[23] Guttes, E., S. Guttes, and H. P. Rusch, *Develop. Biol.*, **3**, 588 (1961).
[24] Prescott, D. M., in *Methods in Cell Physiology* (New York: Academic Press, 1964), vol. 1, p. 365.
[25] Mittermayer, C., R. Braun, and H. P. Rusch, *Biochim. Biophys. Acta*, **91**, 399 (1964).
[26] Burton, K., *Biochem. J.*, **61**, 473 (1955).
[27] Ceriotti, G., *J. Biol. Chem.*, **214**, 59 (1955).
[28] Gornall, A. G., C. J. Bardawill, and M. M. David, *J. Biol. Chem.*, **177**, 751 (1949).
[29] Mohberg, J. E., and H. P. Rusch, *J. Cell Biol.*, **23**, 61A (1964).
[30] Hendler, R. W., *Anal. Biochem.*, **7**, 110 (1961).
[31] Sachsenmaier, W., *Biochem. Z.*, **340**, 541 (1964).
[32] Sachsenmaier, W., and H. P. Rusch, *Exptl. Cell Res.*, **36**, 124 (1964).
[33] Kajiwara, K., and G. C. Mueller, *Proc. Am. Assoc. Cancer Res.*, **5**, 33 (1964).
[34] Jacob, F., S. Brenner, and F. Cuzin, in *Synthesis and Structure of Macromolecules*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 28 (1963), p. 329.
[35] Painter, R. B., and R. E. Rasmussen, *Nature*, **201**, 162 (1964).

*COMPLEMENT FIXATION AND TISSUE CULTURE ASSAYS FOR MOUSE LEUKEMIA VIRUSES**

BY JANET W. HARTLEY, WALLACE P. ROWE, WORTH I. CAPPS, AND ROBERT J. HUEBNER

LABORATORY OF INFECTIOUS DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

Communicated March 1, 1965

Although many of the established laboratory strains of murine leukemia virus have been propagated in mouse tissue culture systems,[1-8] no cytolytic or growth stimulatory responses have been observed, and only low titers of virus have been detected. Other than animal inoculation, the only criterion which has been used to demonstrate growth of virus in tissue culture cells is development of immunofluorescent stainable antigen.[4]

Sarma *et al.*[9] have developed an assay for noncytopathic avian leucosis virus infection of tissue culture based on production of complement-fixing (CF) antigen reactive with sera of hamsters carrying avian leucosis virus-induced tumors. In an effort to develop a comparable system for detection of mouse leukemia viruses, studies were made with sera of rats carrying a transplanted mouse leukemia virus-induced tumor. This report describes successful development of a CF test for tissue culture-grown mouse leukemia viruses and its usefulness for virus titration and neutralization tests.

Materials and Methods.—Antiserum: Standard antiserum pools were obtained from rats carrying a transplanted rat lymphoid leukemic thymus induced by the Rauscher virus.[10] This tumor, which was obtained from Dr. Rauscher, had been induced in an Osborne-Mendel (OM) rat by inoculation of rat-passaged Rauscher virus; it had been carried through 4 subcutaneous transplant passages in OM rats when received in this laboratory. It has been maintained here by subcutaneous implantation of minced tumor tissue into weanling or adult OM rats. The rats were bled weekly by orbital puncture and exsanguinated when moribund.

In initial experiments, sera were tested in CF against extracts of the Rauscher rat tumor and of normal OM rat thymus. Representative patterns of antibody response in animals which developed high titer are shown in Table 1.

For preparing standard reagents, sera which titered 1:80 or greater against the tumor antigen

TABLE 1

CF ANTIBODY TITERS OF REPRESENTATIVE RATS CARRYING TRANSPLANTED RAUSCHER RAT LEUKEMIA

Days after transplantation	Antigen: Rauscher rat tumor	Normal rat thymus	Rauscher METC antigen	Control METC antigen	Rauscher rat tumor	Normal rat thymus	Rauscher METC antigen	Control METC antigen
	Rat #2443-1				Rat #2443-2			
→ *								
19	20	0†	0	0	0	0	0	0
25	(20)	0	0	0	(10)	0	(10)	0
34	80	0	80	0	20	0	(10)	0
40	≥80	<20	80	<20	80	0	10	0
46					≥80	0	20	0
	Rat #2403-1				Rat #2403-7			
10	0	0	0	0	0	0	0	0
→								
20	0	0	0	0	0	0	0	0
29	20	0	10	0	0	0	0	0
40	≥80	≥20	≥80	0	80	0	0	0
48	160	(10)	80	0	160	(20)	80	0

* Arrow indicates period when growth of subcutaneous tumor was first seen.
† 0 = <10. Figures in parentheses indicate partial reaction (1 or 2 + fixation) at this serum dilution.

with no reaction at 1:20 with control antigen were pooled; the pool was absorbed once with acetone-extracted mouse liver powder, Difco (100 mg per ml of serum). Such pools generally titered 1:40 or 1:80 in CF against 4 units of Rauscher virus tissue culture antigen. At a 1:20 dilution, these serum pools reacted with the great majority of 5% extracts of mouse and rat leukemia tissues (Rauscher,[10] Friend,[11] Moloney,[12] WM1-B,[13] and Gross[14] viruses) as well as with spontaneous mouse leukemias (AKR, C_3H/Fg, and C58); these pools of selected sera were almost invariably negative with control mouse and rat tissue antigens (more than 150 specimens). However, in view of the occasional partial CF reactions observed and the relatively frequent occurrence of CF response in the tumor-bearing rats to normal lymphoid-tissue antigens, which necessitated the serum selection procedure described above, at present we cannot view the reactions with tissue antigens as unquestionably specific.

In more recent work, sera were selected for standard pools by screening against antigens prepared from virus-infected and normal control mouse embryo tissue culture (METC) cells. When tested at lower dilution (1:10 or 1:20), these serum pools did react with some control tissue-suspension antigens and, much less frequently, with control METC antigens; however, at the dilution used for screening (1:40), they gave no trace of CF reaction with any of several hundreds of control METC antigens.

Complement-fixation (CF) test: The microtechnique described previously for tumor antigen studies[15] was used. The test employed 1.6–1.8 exact units of complement and 4 units of antiserum.

Tissue cultures: Primary cultures of BALB/c or NIH Swiss mouse embryonic tissue were prepared in this laboratory or obtained from Microbiological Associates, Inc., Bethesda, Md. Embryos of 17–20 days' development were minced whole and trypsinized; the cells were diluted in growth medium consisting of 10% unheated fetal bovine serum in Eagle's basal medium (10 FB-BME) and planted at a concentration of 8–12 × 10^6 cells per 32-oz flask. In some experiments the growth medium was "diploid growth medium (DGM)"[16] consisting of 10 FB-BME with nonessential amino acids, 10^{-7} *M* folinic acid, and 1.4 gm/liter $NaHCO_3$. All media contained 2 mM glutamine, 100 units penicillin, and 100 mcg streptomycin per ml.

To prepare cell suspensions for infection, 5- to 9-day-old primary cultures with confluent cell sheets were dispersed with 0.25% trypsin in Earle's balanced salt solution, diluted with an equal volume of 10 FB-BME, sedimented at 900 rpm for 5 min., and resuspended in 10 FB-BME to 10^6 cells per ml. One volume of inoculum, consisting of animal tissue extracts (10% suspensions in BME, clarified by low-speed centrifugation) or fluid or cell suspension of infected tissue cultures, was added to 10 vol of the mouse embryo cell suspension (usually 1 or 2 ml) and the mixture held at room temperature for 1 hr, with occasional agitation. The cells were then diluted tenfold in 10 FB-BME and planted in 60-mm plastic Petri dishes (Falcon Plastics, Los Angeles, California) at 350,000 cells per dish, or in 32-oz flasks using 4 × 10^6 cells. Cultures were incubated at 37°C,

the plates in a humidified cabinet in an atmosphere of 5% CO_2 in air; fluids were replaced twice weekly, using 10 FB-BME.

Antigen and virus pools: Cultures were harvested by removing the medium and scraping the cells from the surface with a rubber policeman. The cells from one plate were suspended in 0.3 ml of supernatant fluid; the cells from 32-oz bottle cultures were suspended in 2 ml.

Before testing for CF activity, cell suspensions were frozen and thawed 3 times and shaken or pipetted vigorously. Larger volumes of cell suspension antigen were homogenized in a TenBroeck grinder or were sonicated in a Branson Sonifier for 2 min at 6 amp.

Supernatant fluids for use as virus pools were usually harvested at 14–16 days, by decanting the fluid without disturbing the cell sheet. Fluids and cell suspensions were stored at −60°C.

Virus isolation and passage: For routine isolation attempts, cells were exposed to undiluted 10% tissue extracts, as described above. Cell suspension harvests, made after 14 days and 18–22 days of culture, were screened for CF antigen at dilutions of 1:2 and 1:4; a CF reading of 3+ fixation or greater at 1:2 was considered positive. Routinely, at least one passage to fresh cells was made from cultures showing partial or positive CF reactivity; most of the negative cultures were also passed at least once.

Routine serial passages were made at 14–22-day intervals. Cell suspensions were usually diluted 1:2 or 1:10 and supernatant fluids were used undiluted; in most cases the inoculum was frozen and thawed once. The theoretical dilution of the inoculum at each passage was about 10^{-6}.

Virus titration: Titrations were carried out by preparing replicate plate cultures of cells infected in suspension with serial tenfold dilutions of virus; by the infection technique used, each plate received 0.035 ml of inoculum. Harvests were made at intervals through 28 days and the cell suspensions screened for CF activity at dilutions of 1:2 and 1:4. Individual plates were assayed for antigen at 20–21 days, and the titer expressed as the 50% tissue culture infective dose ($TCID_{50}$) (Karber method) per 0.1 ml.

Neutralization test: Virus pools consisting of cell-free supernatant fluids were pretitrated and the dilution of virus corresponding to approximately 300 tissue culture infective doses in 0.1 ml in a 14-day harvest was determined.

Dilutions of inactivated (56°C, 30 min) serum were mixed with an equal volume of virus dilution and held at 37°C for 1 hr; 0.2 ml of the mixture was inoculated into 1 ml of mouse embryo cell suspension containing 10^6 cells. The infected cells were held at room temperature, diluted, and plated in duplicate, as described above. Controls for each test included a virus titration, a titration of a standard positive rat serum, and uninoculated cells. At 14 days one plate of each set was harvested and the cell suspension antigens were tested for CF activity at dilutions of 1:2 and 1:4; virus controls were titrated from 1:2 through 1:16. The remaining plates were reserved for a second harvest at 18–20 days if required. Serum neutralization titers were determined as the highest dilution reducing production of CF antigen to 1+ or less CF reaction of the 1:2 dilution of cell suspension, when compared with a virus control antigen titer of at least 1:8.

Miscellaneous treatments: Test samples were exposed to 10% chloroform (10 min) or to 20% peroxide-free ether (2 hr) at 4°C. Equal volumes of sample and 0.25% trypsin or $M/90$ KIO_4 were held at room temperature for 10–15 min; the trypsin was inhibited by addition of 10 FB-BME and the periodate was neutralized with 10% glycerol.

Results.—Recovery of antigen-inducing agents from leukemic animals: Table 2 summarizes attempts to detect propagation of murine leukemia viruses in METC using CF antigen production as the indicator. Agents inducing CF antigen reactive with the Rauscher rat antisera were detected regularly in leukemic tissues of mice and rats infected with the Rauscher, Moloney, and WM1-B viruses, and in single specimens of Friend virus, "avirulent Friend virus,"[17] and a line of Sarcoma 37 (Barrett S37) known to carry Moloney virus. Tissues of Rauscher and Moloney virus-inoculated mice taken prior to development of leukemia were less regularly positive. In contrast, Gross virus-induced rat leukemias were consistently negative. Also, only one of 11 spontaneous AKR leukemias yielded virus, and other spontaneous and X-ray-induced leukemias were negative, as were normal mouse and rat tissues. The failure to recover virus from the transplanted Rauscher rat tumor

TABLE 2

DETECTION OF CF ANTIGEN IN METC CELLS INOCULATED WITH LEUKEMIC AND NORMAL RODENT TISSUES

Virus or treatment	Type of animal	Material tested	CF Antigen in METC No. of Specimens Positive/No. of Specimens Tested* — Leukemic Animals: Mouse	Rat	Hamster	Non-leukemic Animals: Mouse	Rat
Rauscher	Primary leukemia	Plasma	2/2				
	" "	Spleen	4/4	1†/1		2/3	
	Transplant	Tumor		0/4			
Moloney	Primary leukemia	Thymus, spleen, nodes	1/1			5/8	
	Virus carrier (milk-borne)	" " "	2/2				
	Transplant	Tumor	2/2	2/2	0/1		
Friend	Primary leukemia	Spleen	1†/1				
WM1-B	Primary leukemia	Spleen, liver	1/1				
	" "	Thymus		1/1			
	Transplant	Tumor		1/1			
"Avirulent Friend"	Virus inoculated	Thymus, spleen, nodes				1/1	
Gross virus	Primary leukemia	Thymus, spleen, nodes		0/5			
Sarcoma 37	Transplant	Tumor	1†/1				
None	AKR spontaneous leukemia	Thymus, spleen, nodes	1†/11				
	C_3H/Fg spontaneous leukemia	" " "	0/1				
	C_{58} spontaneous leukemia	" " "	0/5				
X irradiation	C_{57} transplant	" " "	0/1				
	C_3Heb "	" " "	0/2				
	ST "	" " "	0/1				
None	BALB/c normal	" " "				0/4	
	" "	Plasma				0/1	
	NIH "	Thymus, spleen				0/4	
	C_{58} "	Thymus, spleen, nodes				0/4	
	AKR "	Whole embryo				0/1	
	SD "	Thymus, nodes					0/2
	" "	Kidney					0/1

* The majority of specimens were tested in BALB/c or NIH METC. Four of the Gross virus specimens were tested only in C_3H/HeN METC. Nine of the spontaneous and X-irradiation-induced leukemias were tested in C_3H/HeN or AKR METC as well as BALB/c. The single positive AKR leukemia isolate was recovered in NIH METC.

† These isolations were verified by reisolation of virus from the same specimen or from another tissue from the same animal.

line is probably due to the presence of neutralizing antibody in the extracts; one of these extracts induced Rauscher leukemia in mice after a prolonged latent period, and virus was readily recovered in tissue culture from these mice.

The great majority of positive specimens produced definite antigen in first passage cultures, and only rarely did blind passage of a culture with no evidence of antigen give positive results. Isolates were readily established in serial passage if cell suspension inocula were used in early passages; supernatant fluids were satisfactory for transfer in later passages. The Rauscher, Friend, Moloney, and WM1-B viruses have been carried for 12–19 serial METC passages at present; antigen was produced at each passage level, the titers being generally 1:4 to 1:16.

Identification of antigen-inducing agents as murine leukemia viruses: That the factor inducing the antigen was the leukemia virus was indicated by (1) recovery of mouse pathogenic leukemia virus from the Rauscher, Friend, and WM1-B passage lines, but in late METC passages with prolonged latent periods; (2) resistance of mice inoculated with 5th and 6th tissue culture passage Rauscher virus to challenge

with Rauscher mouse passage virus; (3) failure of tissue culture fluids to induce antigen after treatment with chloroform (Rauscher virus), ether (Moloney and Rauscher viruses), or heat (56°C for 30 min) (Rauscher virus); (4) failure of kanamycin and aureomycin in culture media to inhibit antigen formation (Rauscher and Moloney viruses) and inability to cultivate pleuropneumonia-like organisms from the virus passage lines; (5) induction of antigen by tissue culture fluid after filtration through a Selas 03 filter (Rauscher virus); and (6) results of control CF testing. Rauscher tissue culture antigens gave consistently negative CF results when tested against potent antisera from adenovirus type 12 tumor-bearing rats and hamsters, and adenovirus type 18, Rous sarcoma, polyoma, and SV40 tumor-bearing hamsters, as well as antiviral sera for LCM, mouse hepatitis, polyoma, mouse adenovirus, K virus, RV and H-1 rat viruses, and the influenza and parainfluenza groups. The Rauscher rat serum pools did not react with tissue suspension CF antigens for LCM, mouse hepatitis, K virus, or polyoma; one serum pool reacted in CF with RV, and two reacted with H-1 rat virus, as expected from the high prevalence of antibodies to these viruses in laboratory rats.[18, 19]

Properties of the antigen: The CF-reactive antigen in sonicates of Rauscher virus tissue culture cells, as well as that present in mouse and rat leukemic tissue extracts, was sedimented into the lower supernate or pellet by centrifugation at 12,000 to 15,000 $\times$ *g* for 60–90 min. CF activity was destroyed by chloroform treatment, was usually partially reduced by heating at 56°C for 30 min, and was essentially unaffected by trypsin or periodate treatment.

Factors influencing yield of antigen: In general work, the Rauscher virus gave more consistently high titers, and has been used for most of the studies reported here. Several experimental variables have been identified as important in determining amount of antigen produced by infected cultures, namely, manner of infection, maintenance medium, and time and method of harvest. In initial experiments, it was found that cells infected in suspension gave consistently higher antigen titers than plate cultures infected as well-established monolayers (5–6 days old); for this reason the infection in suspension technique was used routinely. However, recent preliminary experiments indicate that cultures infected 18–24 hr after seeding (350,000 cells per plate) may give highly satisfactory results.

The nature of the medium used for growth and maintenance of infected tissue culture cells was important in determining the CF antigen titer attained. METC cells infected in suspension with Rauscher tissue culture virus and grown in BME containing regular or agamma-globulinemic calf or horse serum (10%) instead of fetal bovine serum produced 4- to 32-fold less CF antigen than did the standard medium. Low CF antigen yields were also obtained when infected cultures were maintained in DGM.

With regard to time of harvest, high doses of virus produced detectable antigen within 6 days, with maximum titer by 14 days; antigen remained high for at least 28 days. With lower doses of virus, antigen appeared later and reached highest titers at about 18–21 days.

Most of the antigen was associated with cells but could be extracted by homogenization of cells in a tissue grinder or by sonication. Many fluid harvests contained detectable antigen, but in very low titer.

Although NIH strain cultures were more sensitive than BALB/c cultures for

detection of virus, as described below, the yield of antigen from positive cultures was equivalent in the two systems.

Sensitivity of the assay system: The detection of small amounts of virus was dependent not only on the use of optimal cultural conditions for high antigen yield but also on the mouse strain from which tissue cultures were prepared. Although cultures prepared from BALB/c embryos were used for all of the early experiments, it was later found that the amount of Rauscher, Friend, or Moloney virus detected in tissue culture harvests by titration in NIH Swiss embryo cells was 10–100 times that indicated by BALB/c cultures. Titers in NIH cells of $10^{4.5}$ to 10^{6} $TCID_{50}$ were obtained for several Rauscher virus fluid pools, ranging from the 4th to the 14th METC passage. Similarly, 4th and 5th METC passage fluids of Friend virus titered $10^{5.5}$ and $10^{6.5}$, respectively, while Moloney virus tissue culture pools were consistently lower, usually 10^{3} to 10^{4}. Homogenates or sonicates of Rauscher virus-infected cells titered $10^{5.5}$ to $10^{7.0}$, which was 10- to 100-fold higher than the concurrent fluid harvests. Titration end points were generally attained by 21 days and were usually 1–2 logs higher than the 14-day titer. Occasionally, cell suspensions at titration end points gave only partial fixation; in these cases the presence of virus was readily confirmed by passage of the fluid. Closely comparable results were obtained in repeat titrations; one pool of virus was tested 4 times with no more than 0.5 log variation in the titration end point.

Spleen suspension or plasma from mice infected with Rauscher virus gave tissue culture infectivity titers of $\geq 10^{3.5}$, while a suspension of Moloney leukemic mouse tissue titered $10^{2.5}$.

A number of other tissue culture cells were tested for their ability to develop CF-reactive antigen following inoculation with Rauscher METC passage virus; the standard technique for METC infection was used. Low titers of antigen were produced in primary cultures of mouse kidney, mouse embryonic lung, and rat embryo, and virus was detected in fluids from these cultures on passage to METC. The following cultures were completely negative when cell suspensions were tested at 14 and 21 days: primary whole hamster and chick embryo, bovine embryonic spleen, adult rabbit kidney and African green monkey kidney, and serial lines of mouse liver (NCTC 1469), ferret kidney, and WI-38 diploid human embryonic lung. (All cultures were obtained from Microbiological Associates, Inc., Bethesda, Md., and Flow Laboratories, Rockville, Md.)

Long-term cultivation of virus-infected cells: Continuous lines of uninoculated, Rauscher virus-, and Moloney virus-infected BALB/c embryo tissue culture cells have been carried for more than 20 cell-transfer passages during a 1-year period. High CF antigen titers were detected in cell suspensions throughout the passage series, usually in excess of 1:32 for the Rauscher virus line and at least 1:16 for the Moloney virus line. Fluid harvested from 19th cell passage of the Rauscher virus line had a tissue culture infectivity titer of 10^{5} and a sonicated cell suspension titered 10^{7}. No antigen was detected at any time during serial passage of the control cells. Manaker *et al.*,[1] using induction of leukemia in mice as the indicator of virus growth, have described the release of Moloney virus throughout 4 years' continuous passage of virus inoculated BALB/c spleen tissue cultures.

Neutralization tests: The tissue culture system also provided a satisfactory indicator for virus neutralization tests (see Table 3). Seventeen of 19 rat sera with

TABLE 3

REPRESENTATIVE TISSUE CULTURE NEUTRALIZATION TEST

Virus* Dilution	Serum	Dilution	CF Reaction† Antigen Dilution 2	4	8	16	Serum CF Titer Antigen: Rauscher METC	Control METC
$10^{-1.5}$	Medium only		4	4	4	3		
$10^{-2.5}$	" "		4	4	2	1		
$10^{-3.5}$	" "		3	2	1	0		
$10^{-4.5}$	" "		0	0				
$10^{-1.5}$	Rauscher rat serum #26914	1:5	4	4			20	<10
"	Rauscher rat	1:40	0	0			40	<10
"	serum pool	1:80	3	1				
"	11	1:160	4	3				
"	Rauscher rat	1:20	0	0			80	<10
"	serum #36974	1:40	0	0				
"		1:80	3	2				

* Rauscher leukemia virus, METC fluid.
† Degree of CF reactivity of 14-day harvest of METC cultures. 4+ = complete fixation of complement.

CF titers of 1:40 or greater inhibited CF antigen production by Rauscher virus; neutralization titers ranged from 1:10 to 1:80. In contrast, none of 17 sera from uninoculated rats or tumor-bearing rats without CF antibody gave positive neutralization at 1:5 or 1:10 dilution.

Rauscher rat-serum pools also inhibited CF antigen production by Friend and Moloney METC passage viruses in tissue culture neutralization tests.

The results of neutralization tests will be reported in detail later.

Discussion and Summary.—Sera of rats carrying a transplanted leukemia induced by Rauscher virus react in the CF test with METC cells infected with Rauscher, Moloney, Friend, WM1-B, and "avirulent Friend virus," with extracts of mouse and rat tumors induced by these viruses and the Gross virus, and with spontaneous mouse leukemias. In addition to indicating that murine leukemia viruses share at least one major antigen, this finding has provided the basis for rapid (2–4 weeks), sensitive, and reproducible tissue culture isolation, titration, and neutralization tests for all laboratory-adapted strains of murine leukemia virus tested, except Gross virus. The present tissue culture procedure does not appear to provide a sensitive means of detecting naturally occurring strains, possibly because of their narrower host range.

Tissue culture cell harvests contain 1–2 logs higher titers of virus and antigen than culture fluids, providing a simple means of obtaining high titer preparations. The CF antigen probably represents the virion, since they are of comparable size, and rat sera with high CF antibody also contain high titer neutralizing antibody.

The generosity of Dr. F. J. Rauscher in supplying the transplanted rat tumor, which made these studies possible, is gratefully acknowledged. We are indebted to Drs. John H. Pope and Ludwik Gross for supplying their virus strains for use in these studies, to Dr. Lloyd W. Law for supplying the Moloney virus carrier mice and many of the spontaneous leukemias, to Dr. John B. Moloney for the Moloney rat and hamster tumors and Sarcoma 37, and to Miss Delta E. Uphoff for the X-ray-induced leukemias. Mr. H. C. Turner supervised the CF testing, and Mr. William T. Lane and Mr. Charles W. Shifler rendered valuable technical assistance.

* This research supported by NCI Field Studies.

[1] Manaker, R. A., P. C. Strother, A. A. Miller, and C. V. Piczak, *J. Natl. Cancer Inst.*, **25**, 1411 (1960); Manaker, R. A., E. M. Jensen, and W. Korol, *J. Natl. Cancer Inst.*, **33**, 363 (1964).

[2] Gross, L., *Proc. Am. Assoc. Cancer Res.*, **3**, 231 (1961).

[3] Ginsburg, H., and L. Sachs, *Virology*, **13**, 380 (1961); Ginsburg, H., and L. Sachs, *J. Natl. Cancer Inst.*, **27**, 1153 (1961); *ibid.*, **28**, 1391 (1962).
[4] Osato, T., E. A. Mirand, and J. T. Grace, *Jr., Nature*, **201**, 52 (1964).
[5] Prigozhina, E. L., and A. A. Stravovskaya, *Acta Virol.*, **8**, 277 (1964).
[6] Sinkovics, J. G., and C. D. Howe, *J. Infect. Diseases*, **114**, 359 (1964).
[7] Peries, J., J. P. Levy, M. Boiron, and J. Bernard, *Nature*, **203**, 672 (1964).
[8] Rowe, W. P., *Science*, **141**, 40 (1963).
[9] Sarma, P. S., H. C. Turner, and R. J. Huebner, *Virology*, **23**, 313 (1964).
[10] Rauscher, F. J., *J. Natl. Cancer Inst.*, **29**, 515 (1962).
[11] Friend, C., *J. Exptl. Med.*, **105**, 307 (1957).
[12] Moloney, J. B., *J. Natl. Cancer Inst.*, **24**, 933 (1960).
[13] Pope, J. H., *Austral. J. Exptl. Biol.*, **39**, 521 (1961); *ibid.*, **40**, 263 (1962).
[14] Gross, L., *Proc. Soc. Exptl. Biol. Med.*, **76**, 27 (1951).
[15] Huebner, R. J., W. P. Rowe, H. C. Turner, and W. T. Lane, these PROCEEDINGS, **50**, 379 (1963).
[16] Ward, T. W., personal communication.
[17] Pope, J. H., *Austral. J. Exptl. Biol.*, **41**, 349 (1963).
[18] Kilham, L., and L. J. Olivier, *Virology*, **7**, 428 (1959).
[19] Moore, A. E., *Acta, Unio Intern. Contra Cancrum*, **19**, 273 (1963).

---

*THEORY OF THE FLOW OF ACTION CURRENTS IN ISOLATED MYELINATED NERVE FIBERS, I**

BY R. LORENTE DE NÓ AND V. HONRUBIA†

THE ROCKEFELLER INSTITUTE

Communicated March 24, 1965

The systematic presentation of the already outlined theory of the isolated nerve fiber[1] is begun in this communication.

Technique.—To the details already given[1] the following should be added.

The dc amplifier (Bioelectric Instruments, Inc.) that has been used includes a device to compensate for the input capacity. Under the conditions of the experiments, however, the input capacity could never be fully compensated, and therefore the amplifier always acted as a shunt for the action currents. From the results of placing various ohmic shunts across the amplifier it may be estimated that for the action currents the effective input resistance of the amplifier never was higher than 20–30 megohm, and that it often must have been considerably smaller. The presence of the amplifier, therefore, always modified the mode of propagation of the nerve impulse. After this role of the amplifier had been understood, it became apparent that the most powerful method of analysis consists precisely of modifying the mode of propagation of the impulse by means of various sets of shunts placed between the Ringer pools. Several of the situations that have been encountered will be discussed below in reference to the diagrams in Figure 3.

Diagrams *I* to *V* (Fig. 1) indicate the five manners in which the amplifier has been connected to the preparation to record the action currents of impulses which are propagating themselves from right to left. Since in the Ringer pools the flows of action currents do not establish measurable potential differences, the amplifier can and does measure only the potential differences created by the flow of action currents in the external longitudinal conductors of those segments of the isolated fiber which are present in the vaseline gaps. Ordinarily, the external longitudinal resistances were practically equal in the two gaps. Thus, except in those rare instances in which a short broken segment of another fiber was present in one of the gaps, the external longitudinal resistances in the two gaps may be taken as having had a uniform value r_e per unit length.

The symbols given in Figure 1, *I* and *IV*, have the following meaning. The isolated fiber itself is taken as the x-axis: x_1 to x_4 are the end points of the two gaps, and $i_1(x,t)$ and $i_2(x,t)$ are the