of 68,000 and 60,000, respectively; hence, each should be similarly restricted by the dimensions of the pores in the gel. The hemoglobins and the *Drosophila* homogenate were both placed on 5-percent polyacrylamide, and electrophoresis was performed (*3*) in 0.05*M* tris (adjusted to *p*H 8.6 at 25°C with H_3PO_4) at 18 volt/cm for 3 hours. Hemoglobin was stained with amido black; alcohol dehydrogenase was located by the reduced tetrazolium procedure (*1*). The distance (in millimeters) from the origin for hemoglobins A, S, and C was 62, 47, and 32, respectively; for *Drosophila* ADH_1, ADH_3, and ADH_5, it was 65, 51, and 31, respectively. Comparative hemoglobin migrations show that a single charge-unit difference would result in a migration difference of 7.5 mm. The migration difference between ADH_1 and ADH_5 corresponds to 4.5 charge units. In that NAD would contribute one charge unit in the oxidized form but would contribute two charge units in the reduced form, the correlation of 3.5 NAD and 4.5 charge units is not unreasonable.

Multiple forms of enzymes that are the result of chemical modification of the basic protein have been observed. Malate dehydrogenase from mitochondria exists in five forms that are interconvertible by treatment with iodine or acid (*7*). Lactate dehydrogenase of the muscle (LDH_5) consists of subbands that can be interconverted by mercaptoethanol (*3*) or formaldehyde (*8*). Sodium borohydride treatment of an esterase (from maize) causes the appearance of enzyme species with electrophoretic migration rates similar to those of naturally occurring isoenzymes (*9*). Alcohol dehydrogenase of horse liver can be altered in electrophoretic migration by treatment with NAD (*10*). Alcohol dehydrogenase of *Drosophila* also may be altered by NAD treatment to a form with both a different electrophoretic mobility and a different heat stability. The increased stability may indicate that a conformation change in protein structure occurs when ADH_5 binds the NAD.

K. BRUCE JACOBSON

Biology Division,
Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37830

References and Notes

1. E. H. Grell, K. B. Jacobson, J. B. Murphy, *Science* **149**, 80 (1965).
2. ———, *Ann. N.Y. Acad. Sci.*, in press.
3. P. J. Fritz and K. B. Jacobson, *Biochemistry* **4**, 282 (1965).
4. H. Ursprung and L. Carlin, *Ann. N.Y. Acad. Sci.*, in press.
5. M. M. Ciotti and N. O. Kaplan, in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1957), vol 3, p. 890.
6. K. B. Jacobson and J. B. Murphy, in preparation.
7. G. B. Kitto, P. M. Wassarman, N. O. Kaplan, *Proc. Nat. Acad. Sci. U.S.* **56**, 578 (1966).
8. N. Ressler and C. Tuttle, *Nature* **210**, 1268 (1966).
9. D. Schwartz, *Proc. Nat. Acad. Sci. U.S.* **52**, 222 (1964).
10. J. S. McKinley-McKee and D. W. Moss, *Biochem. J.* **96**, 583 (1965).
11. Sponsored by AEC under contract with the Union Carbide Corporation. Human hemoglobins A, C, and S were made available by Dr. A. Chernoff.

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Titration Patterns of a Murine Sarcoma–Leukemia Virus Complex: Evidence for Existence of Competent Sarcoma Virions

Abstract. *Stocks of murine sarcoma virus show titration patterns ranging from one- to two-hit kinetics. The comparison of various titrations of this virus, both with and without added helper virus, to theoretical model systems composed of defined constituents, suggests the existence of a sarcoma virus that does not need coinfecting murine leukemia virus to be manifested as a focus-forming unit. The behavior of such nondefective particles is compatible with a postulated leukemia-sarcoma virus hybrid.*

Harvey (*1*) and Moloney (*2*) have reported the isolation, from passage lines of the Moloney leukemia virus, of agents that are sarcomagenic for mice, rats, and hamsters (*3*). The Moloney isolate of the murine sarcoma virus (MSV) has been shown to be capable of inducing foci of morphologically altered cells in infected mouse (*4*) and rat (*5*) cell cultures and can thus be readily titrated in vitro. The titration pattern of some stocks of MSV indicated that the infection of mouse cells was defective and required simultaneous infection with a murine leukemia virus for production of infectious progeny MSV. The presence of excess leukemia virus in stocks of MSV was directly demonstrated by production of complement-fixing leukemia virus antigen in mouse cultures infected with dilutions of the virus stocks beyond the focus-inducing end point (*4*). Thus stocks of MSV were shown to contain both sarcomagenic and leukemogenic virions.

Hamster tumors induced by MSV do not contain detectable infectious MSV. However, fully infectious MSV can be recovered by inoculating the hamster tumor cells either into intact mice or onto mouse embryo cultures that are infected with a murine leukemia virus (*3*).

The murine sarcoma-leukemia virus complex thus appears analogous to the avian Rous sarcoma (defective strain)–leukosis virus complex in its mixed virion composition, its ability to induce tumors in heterologous hosts, and in the recoverability of the virus from such heterologous tumors by similar procedures (*6*). Studies of the murine sarcoma virus appear potentially important in the comparative virology of sarcomagenic viruses in different species and as models for the possible recovery of the components of a human leukemia-sarcomagenic viral complex.

Hartley and Rowe (*4*) have pointed to two differences between the murine and avian viral complexes. The formation of the focal lesion produced by MSV in mouse cells requires the release of progeny infectious MSV, whereas the focus produced by Rous sarcoma virus (RSV) on chicken cells apparently can arise solely from cellular divisions originating in the initially transformed cell and in the absence of release of infectious virus. Also, the murine viral complex has a very high ratio of leukemia virion to sarcoma virion (up to 1000/1), whereas in the avian viral complex the leukosis virion/sarcoma virion ratio is much lower (10/1).

Inability of the MSV-induced focus to arise by cellular division alone has been confirmed in this laboratory (*7*). We have obtained evidence of some degree of viral defectiveness in focus assay on Swiss mouse embryo cells of a variety of stocks of murine sarcoma virus, obtained from either virus-induced mouse tumors or infected mouse cultures. The criterion of defectiveness employed was the apparent decrease in projected viral titer (number of foci × dilution factor) as judged by focus production on plating serial dilutions of the virus on mouse indicator cells (that is, "deviant" titration) and the increase in projected sarcoma virus titer, that

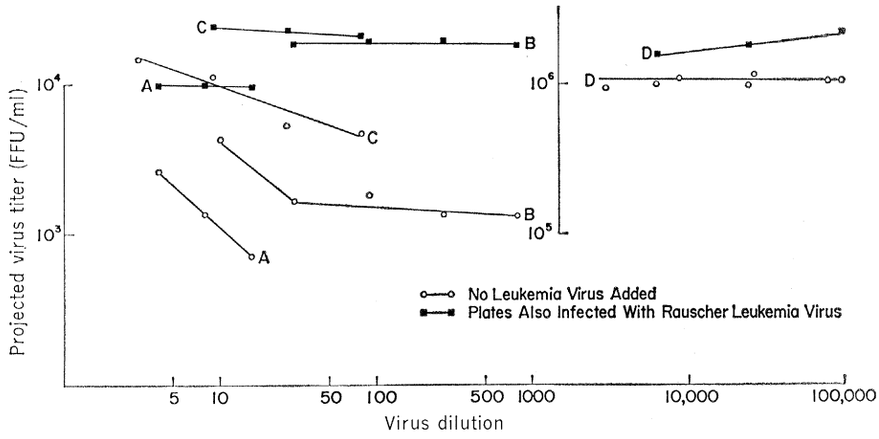

Fig. 1. Titrations of various stocks of MSV. The methodology of the focus assay on secondary mouse embryo cells, and the plotting of the data, has been described (*4, 7*). Optimal dilutions of Rauscher leukemia virus were added to alternate sets of plates at the time of MSV infection.

did not vary with dilution, on co-infection with a constant amount of a high-titer infectious murine leukemia virus. In this report we present some of the data on the quantitative aspects of these titrations. We believe that this data is best interpreted by the postulate that these viral stocks contain leukemia virions, defective sarcoma virions, and, in addition, a variable but significant fraction of sarcoma virions that can induce foci without necessity of co-infection with a leukemia virus. The possible nature of this class of virions, which we designate as "competent," is discussed.

The focus-assay titration patterns of four selected MSV stocks with and without murine leukemia virus are shown in Fig. 1. The titration pattern (*A*) of the MSV harvested 2 days after infection of the 3T3 mouse cell line (*8*) closely approaches that expected for a "two-hit" infection and thus may correspond to a virus stock consisting almost solely of defective sarcoma virions and leukemia virions. We have observed this deviant type of titration pattern consistently in early harvests of infected 3T3 cells, occasionally in early viral harvests from infected mouse embryo cells, but otherwise very infrequently. A quite different pattern (*D*) is that given by virus extracted from the virus-induced mouse tumor. This high-titer virus stock [10^6 focus-forming units (FFU) per milliliter] shows no deviation in projected titer on plating the final serial dilutions that yield nonoverlapping foci. It could readily be mistaken for a nondefective stock were it not for a small increase in virus titer as revealed by multiple platings of the viral dilutions in the presence of added potent Rauscher leukemia virus. The other tumor extract virus (*9*) also shows an apparent invariance of titer on plating the final serial dilutions, but the focus-yielding titer is considerably elevated in the presence of leukemia virus (*B*). The virus harvested from infected mouse embryo culture shows (*C*) a significant dependence of apparent titer on dilution, which is eliminated on plating in the presence of added leukemia virus, but the magnitude of this deviation falls considerably short of two-hit kinetics.

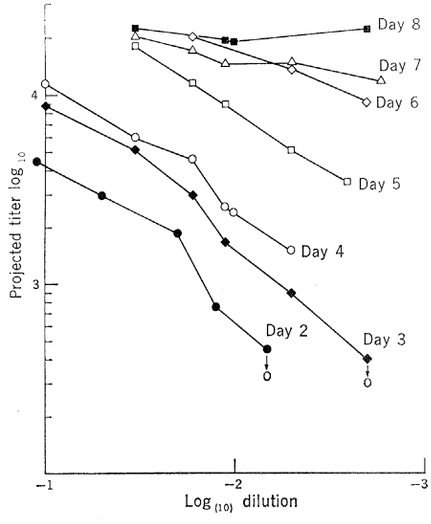

Fig. 2. Growth of MSV in 3T3 cells, shown as titration patterns of sarcoma virus harvested on successive days after infection. The medium for both cell growth and virus infection was McCoy's 5A with 15 percent fetal calf serum. The virus was collected as mixed supernates and cell lysates, freeze-thawed once, and centrifuged (1000 rev/min for 10 minutes) before assay on mouse embryo cells.

The high-titer virus obtained from the mouse tumor and the early viral harvest from infected 3T3 cells, which respectively show a nondeviant and a highly deviant titration pattern, also differ in another respect. Intact cells or cell homogenates, obtained from cultures previously inoculated with greater than twofold dilutions of the tumor-extracted virus beyond the initial focus-inducing end point, fail to yield foci when plated on fresh mouse indicator cultures, but do give rise to propagation of leukemia virus as judged by the development of measureable "helper" activity.

When cell homogenates from plates that had been previously inoculated with dilutions of the 3T3 infected cell harvest beyond the focus-inducing end point were plated on indicator mouse cells only leukemia virus was recovered. However, foci did develop when whole cells from such plates were passaged. These findings suggest that infection of mouse cells with a sarcoma virus having a linear, nondeviant titration pattern leads to the full expression (within a factor of 2) of all potential sarcoma virions, whereas infection with a virus, having a deviant titration pattern, leads to many infections that remain silent unless the cell is subsequently superinfected with a leukemia virus.

The titration of MSV stocks harvested on sequential days after infection of the 3T3 mouse cell line is shown in Fig. 2. All of these stocks that were harvested after day 3 were found to be restorable to essentially the same virus titer when plated in the presence of added leukemia virus. These virus stocks have intrinsic interest as indicator systems for murine leukemia viruses and their use as a component in an in vitro assay system for leukemia viruses will be reported in detail elsewhere (*8*). In the context of this report the progressive decline in the degree of deviation of these serially harvested stocks from completely deviant titration patterns, while the same restorable focus titer is maintained, should be noted.

A theoretical reconstruction of titration patterns for cells infected with only defective sarcoma virus and variable amounts of leukemia helper virus is shown in Fig. 3. The model contains two restraints in that the number of cells (10^5 per plate) and of sarcoma

virions (10^4/ml) are maintained at constant values. It will be observed that at low ratios of leukemia virions to sarcoma virions only a fraction of available sarcoma virions are expressed. At high ratios, all the sarcoma virions plated in the initial dilutions are expressed, but at higher dilutions only a portion of the sarcoma virions are again expressed. The significant feature of this theoretical model is that it shows that the titration pattern does not vary with dilution when the cells are completely infected with leukemia virus, but that there is an abrupt change to a two-hit titration pattern once there is insufficient leukemia virus to infect all the cells.

A different theoretical reconstruction is shown in Fig. 4. In this model the total number of sarcoma virions and the number of cells are again kept constant. A further restraint is imposed in that the ratio of leukemia virions to sarcoma is maintained constant at a value (50) which the previous model had shown sufficient for complete expression of all potential sarcoma virions in undiluted platings of the virus stock. Furthermore, it is assumed that a portion of the sarcoma virions are competent, that is, that on infecting a cell they produce foci without superinfection of any of the leukemia virions present in the virus mixture. Figure 4 represents the calculated titration patterns for sarcoma virus stocks where the content of such competent sarcoma virions is varied between 0 and 70 percent. It may be noted that under these conditions the projected titer of a virus stock became independent of dilution when the content of competent sarcoma virions reached 40 percent. The general resemblance of the family of titration patterns with those observed with sequential harvests of virus from infected 3T3 cells (Fig. 2) should be noted.

In the course of our studies on the quantitative aspects of the use of sarcoma virus stocks that showed deviant titration patterns as indicator systems for leukemia virions, we examined the effect of the concentration of added exogenous leukemia virus on the titration pattern of the indicator sarcoma virus. The experimental titration patterns obtained when the same serial dilutions of a common leukemia virus were added to platings of two different indicator sarcoma virus stocks, which had essentially the same potential titer of expressible foci but which differed in the degree of deviancy of their titration patterns, are shown in Fig. 5.

The calculated patterns that are obtained when increasingly potent concentrations of the same leukemia virus stocks are added to each plating of dilutions of two indicator sarcoma virus stocks (1 percent and 15 percent assigned content of competent sarcoma virions, respectively) are shown in Fig. 6. The resemblance of the theoretically deduced titration patterns to the experimental data in Fig. 5 should be noted.

We interpret the titration data presented here as implying that stocks of murine sarcoma virus contain competent, in addition to defective, sarcoma virions and murine leukemia virions. The amount of competent sarcoma virion appears to vary significantly in various stocks of the virus. In early harvests from infected 3T3 cells the competent sarcoma virion is of the order of 1 percent of the total sarcoma virions. In late (8 days after infection) viral harvests from infected cells the content of competent virion appears to be of the order of 40 percent or greater. Virus stocks obtained from virus-

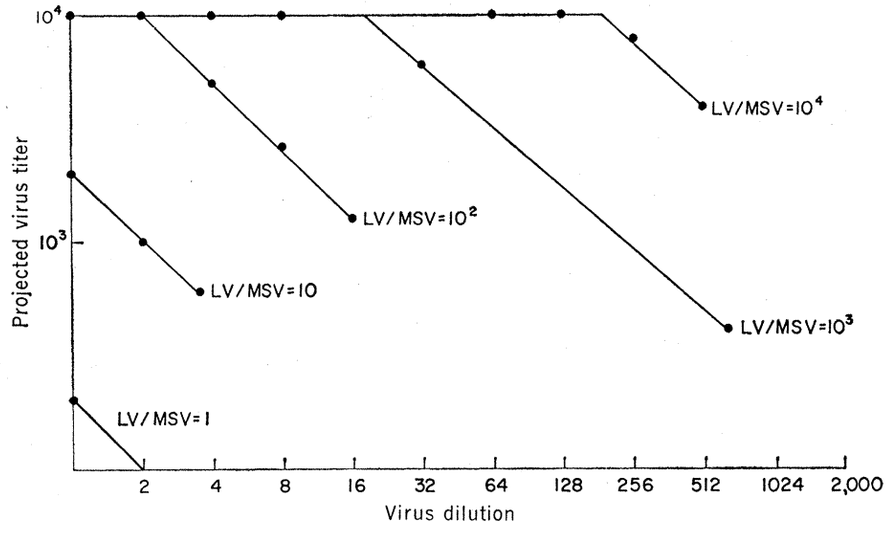

Fig. 3. Model MSV titration patterns. It is assumed that all MSV virions are defective and the potential maximum virus titer is 10^4 FFU per milliliter. Each titered stock contains variable amounts of murine leukemia virus (*LV*). The inoculum volume is 0.2 ml dispersed on 10^5 cells per plate.

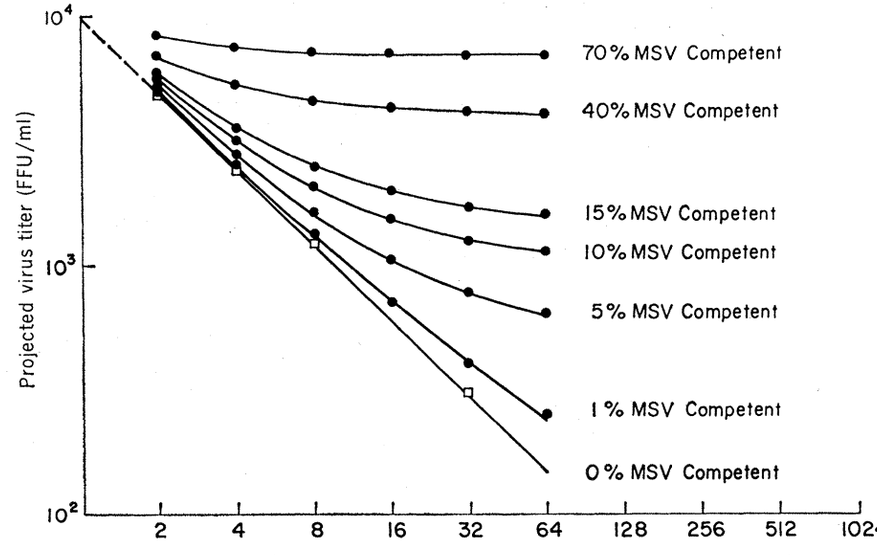

Fig. 4. Model MSV titration patterns. It is assumed that the virus stocks contain variable amounts of "competent" MSV virions (that is, that do not require helper virus for focus formation). The MSV titer of each stock is 10^4 potential FFU per milliliter in conjunction with 5×10^5 leukemia virions per milliliter. The inoculum volume is 0.2 ml distributed among 10^5 cells per plate.

induced tumors in BALB/c mice yield titration patterns that indicate the presence of 70 percent competent content of sarcoma virion.

The competent sarcoma virions, the leukemia virions, and the defective sarcoma virions share similar or identical exterior antigens. Isologous immune serums that specifically neutralized the leukemia virus also neutralized the focus-inducing capacity of the highly competent sarcoma virus isolated from the mouse tumors (*7*) and both the overt and helper virus restorable titers of defective virus stocks obtained from infected 3T3 cells (*8*).

We have examined the virus shed by individual foci that have arisen on infection of mouse embryo fibroblasts (MEF) with terminal dilutions of highly competent sarcoma virus. Such virus stocks yielded, on further passage, either defective or competent sarcoma virus progeny according to the infected cell type and time of viral harvest. Thus competence is not a permanent intrinsic trait which is transmitted to all progeny sarcoma virus. When MEF and 3T3 cells, respectively, were infected with Rauscher leukemia virus only, helper activity developed rapidly in the former but quite slowly in the latter (*8*). Hence, the development of competent sarcoma virions may depend rather on the concentrations of the leukemia and sarcoma virions and on the nature of the infected cell.

The requirement of release of infectious virus for focus development, the presence of common exterior antigens on both the competent sarcoma virus and leukemia virus, and the data on the genetic impermanence of competent virus, taken together, imply that the competent virion consists of a temporary association of both the leukemia virus and defective sarcoma virus genomes in an effective single particle. Such competence could result from trivial clumping of the two necessary coinfecting virus particle types (*10*). This, however, seems unlikely because the methodology of virus preparation prior to assay was identical at all virus harvest times in both the MEF and 3T3 cell systems. Furthermore, the titration pattern of the 3Pl virus stock, which indicated an intermediate level of competent sarcoma virions, was unaltered by procedures that could effect particle dispersion and segregation, such as repeated freezing and thawing, cycles of varying low-speed centrifugation and prolonged sonication. We consider that the experimental data is consistent with the formulation of the competent virion as a hybrid of a defective sarcoma and a leukemia virion. We are unaware of any currently known example of a viral hybrid involving an RNA oncogenic virus, although hybridization is a well-known phenomenon among DNA-containing viruses. Infection of a mouse cell with such a single hybrid virion could yield both infectious sarcoma virions and leukemia virions and probably some further hybrids, and so the cycle of

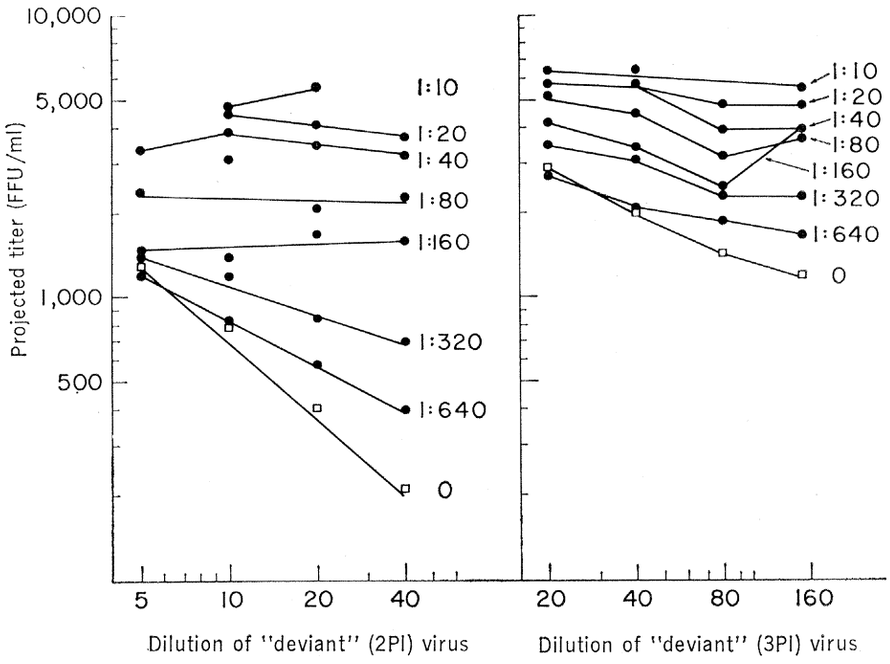

Fig. 5. Experimental MSV titration patterns illustrating the effect of addition of aliquots (0.1 ml) of serial twofold dilutions of the same stock of Rauscher virus during infection of mouse embryo cells with appropriate dilutions of two different MSV stocks (2Pl and 3Pl, respectively). The dilution of added leukemia virus is indicated next to each titration pattern.

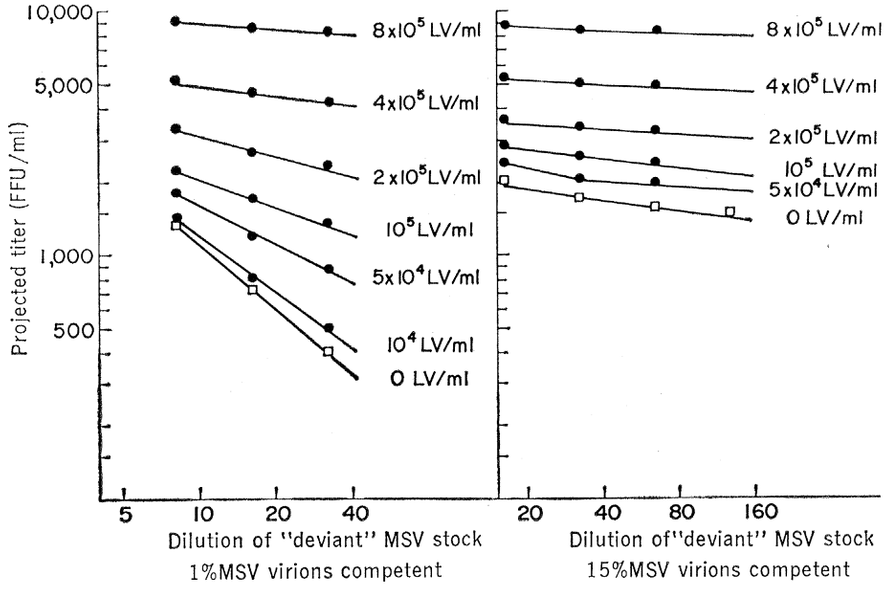

Fig. 6. Model MSV titration patterns. Two different MSV stocks, containing 1 percent and 15 percent competent virions respectively, are titered in the presence of serial dilutions of exogenous leukemia virus. The assumption is that each stock contains a total of 10^4 potential FFU per milliliter. The 0.2 ml volume of the dilutions of each of the sarcoma virus stocks plated are also supposed to contain the indicated amounts of exogenous leukemia virus (*LV*).

infection would continue and be manifest as a focus.

The biological importance of competence arises from the increased probability of survival which it imparts to the sarcoma genome. We have experimentally observed that on serial 3-day passages of the sarcoma virus in 3T3 cells (when the harvested virus contained few competent virions) the absolute titer of recoverable sarcoma virus rapidly declined to extinction (*8*).

TIMOTHY E. O'CONNOR
PETER J. FISCHINGER
Section of Molecular Virology, Leukemia and Lymphoma Branch, National Cancer Institute, Bethesda, Maryland 20014

References and Notes

1. J. Harvey, *Nature* **204**, 1104 (1964).
2. J. Moloney, *Nat. Cancer Inst. Monogr. 22*, 139 (1966).
3. R. Huebner, J. Hartley, W. Rowe, W. Lane, W. Capps, *Proc. Nat. Acad. Sci. U.S.* **56**, 1164 (1966).
4. J. Hartley and W. Rowe, *ibid.* **55**, 780 (1966).
5. R. Ting, *Virology* **28**, 783 (1966).
6. P. Sarma, W. Vass, R. Huebner, *Proc. Nat. Acad. Sci. U.S.* **55**, 1453 (1966).
7. T. O'Connor, in *Perspectives in Leukemia*, W. Dameshek and R. Dutcher Eds. (Grune and Stratton, New York, in press).
8. P. Fischinger and T. O'Connor, in preparation. The 3T3 cells were a gift from Dr. Green [G. Todaro and H. Green, *J. Cell Biol.* **17**, 299 (1963)].
9. M. A. Chirigos, K. Perk, W. Turner, B. Burka, M. Gomez, in preparation.
10. M. Pring, *Proc. Nat. Acad. Sci. U.S.* **58**, 607 1967.
11. The technical assistance of Mrs. Carolyn Moore and Ellis Sheets is gratefully acknowledged.

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Pregnancy Following Coital-Induced Ovulation in a Spontaneous Ovulator

Abstract. *Ovulation induced in the immature rat by pregnant mare's serum can be prevented by chlorpromazine. Coitus induces ovulation in such animals, and ovulation so induced may result in pregnancy. Both implantation and pregnancy appear to be normal although the duration of gestation may be prolonged. Decidual response also is produced in the immature rat after coitus-induced ovulation. We suggest that the reflex release of luteinizing hormone may occur in Primates after coitus.*

Reflex ovulation following coitus and electrical or mechanical stimulation of the uterine cervix has been demonstrated in both immature and mature rats (*1*). Hence the necessary neural pathways for induction of reflex ovulation exist in an animal generally considered to be a spontaneous ovulator.

In earlier studies (*1*) we used immature rats treated with pregnant mare's serum (PMS) and blocked the PMS-induced ovulation with chlorpromazine; the rats were then permitted to mate or were exposed to either electrical or mechanical stimulation of the uterine cervix. Ovulation occurred in all groups; the criterion for ovulation, and hence endogenous release of luteinizing hormone, was the presence of ova in the oviduct (*2*). This study was designed to demonstrate that these ova can be fertilized, will implant, and will undergo normal development.

Immature 28-day-old Purdue-Wistar rats were injected between 0800 and 1000 hours with 30 or 45 I.U. (international units) of PMS; 51 hours later each rat was injected with 0.75 mg of chlorpromazine per 100 g of body weight, this dose being sufficient to block the release of luteinizing hormone and prevent ovulation. Control rats received the same treatment except for the injection of chlorpromazine. The animals were then placed with mature male rats overnight, and vaginal smears were taken next morning to determine the presence or absence of sperm. The rats were either autopsied on day 9 after mating to determine whether implantation had occurred, or permitted to go to term or close to term.

Mating occurred in 50 percent of the rats treated with 30 I.U. of PMS. The incidence of mating was increased to 83 and 87 percent in two separate groups by treatment with 45 I.U. of PMS, and dropped to 55 and 58 percent in two groups after treatment with 45 I.U. of PMS and the chlorpromazine (Table 1). Thus chlorpromazine appeared to interfere partially with the female's receptivity of the male, but approximately half of the females still mated.

Implantation of the blastocysts was noted in 100 percent of the mated rats treated with 30 I.U. of PMS and in 75 percent of the rats treated with 45 I.U. of PMS. Treatment with 45 I.U. of PMS and chlorpromazine decreased the percentage of rats with implantation sites to 43 percent, but the difference was not significant ($P > .05$). The percentages that we report of mated rats with implantation sites are comparable to the values obtained by others (*3*): our averages were 22.6 ± 2.4 percent for rats treated with 30 I.U. of PMS and 25.4 ± 2.4 percent for rats treated with 45 I.U. of PMS.

A number of rats were permitted to go to term or were killed on day 25 after mating if parturition had not occurred. Of those treated with PMS alone, 54 percent became pregnant. Of the 11 rats treated with PMS and chlorpromazine, 55 percent mated and 33 percent became pregnant. The difference is not significant ($P > .1$).

The tendency for fewer rats with implantation sites on day 9 or fewer pregnancies at term following treatment with both PMS and chlorpromazine, than with PMS alone, may reflect a direct effect of chlorpromazine on the ovary. Others have noted that chlorpromazine and similar drugs reduce or inhibit deciduomata as well

Table 1. Implantation and pregnancy after mating in the PMS-treated, immature rat, with and without blockage by chlorpromazine (CPZ) of spontaneous release of luteinizing hormone. Numbers of rats appear in parentheses.

Rats			Mated rats	
Treated with:		Showing sperm in vagina (%)	With implantation (%)	With fetuses or young (%)
PMS (I.U.)	CPZ			
30(18)	No	50	100	
45(12)	No	83*	75	
45(12)	Yes	58	43†	
45(15)	No	87		54
45(11)	Yes	55		33‡

* One additional rat had blastocysts on day 9 although no sperm were found in the vaginal tract on the morning after mating. † Not significantly different from controls without CPZ ($P > .05$). ‡ Not significantly different from controls without CPZ ($P > .10$).

Table 2. Decidual reaction in the immature rat after treatment with PMS or chlorpromazine (CPZ), or both, and mating. Number of rats appear in parentheses.

Rats			Traumatized horn: normal horn (av. wt %)
Treated with:		Having deciduoma (%)	
PMS (I.U.)	CPZ		
45(9)	No	100	192.0
45(8)	Yes	88	92.4*

* Significantly different from controls without CPZ ($P < .05$).