In the wet season trials 1st instar *An. gambiae* larvae were recovered 8 and 10 days after treatment respectively in plots treated with 1 percent Abate sand granules and with an emulsion formulation at applied concentrations of 1.0 ppm. The presence of only 1st instar larvae in the emulsion treated plots during a 24-day observation period suggested a sorption-release activity of Abate maintaining sufficient insecticidal effect to kill larvae before reaching 2nd or 3rd instar.

In the dry season trials 1st instar *An. gambiae* larvae were first recovered from field plots treated with Abate 1 percent floating granules at the applied concentration of 1.0 ppm, 19 days after treatment with vermiculite and perlite formulations and 13 days in the krobite treated plots.

Further evidence of a sorption-release activity was suggested by the lower mortality rates of third instar *An. gambiae* larvae bioassayed in plot water samples than the rates observed in bioassays made in the plots themselves.

ASSAYS FOR THE FRIEND MURINE LEUKEMIA VIRUS (FLV) COMPLEX IN THE STABLE FLY, *STOMOXYS CALCITRANS*

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INTRODUCTION. In 1956, Friend (1956) isolated a Type C virus pathogenic to mice which had been inoculated with cell-free extracts prepared from the Ehrlich ascites mouse carcinoma. The agent, originally considered as a single virus, caused a leukemia-like disease syndrome similar to erythroblastosis, with characteristic enlargement of spleen and liver; subsequently the Friend murine leukemia virus (FLV) was found to consist of a complex of viruses. The component which induces lymphatic leukemia *in vivo* is known as the Friend lymphatic leukemia virus (FLLV or LLV).

Axelrad and Steeves (1964) developed a rapid quantitative assay for Friend leukemia virus based on enumeration of macroscopic surface spleen foci following 30–60 seconds immersion of the extirpated spleen in a fixative composed of ethyl alcohol, glacial acetic acid, formaldehyde and water. The spleen focus-forming virus is "defective" making it incapable of undergoing its entire infectious cycle and of inducing erythroleukemia in the absence of co-infection with LLV (Steeves and Eckner, 1970). Thus LLV serves as a helper virus, supplying factors missing from the SFFV genome, which then permits the production of infectious SFFV virus. In the Mirand strain of the FLV complex, which induces spleen focus formation and polycythemia, there is also present lactic dehydrogenase–elevating virus (LDV) (Riley *et al.*, 1960).

Since 1965, experiments have been carried out at the University of North Dakota to determine the extent to which various murine, avian, and feline oncogenic viruses could be transmitted mechanically or biologically by a variety of blood-sucking arthropods. The complex nature of the

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FLV made it necessary to consider a number of factors when attempting to transmit this virus biologically, or when determining its viability or extrinsic incubation period. The effect of the insect host on the ingested viral complex did not arise when simple mechanical transmission on the stylets of the proboscis was involved.

Prior studies concerning extrinsic incubation of FLV in the stable fly, *Stomoxys calcitrans* involved feeding 1–3 day old male and female flies fully on FLV-infected mice or on sterile sponges soaked with FLV-infected blood or blood-spleen mixtures, the viral titers having been determined previously. At various time intervals 35 infected flies were homogenized. The homogenate was diluted appropriately, and 0.2 ml was injected intraperitoneally (ip) into each of five 4–6 week old BALB/c mice. The mice were autopsied when spleens were large and palpable or at 21 days postinjection. Mice with spleen weights of 275 mg or more, and whose spleens developed 5 or more external foci when tested, were considered positive of Friend disease. Although 180 mg spleen weight end-points are most commonly accepted as indicators of Friend-induced splenomegaly (Chirigos *et al.*, 1967) it was found that the presence of insect material in test material occasionally produced splenomegaly, even in the absence of FLV (Luecke *et al.*, 1973).

It was important to determine whether apparent virus decay patterns in the stable fly (Rehacek *et al.*, 1973) were due to total inactivation or represented inactivation of only a portion of the FLV complex. Therefore, a collaborative study was undertaken with the National Cancer Institute and the sensitive XC test was employed in order to monitor individual components of the FLV complex. Spleens subjected to XC testing cannot be subjected to fixation procedures involved in foci enumeration and therefore it was decided to include spleens in this study which at autopsy weighed more than 780 mg (i.e. the most commonly accepted parameter for non-arthropod studies). This test was developed by Klement *et al.* (1969). They showed that a cultivable line of cells derived from a rat lymphoma which had been induced by the Prague strain of Rous Sarcoma virus (XC cell), was stimulated to form syncytia when exposed to mouse cells infected with murine oncogenic RNA virus. This reaction, known as the XC reaction, forms the basis of both a quantal and quantitative assay of murine leukemia virus *in vitro*, and is referred to as the mixed cytopathogenic or XC assay (Rowe *et al.*, 970; Bass and Turner, 1972).

The present paper deals with preliminary results when using this XC test in the determination of the extrinsic incubation characteristics of the FLV complex in the stable fly. Comparisons of XC, splenomegaly, and back titration studies were made.

MATERIALS AND METHODS: *Mice:* All animals used as donors, as well as in bioassay procedures were 4–6 week-old BALB/c males from the A. R. Schmidt Co. (ARS/Sprague Dawley, Life Sciences Division, Mogul Corporation) Madison, Wisconsin.

Virus: Seed FLV consisted of a 50 percent homogenate of splenic tissue from a female DBA mouse infected with FLV obtained from Dr. C. Friend. This virus had undergone 69 passages in BALB/c mice in the University of North Dakota laboratory. The titer of the virus was $10^{-5.53}$ 50 percent end-point spleen enlarging dose (SED_{50}), and spleen focus-forming units (SFV) per ml in BALB/c mice.

Insects: Stomoxys calcitrans were obtained from Dr. Calvin M. Jones, USDA, Lincoln, Nebraska. The larvae were reared in CSMA standardized fly larval medium (Ralston Purina, Kansas City, Missouri). The adult flies were maintained in 1 $ft.^3$ cages at 27° C. and 60 percent relative humidity. Both the normal colonies used for egg production and the experimental flies were fed daily on normal citrated human blood.

Bioassay procedure: Approximately

6,000 mixed male and female *Stomoxys calcitrans* 2–3 days old were allowed to feed fully on an FLV-meal prepared by homogenizing 44 infected spleens weighing a total of 103.8 gms. in a Virtis "45" homogenizer using 103.8 ml of cold saline; an additional 103.8 ml of whole citrated blood was then added, and mixed thoroughly. Gauze sponges were soaked in this mixture and introduced into the fly cages. Flies judged to be fully fed by observation of the blood-bloated abdomen were aspirated into 1 ft.^{3} cages. They were maintained at 16° C., and offered whole human citrated blood daily.

Beginning on day 0 (1 hour postfeeding) and at 5, 7, 11, 14, and 21 days post-FLV-feeding, 10–15 groups of 35 flies each were homogenized in Ten-Broeck homogenizers with 2.0 ml cold saline containing 1,000 units of penicillin/ml and 5 mcg of streptomycin/ml. The homogenates were each centrifuged at 2400 rpm for 5 minutes. The supernatant fluids were recovered and considered as the 10° (undiluted) fraction. Serial 10-fold dilutions in saline containing penicillin and streptomycin were also prepared for the 0 and 5 day study. Two-tenths of a ml was injected ip into each of five recipient BALB/c mice per dilution. This procedure was repeated for each of the 10–15 groups tested each day of assay. Recipient mice were autopsied 21 days later, this being the standard protocol. This procedure was employed at 0 and 5 days for dilutions between 10^0 and 10^{-5}. The day 7, 11, 14, and 21 studies included mice injected only with 10^0 preparations. Fifty mice (10 groups of 5) each were used for the 7 and 11 day studies, but 75 mice (15 groups) were used for the 14 and 21 day studies. Since possible virus inactivation might result from action of the fixatives used in the spleen focus assay, only 5 animals were randomly selected from each group for spleen weight *plus* splenic foci determinations. All remaining spleens (i.e. the specimens assayed, as well as those for possible future XC testing) were weighed. Those weighing 180 mg or more, were prepared as 10 percent homogenates in physiological saline containing penicillin, streptomycin and 4 percent fetal bovine serum, and frozen at −80° until the XC test could be performed.

Cell cultures: National Institutes of Health (NIH) Swiss mouse embryo fibroblast (MEF) cells and the XC cell line, provided by Dr. W. Rowe, NIH, Bethesda, Md., were grown in Eagle's Minimum Essential Medium with Earle's salts, 1 percent L-glutamine (200 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 0 percent fetal calf serum.

XC assay: The procedure for the XC assay for murine leukemia viruses was identical to the procedure described by Rowe *et al.* (1970). Briefly, culture plates were seeded with 3.5 x 0^5 mouse embryo fibroblast cells and incubated at 37° C. for 24 hours in a humid atmosphere containing 5 percent CO_2. Cultures were treated with DEAE-dextran (50 μg per plate) for 1 hour at 37° C. Cultures were washed once with growth medium and inoculated with 0.4 ml of various dilutions of virus preparations, i.e. of a 10 percent spleen homogenate prepared from a BALB/c mouse injected 21 days earlier with an homogenate of FLV-infected *Stomoxys calcitrans* held for a certain time interval after the infected blood meal. Then 3.6 ml of growth medium was added to each plate, and the cultures were incubated for an additional 6 days, after which they were exposed to ultraviolet light (60 erg/mm/second for 30 seconds). The cultures were then seeded with 1 x 10^6 XC cells in 4.0 ml volume, and 4 days later were fixed with methanol and stained with Papanicolau hematoxylin stain. They were then examined macroscopically and microscopically for multinucleated giant cells or syncytia, designated as XC plaques, and the titer of the virus preparation was reported as $\log_{10}$ plaque forming units (PFU) per ml.

PROCEDURE AND RESULTS AT VARIOUS TIMES POSTFEEDING. *Test for FLV titers:*

1 hour and 5 day studies. Fifty 4–6 week-old BALB/c mice were each injected ip with 0.2 ml of various dilutions (10^{0}–10^{5}) of homogenates prepared from *Stomoxys calcitrans* which had fed either one hour (0 days) or 5 days earlier. All animals were autopsied 21 days later, and spleen weight and foci were recorded. The ID_{50} was found to be $10^{-3.5}$ per ml at 0 day, and $10^{-0.9}$ per ml at 5 days. These animal bioassay results are similar to those found in the University of North Dakota laboratory during previous extrinsic incubation studies, and indicated that the FLV was of satisfactory titer for extended viability studies. The 50 percent infectious dose (ID_{50}) was estimated by the method of Reed and Muench *et al.* (1938).

Seven day study: Fifty 4–6 week-old BALB/c mice were injected ip with 0.2 ml of a 10^{0} dilution of *Stomoxys calcitrans* (35 flies/2.0 ml diluent) fed an FLV meal 7 days earlier. At autopsy, 21 days later, one cage of 5 mice was selected for spleen weight plus spleen foci determinations. Spleen weights ranged between 121 mg–130 mg, and *none* had foci characteristic of FLV involvement. Among 12 additional mice with spleen weights between 124 mg–946 mg, eight had significant spleen focus development (ranging from 5 to confluent). Altogether 20 of 50 animals had spleens weighing more than 180 mg.

One 210 mg spleen on which spleen focus assay was not done was processed and frozen for XC testing (Table I). The four cage mates of this mouse developed spleens of 127 mg, 135 mg, 138 mg, and 193 mg, but splenic foci were *not* determined due to possible virus inactivation as a result of the spleen-fixation procedure involved. The 210 mg spleen material was divided before freezing (i.e. split sample), one aliquot for XC testing, the other for back titration in mice. Results of the XC test indicated $>0^{5}$ plaque forming units (PFU/1.0 ml). Of 15 animals inoculated with this material (Table I) *none* showed evidence of a leukemic response (spleen weights of 143 mg to 278 mg, *none* with foci). Five of these 15 mice were not autopsied until 50 days after injection. Their spleens represented the 5 largest in this back titration, (214 mg–278 mg) but showed no evidence of splenic foci.

Eleven day study: Fifty 4–6 week-old BALB/c mice were injected ip with 0.2 ml of a 10^{0} dilution of *Stomoxys calcitrans* (35 flies/2.0 ml diluent) fed an FLV meal 11 days earlier. At autopsy, 21 days later, in one cage of 5 mice spleen weights ranged between 127 mg–153 mg and *none* had foci, all therefore being negative. Eight of the 50 were found to have spleens over 180 mg. These were homogenized, divided (split sample), and frozen at −80° C. The largest spleens, weighing 234 mg, 379 mg, and 241 mg were prepared for XC assay.

a. *234 mg spleen.* Cage mates of the mouse with the 234 mg spleen were found to have spleens of 141 mg, 189 mg, 206 mg and 379 mg. Spleen foci were *not* determined on these. XC PFU were 10^{5}/ml. Back titration studies were performed, and of 15 animals inoculated with the 234 mg spleen, all showed evidence of a leukemic response (spleen weights 626 mg–2060 mg, and all had too numerous to count (TNTC) or confluent foci). Ten of the 15 animals in this group were autopsied at 21 days post-inoculation and the remaining 5 were dead by the 48th day. Thus the LLV and SFFV components of FLV retained their potency to the second animal passage and had considerable viability after 11 days in the insect.

b. *379 mg spleen.* Cage mates of the mouse with the 379 mg spleen were those in the above paragraph. Of 15 animals inoculated in back titration studies, all demonstrated a leukemic response (spleen weights 1439 mg–1995 mg, and all had confluent foci). Only 3 animals survived the 21 day period. All 12 others were dead from leukemia on or before the 20th day. XC PFU were 2.73×10^{7} ml.

TABLE 1.—*In vivo* and *in vitro* results following inoculation of BALB/c mice with Friend murine leukemia virus complex incubated for 7, 11, 14, and 21 days in the stable fly, *Stomoxys calcitrans* (Linnaeus).

In vivo assay including spleen weights and spleen foci				*In vitro* XC assay		*In vivo* back titration of XC test spleens	
FLV Incubation *in Stomoxys calcitrans* (days)	No. of positive BALB/c recipients (spleen wt. >180 mg) / Total injected	Spleen weight range of positives (mg) / Total range (mg)	No. of BALB/c with splenic foci / Total tested for foci	Test spleen weight (mg)	Virus titer XC PFU/ml	Back titration range (mg) 21 days / Back titration range (mg) 50 days	Back titration results at 21 days / Results at 50 days
7	20/50	190–946/97–946	8/17	210	$>10^5$	(10 BALB/c) 143–206 / (5 BALB/c) 214–278	No foci / No foci
11	8/50	185–379/86–379	0/5	a) 234	$>10^5$	(15 BALB/c) 626–2060 / All dead before 50 days	All with confluent foci
				b) 379	2.73×10^7	(15 BALB/c) 1439–1995 / All dead before 50 days	All with confluent foci
				c) 241	$>10^5$	(10 BALB/c) 161–240 / (5 BALB/c) 218–1921	0–1 foci / 0–confluent foci
14	1/75	617/89–617	0/5	617	$>10^5$	(15 BALB/c) 582–2137 / All dead before 50 days	All with confluent foci
21	7/75	181–252/81–252	0/5	252	$<10^{2.5}$	(10 BALB/c) 102–132 / (5 BALB/c)	No foci / No foci

The LLV and SFFV were also viable after 11 days in the stable fly.

c. *241 mg spleen.* Cage mates of the mouse with the 241 mg spleen were found at autopsy to have spleens of 135 mg, 145 mg, 191 mg and 226 mg. Spleen foci were not determined. XC PFU were $>10^5$/ml. Back titration studies were performed in 15 animals. Ten mice were autopsied at 21 days post-injection, and none showed evidence of leukemia (spleen weights 161 mg–240 mg with 0 or 1 focus per spleen); however, three of five animals held for 50 days were positive for leukemia (spleen weights of 218 mg–1921 mg with 15 to TNTC foci). It appears here that the standard 21-day holding period prior to autopsy may be too short for completing such a study.

Fourteen day study: Seventy-five 4–6 week old BALB/c mice were injected ip with 0.2 ml of a 10^0 dilution of *Stomoxys calcitrans* (35 flies/2.0 ml diluent) fed an FLV-meal 14 days earlier. At autopsy 21 days later, in 1 cage of 5 mice spleen weights ranged between 141 mg–178 mg, and *none* showed foci. Only *1* of the remaining 70 mice had a spleen exceeding 180 mg; this spleen, weighing 617 mg (Table 1) was homogenized, divided (split sample), and used in the VC test. Cage mates of this mouse had spleens of 126 mg, 132 mg, 137 mg and 142 mg, and no spleen foci were determined. Results of the XC test on the 617 mg spleen were $>10^5$ PFU/ml. Of 15 animals back-titrated with this spleen, all showed evidence of a leukemic response (spleen weights 582 mg–2137 mg, and all had confluent foci). Only 3 of the 15 mice survived to the 21st day; all others died on or before the 50th day post-injection. Thus there was only one positive result among 75 mice inoculated with a preparation made from flies fed on FLV 14 days earlier, indicating a possible persistent infection in a small percentage of flies.

Twenty-one day study: Seventy-five 4–6 week-old BALB/c mice were each injected ip with 0.2 ml of a 10^0 dilution of *Stomoxys calcitrans* (35 flies/2.0 ml diluent) fed on FLV meal 21 days earlier. In 1 cage of 5 mice 21 days later, spleen weights were 120 mg–148 mg, and *none* had foci. Seven of the 75 mice had spleen weights between 181–252 mg (Table 1). The largest was prepared as described earlier. Cage mates of the mouse with the 252 mg spleen had spleens of 120 mg, 155 mg, 181 mg, and 208 mg. Splenic foci were not determined. Less than $10^{2.5}$ PFU/ml were noted in the XC test. Back titration studies were performed in 15 animals. None of 10 mice 21 days later showed any FLV infection (spleen weights 102 mg–127 mg, none with foci). The 5 remaining mice were observed for 50 days, at which time the spleen weights ranged 91 mg–214 mg, and none had foci. Thus results were completely negative in the second *in vivo* passage. However, since 7/75 first passage animals had spleen weights between 180 mg and 252 mg with no foci, possibly LLV may have survived in the flies after 21 days, but not the SFFV.

DISCUSSION. The studies described in this paper have involved a combination of two methods of determining FLV activity and viability in insects: (a) the standard *in vivo* bioassay by inoculating into susceptible mice homogenates of virus-infected insects after various days post-feeding, and (b) the *in vitro* XC assay for quantitative detection of murine leukemia virus, using an extract of the spleen of a mouse that had been inoculated with infected insect homogenate. In this present study there was no attempt to determine where the virus was distributed within the fly. It appears, however, that in various individual insects, the virus retains its potency and replicates. In some cases the spleen focus-forming virus (SFFV) component of FLV is inactivated, and in other cases it is not. The 7, 11, and 14 days of FLV viability in the stablefly is evident from the data given. At 21 days the SFFV component is inactivated, with no positive spleen foci results, and a drop in XC

assay virus titer to $<10^{2.5}$, PFU/ml from a consistent potent level of $>10^5$. The FLV complex, therefore, appears to become inactivated in the stable fly between 11 and 21 days.

SUMMARY: Friend murine leukemia virus complex, comprising the defective lymphatic leukemia virus component (SFFV), was detected in spleens of BALB/c mice inoculated with homogenates of the stable fly, *Stomoxys calcitrans*, at a titer greater than 10^5 XC assay plaque forming units (PFU) per ml, after 11 days, and in one instance up to 14 days, in the insects. During this period varying numbers of mice developed Friend leukemia, using the criteria of (a) at least 180 mg spleen weight plus, (b) at least 5 spleen foci. Replication or delayed decay of the FLV components appeared to occur in certain lots of the insects up through 11 days, after which both components appeared to deteriorate, except in 1 out of 75 mice inoculated with 14-day stable fly-incubated virus. At 21 days the XC titer dropped to $<10^{2.5}$ PFU/ml, with concurrent and complete inactivation of the SFFV.

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