivocally that there were marked differences between these two viruses (Fig. 2).

A second isolate of SIV from *S. pruinosa* from Tia, N.S.W., in October 1963 was serologically identical with the original isolate, even though the latter had been cultured by several passages through *Galleria*. In view of the several similarities between SIV and the adenoviruses, and the observation that the latter contain a common antigen (Pereira *et al.* 1963), it seemed worth while to determine whether SIV contained an antigen common to that found in adenovirus. The results (Fig. 2) demonstrate that the antigens of SIV are not related to the antigens of adenovirus 5.

(vi) *Host Range of SIV*.—No systematic attempt has been made to determine the host range of SIV, but several incidental observations are of interest. The virus was found, as stated above, in the scarabaeid *S. pruinosa* which is a pest species over much of eastern Australia. The virus was infectious by inoculation into larvae of the larger *Anaplognathus* sp. and of *Anodontonyx nigrolineatus* (Boisd.). The latter appeared to be very susceptible to oral infection, and it was generally killed if high concentrations of virus were injected. Both *Anodontonyx* and *Sericesthis* were readily infected when virus preparations were placed on the mouthparts of CO_2-anaesthetized larvae. *A. nigrolineatus* occurred in association with infected *S. pruinosa* but was not found infected in the field (Roberts, personal communication). Attempts to infect *Tenebrio molitor* L. larvae and adults either by feeding or inoculation were unsuccessful. Attempts to infect *Periplaneta americana* (L.) and *Ctenolepisma longicaudata* Esch. by injection were unsuccessful. *G. mellonella*, *Pieris rapae* (L.), and *Bombyx mori* (L.) were readily infected by inoculation, but attempts to infect them by feeding were unsuccessful. When SIV was added to the medium in which second instar larvae of *Aedes aegypti* (L.) were developing, after 2 weeks the fat-body in two living larvae became iridescent blue when viewed through the cuticle, indicating infection by SIV. Thus SIV is similar to TIV in that it is capable of infecting some species of Coleoptera, Diptera, and Lepidoptera.

IV. Discussion

SIV is morphologically indistinguishable from TIV. The two viruses are similar in size, shape, sedimentation coefficient, host range, and in the possession of DNA. These characters demonstrate the close similarity of the two viruses. However, SIV differs from TIV in several respects. It contains a higher percentage of DNA. Three estimations of the DNA content of TIV have been published. Smith (1958) reported it to be about 15%, Thomas (1961) found 12·4%, and Allison and Burke (1962) reported a value of 16%. These reports are less than the 17·6% found for SIV. SIV also has serological differences from TIV, each virus having three antigens detectable in gel diffusion tests, none of which is common to both viruses. SIV also appears to be more damaged in distilled water than TIV, but it would seem desirable to examine the effect of buffers on TIV. TIV has been reported to have 812 structural units (Smith and Hills 1962), but we were unable to demonstrate this structure in SIV. For the present it seems desirable to treat SIV and TIV as distinct viruses belonging to the same group.

Thermal lability, ether insensitivity, and pH stability are three characteristics which SIV shares with the adenovirus group. This group consists of a series of viruses with varying numbers of structural units, and TIV was assigned to this

group by Lwoff, Horne, and Tournier (1962). SIV is very different structurally from the adenovirus group and has no serological relationship detectable in gel diffusion tests.

The ultraviolet absorption spectrum of SIV differs from that expected for a nucleoprotein of the composition described for this virus. The ratio of absorbance at 260 mμ to that at 280 mμ of 1·23 is lower than expected on the basis of the amount of DNA in the particle. This may be explained on the basis of the "enhanced mutual shading" effect described by Duysens (1956). Ultraviolet absorption spectra for TIV have been reported by Thomas (1961) and by Thomas and Williams (1961).

The evidence of several experiments suggests that infectivity (as measured by inoculation into *Galleria*) is unaffected by the presence of the particle membrane. Removal of or damage to the membrane by water, by high pH, or by sonic disruption does not reduce infectivity.

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

Fig. 1.—SIV prepared in standard buffer, fixed in osmic acid, and examined in phosphotungstic acid, pH 7·0. Compare with Plate 2, Figure 1.

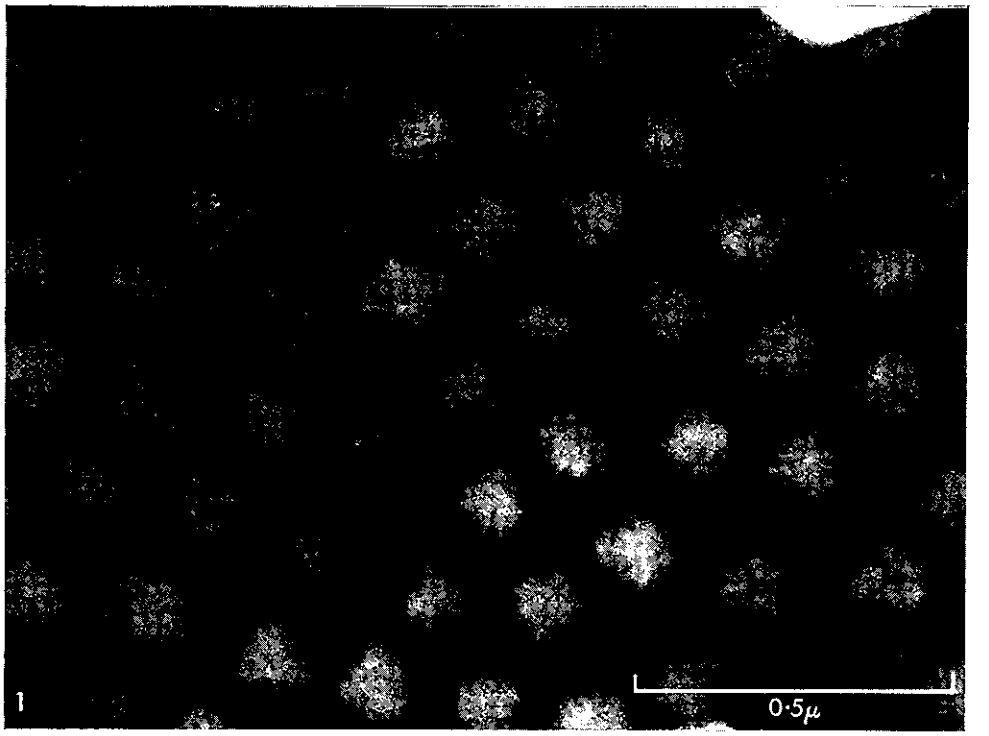
Fig. 2.—Section of SIV virus particles, stained with uranyl acetate, showing central core containing DNA. Note regularity of arrangement of virus particles.

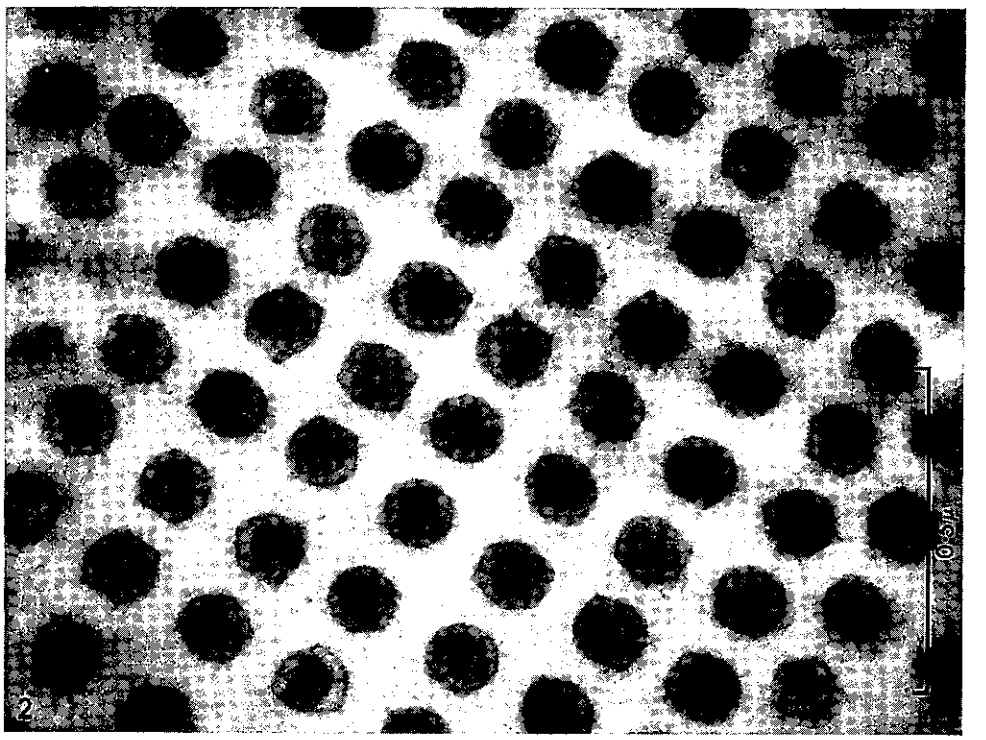
PLATE 2

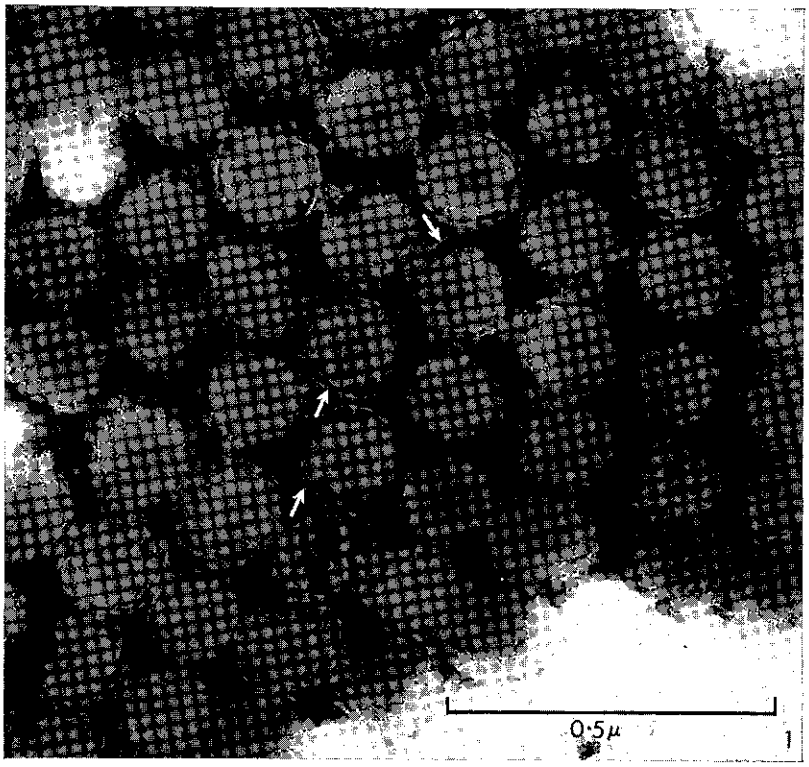
Fig. 1.—SIV prepared in distilled water, and examined in phosphotungstic acid. Note damaged viral membrane (arrows).

Fig. 2.—Ouchterlony gel diffusion plate showing serological difference between SIV and TIV. SIV antiserum in centre well. Peripheral wells: *1*, SIV pH 10·5; *2*, SIV pH 11·5; *3*, TIV pH 10·5; *4*, TIV pH 11·5; *5*, SIV intact virus; *6*, brei from *Galleria* larva infected with SIV.

PROPERTIES OF AN IRIDESCENT VIRUS






0·5μ
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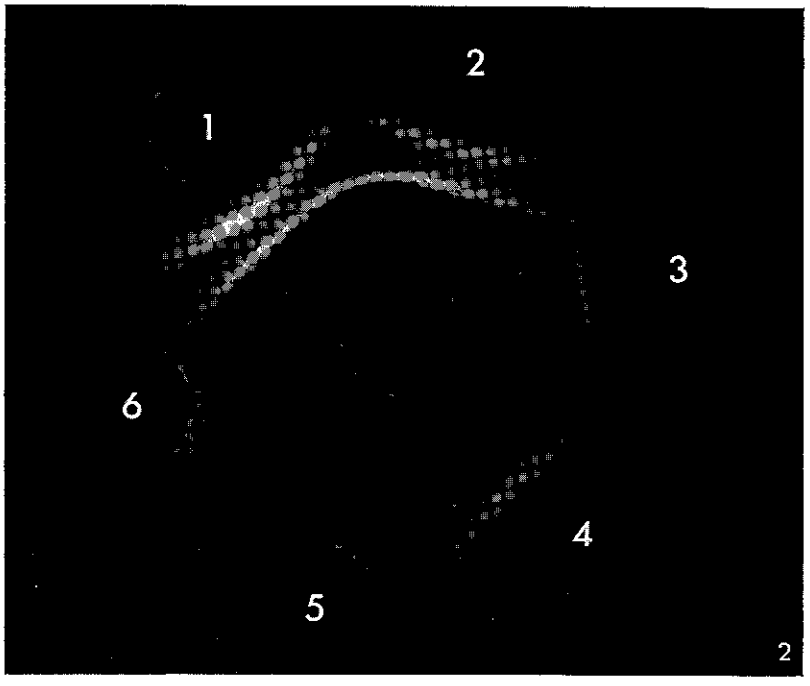
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