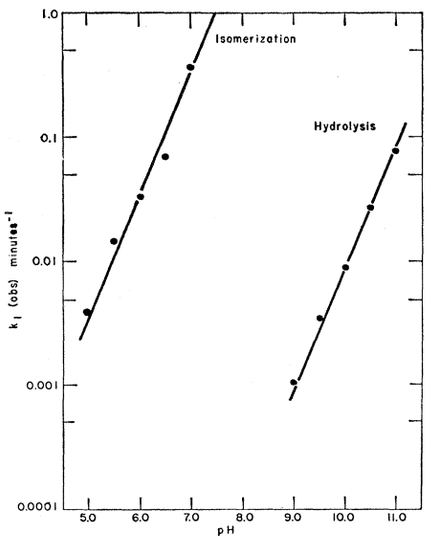

Fig. 2. Rate of isomerization and hydrolysis of 2′-*O*-(*N*-acetylvalyl)-adenosine as a function of *p*H at 15°C. The solid lines are calculated from the second-order rate constants given in the text.

during the isolation of the aminoacyl adenosine. If it is assumed that aminoacyl migration will show the same *p*H dependence as does the hydrolysis of aminoacyl sRNA (*10*), then we feel that so far as base-catalyzed migration is concerned, the initial incubation conditions for the present system and for that developed by Zachau and coworkers are very similar.

We have determined the rate of acyl migration in 2′-*O*-(*N*-acetylvalyl)-adenosine under various conditions. The band from a thin-layer chromatogram corresponding to 2′-*O*-(*N*-acetylvalyl)-adenosine (containing C^{14} in either the acetyl or the valyl group) was scraped off and eluted with 0.01*M* formic acid. The sample was divided into a number of small tubes, lyophilized to remove the volatile (formic acid) buffer, resuspended in appropriate buffer, and incubated at 15°C. Portions were removed at intervals, the volume of each was reduced, carrier *N*-acetylvalyladenosine was added, and the sample was again chromatographed. The first-order rate constants for migration k_1(obs) were obtained from a graph where log [percent 2′ (obs) − percent 2′ (eq)] was plotted against time for each *p*H value. Figure 2 shows a graph of the k_1(obs) values plotted as a function of *p*H. The rate dependence demonstrates that, at least over the range of *p*H 5 to to *p*H 7, migration is base-catalyzed and exhibits a strict dependence on hydroxide-ion concentration. Also plotted in Fig. 2 are the k_1(obs) values for the base-catalyzed hydrolysis of 2′ (or 3′-)-*O*-(*N*-acetylvalyl)-adenosine at 15°C (*7*). The second-order constants for the two rate equations at 15°C are:

rate = k_2 [2′ (or 3′-)-*O*-(*N*-acetylvalyl)-adenosine] [OH]

k_2(obs) migration = 3,600,000 M^{-1}min^{-1}

k_2(obs) hydrolysis = 89 M^{-1}min^{-1}

The ratio of the k_2(obs) for migration to the k_2 for hydrolysis is 40,000. This ratio is considerably higher than the ratio of the base-catalyzed migration to the base-catalyzed hydrolysis of β-glycerol acetate (*6*). Presumably this high ratio reflects the more favorable steric arrangement of the *cis* hydroxyl groups of adenosine for acyl migration (*6, 11*).

The similarity of the mechanism of migration (intramolecular alcoholysis) to alcoholysis and to hydrolysis (*12*) suggests that the aforementioned ratio of the rate of migration to the rate of hydrolysis may be used to obtain the $t_{\frac{1}{2}}$ (half-life) of migration for aminoacyl sRNA from the $t_{\frac{1}{2}}$ of hydrolysis of various aminoacyl sRNA molecules (*13*). In this way the values for $t_{\frac{1}{2}}$(obs) of migration at *p*H 7.25 and 37°C are estimated to range from 0.01 second for glycyl sRNA to 0.1 second for valyl sRNA. This very rapid migration to the equilibrium mixtures of 2′- and 3′-acyl isomers suggests that in the cell the pool of free aminoacyl sRNA as an intermediate in protein synthesis is present as an equilibrium mixture of 2′- and 3′-acyl isomers, as has also been suggested by Wolfenden *et al.* (*6*). The acyl migration lifetimes are less than, or on the same order as, the estimated lifetimes of aminoacyl sRNA during protein synthesis (*14*); indicating that migration to equilibrium must take place in vivo. At present there is no experimental evidence concerning the initial site acylation of sRNA, while studies on puromycin analogs (*15*) indicate that the 3′-isomer is used at the ribosomal level.

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References and Notes

1. H. G. Zachau, G. Acs, F. Lipmann, *Proc. Natl. Acad. Sci. U.S.* **44**, 885 (1958); L. I. Hecht, M. L. Stephenson, P. C. Zamecnik, *ibid.* **45**, 505 (1959); J. Preiss, P. Berg, E. J. Ofengand, F. H. Bergmann, M. Dieckmann, *ibid.*, p. 319.
2. P. C. Zamecnik, *Biochem. J.* **85**, 257 (1962); D. W. Urry and H. Eyring, *Arch. Biochem. Biophys., Suppl.* **1**, 52 (1962).
3. W. Frank and H. G. Zachau, *Z. Physiol. Chem.* **331**, 258 (1963).
4. C. S. McLaughlin and V. M. Ingram, *Federation Proc.* **22**, 350 (1963).
5. J. Sonnenbichler, H. Feldmann, H. G. Zachau, *Z. Physiol. Chem.* **334**, 283 (1963); H. Feldmann and H. G. Zachau, *Biochem. Biophys. Res. Commun.* **15**, 13 (1964).
6. R. Wolfenden, D. Rammler, F. Lipmann, *Biochemistry* **3**, 329 (1964).
7. C. S. McLaughlin, Ph.D. thesis, Massachusetts Institute of Technology (1964).
8. MN-Cellulose Powder 300 G (Macherey Nagel and Co., Germany).
9. H. G. Zachau, H. Feldmann, W. Frank, W. Karau, J. Sonnenbichler, in *Symposium on Nucleic Acids* (Pergamon Press, New York, in press).
10. R. Wolfenden, *Biochemistry* **2**, 1090 (1963).
11. L. Anderson and H. Lardy, *J. Am. Chem. Soc.* **72**, 3141 (1950); E. van Tamelen, *ibid.* **73**, 5773 (1951).
12. O. E. van Lohuigen and P. E. Verkade, *Rec. Trav. Chim.* **79**, 133 (1960); M. L. Bender and W. A. Glasson, *J. Am. Chem. Soc.* **81**, 1590 (1959); R. W. Taft Jr., M. S. Newman, F. H. Verhoek, *ibid.* **72**, 4511 (1950).
13. N. Coles, M. Bukenberger, A. Meister, *Biochemistry* **1**, 317 (1962).
14. J. B. Lingrel and H. Borsook, *ibid.* **2**, 309 (1963); J. E. Darnell, S. Penman, K. Sherrer, V. Becker, *Cold Spring Harbor Symp. Quant. Biol.* **28**, 287 (1963); E. T. Bolten, *Carnegie Inst. of Wash. Year Book* **58**, 275 (1959).
15. D. Nathens and A. Neidle, *Nature* **197**, 1076 (1963).
16. Supported by USPHS grant (AM-03409) from NIH and by a predoctoral fellowship from NIH to C.S.M. We thank Drs. Wolfenden, Rammler, and Lipmann for allowing us to see the manuscript of their paper (*6*) before publication.

26 June 1964

Rubella Antibodies in Human Serum: Detection by the Indirect Fluorescent-Antibody Technique

Abstract. *Antibodies for rubella virus were detected in human serum and titrated by the indirect method of immunofluorescence; a chronically infected, continuous line of monkey kidney cells was used as antigen. Positive reactions were obtained with serums from convalescent patients or persons who had been exposed to the virus while serums from patients in the acute stage of the disease and those from unexposed individuals were negative.*

The demonstration and titration of antibodies for rubella virus has been dependent upon the neutralization technique which relies upon either the interference of the rubella agent grown in tissue culture with another virus, ECHO-11 (*1*), or the inhibition of specific cytopathogenic activity in primary

Table 1. Fluorescent-antibody titers of human serums with rubella-infected and normal cells.

Serum	Infected cells	Normal cells
	Clinical disease	
Acute	1:2	Negative
Convalescent	1:16	Negative
Acute	Negative	Negative
Convalescent	1:16	Negative
Acute	Negative	Negative
Convalescent	1:16	Negative
Acute	Negative	Negative
Convalescent	1:16	Negative
Acute	Negative	Negative
Convalescent	>1:16	Negative
Convalescent	>1:16	Negative
Convalescent	>1:32	Negative
	Subclinical	
Post-exposure	1:8	Negative
Post-exposure	>1:8	Negative
Post-exposure	1:16	Negative
Post-exposure	>1:16	Negative
	Infants	
No exposure	Negative	Negative
No exposure	Negative	Negative

human amnion tissue (*2*). These slow and cumbersome procedures are in large part responsible for the limited progress in the laboratory diagnosis of this disease and on the evaluation of experimental vaccines. Attempts to demonstrate rubella virus by the direct method of immunofluorescence have been unsuccessful, probably because of the difficulty in obtaining specific antiserum in sufficiently high titer to conjugate with fluorescein. Unsuccessful attempts with the indirect method have also been reported (*3*). However, since the indirect method has proved satisfactory with certain other agents including the enteroviruses (*4*) and with measles virus (*5*), and since an unusually reliable source of antigen was available, this procedure was attempted. The devolpment of a rapid and specific test for rubella antibodies by the indirect method of fluorescent microscopy is described here.

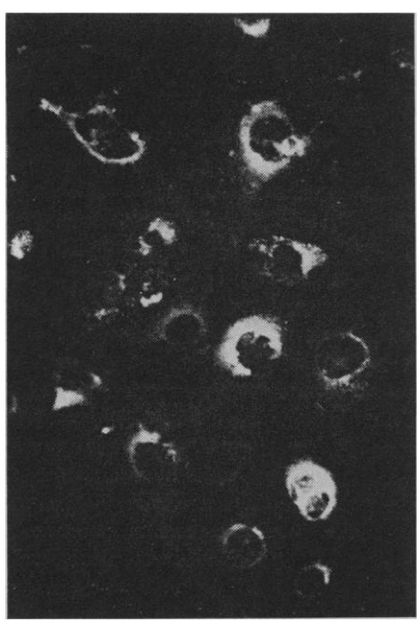

Fig. 1. Rubella-associated (RA) cells stained with fluorescein-labeled antibody to human γ-globulin by indirect technique after a 1:4 dilution of human serum from patient convalescing from rubella infection (× 180).

Serums from acutely ill and convalescent patients were collected at the University of Michigan Student Health Service through the cooperation of the director, Dr. Morley Beckett. Fluorescein-labeled goat anti-serum to human γ-globulin was obtained commercially (*6*) and further purified by fractionation on a diethyl aminoetheyl (DEAE) column (*7*). After equilibration with 0.0175*M* phosphate buffer at *p*H 6.3, the DEAE was packed into glass columns under air pressure. Undiluted, conjugated serum (15 ml) was placed in the column (1 × 20 cm) and eluted with sodium phosphate buffer with increasing concentrations of NaCl. The fraction eluted at 0.125*M* NaCl in 0.0175*M* sodium-phosphate buffer contained the bulk of the labeled specific globulin. A 1:16 dilution of this fraction gave optimum specific reaction and a minimum of nonspecific background stain. The source of antigen consisted of coverslip cultures of continuous-line monkey kidney cells (LLC-MK2) which were chronically infected with rubella virus. This rubella-associated line of cells (RA) has been subcultured for 25 generations over a 2-year period and possesses certain biological and morphological features which differentiate it from an uninfected line of the same kind of cells. The persistence and growth curve of rubella virus in these cells has been described (*8*).

The time for removal of coverslip cultures was critical. Cultures removed during the first 3 days were unsatisfactory because of background nonspecific staining, as were older cultures removed between the 8th and 12th days after seeding. The optimum period between the 4th and the 7th days resulted in uniform monolayers of better staining cells. This period also corresponded exactly with that of maximum virus production (*8*). Chronically infected and normal coverslip cultures were washed in phosphate-buffered saline at *p*H 7.3, fixed in acetone for 20 minutes (with a change of fresh acetone after 10 minutes), and dried in air for a minimum period of 30 minutes. One or two drops of appropriate dilutions of the human serum were then added to the coverslips. After incubation for 1 hour at 37°C, the cells on the coverslips were washed by immersing each three times for 10 minutes in phosphate-buffered saline then stained for 1 hour at 37°C with the fluorescein-labeled, goat antiserum to human γ-globulin. The preparations on the coverslips were again washed three times for 10 minutes each, mounted on glass slides, and examined under a binocular microscope with a darkfield condenser, an Osram HBO-200 being the light source. The filter system included a UG-5 exciter and an OG-4 and OG-5 barrier-filter combination. The reactions were graded according to the brilliance of fluorescence, and titration endpoints were taken as the highest serum dilution giving a 3-plus or 4-plus stain.

Table 1 presents the fluorescent antibody titers obtained with pairs of acute and convalescent serum, and with serums from other convalescent cases of clinically diagnosed rubella; virus was recovered from three of these patients. In all instances specific reactions were recorded on infected cells with serums obtained from convalescents approximately 1 month after onset of the disease. In contrast specimens from patients acutely ill taken within 48 hours of the appearance of the rash were negative except for one which had a very low titer. A serum dilution of at least 1:8 was best for differentiation of reaction. Specimens from both acutely ill and convalescent patients were completely negative with normal, uninfected cells. Figure 1 illustrates the reaction of a serum from a convalescent patient with infected cells. It will be noted that the specific stain is intracytoplasmic. Serum was also obtained from four additional women, including two who were pregnant, 4 to 6 weeks after known exposure to cases of rubella. These specimens were all positive, possibly as a result of subclinical infection. Two serums from 7-month-old infants were completely negative.

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References and Notes

1. P. D. Parkman, E. L. Buescher, M. S. Artenstein, *Proc. Soc. Exptl. Biol. Med.* **111**, 225 (1962).
2. T. H. Weller and F. A. Neva, *ibid.* **111**, 215 (1962).
3. B. Sigurdardottir, K. F. Givan, K. R. Rozee, A. J. Rhodes, *Can. Med. Assoc. J.* **88**, 128 (1963).
4. J. L. Riggs and G. C. Brown, *Proc. Soc. Exptl. Biol. Med.* **110**, 833 (1962).
5. G. C. Brown, unpublished data.
6. Baltimore Biological Laboratory.
7. J. L. Riggs, P. C. Loh, W. C. Eveland, *Proc. Soc. Exptl. Biol. Med.* **105**, 655 (1960).
8. H. F. Maassab, J. A. Veronelli, A. V. Hennessy, American Society for Microbiology, 64th Annual Meeting, May, 1964, *Bacteriol. Proc.* (1964), p. 144.

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Poliovirus: Guanidine Dependence and Loss of Neurovirulence for Monkeys

Abstract. *Guanidine-dependent polioviruses are obtained in vitro by subculturing Brunenders, Mahoney, and Sabin strains in the presence of increasing concentrations of guanidine. Mahoney viruses dependent on guanidine lose virulence (as indicated by paralysis) for monkeys inoculated intramuscularly or intracerebrally. Protection against virulent Mahoney viruses is induced by treatment with guanidine-dependent strains, and serum antibodies against the virulent strains are present in the treated animals.*

Guanidine inhibits the growth of poliovirus in HeLa cells (*1*). This property of guanidine was recognized independently by Rightsel *et al.* (*2*) and by Melnick *et al.* (*3*).

Table 1. Guanidine dependence induced in poliovirus 1, Brunenders strain (1S), propagated in HeLa cells. The results are expressed in cytopathic units which were calculated by the end-point method.

Modified poliovirus strain	Guanidine HCl in culture medium (μg/ml)		
	0	200	1000
1S	10^8	10^3	10^3
4G*	10^6	10^6	10^6
28G†	10^5	10^9	10^7
52G‡	10^3	10^8	10^8
108G§	10^3	10^3	10^6

* 4G is 1S propagated once with guanidine HCl at 62.5 μg/ml and three times at 250 μg/ml. † 28G is 4G propagated 24 times with guanidine HCl at 250 μg/ml. ‡ 52G is 28G propagated 24 times with guanidine HCl at 1000 μg/ml. § 108G is 52G propagated 56 times with guanidine HCl at 1000 μg/ml.

Experiments originally designed to clarify the nature of the guanidine inhibition revealed that poliovirus strains, after a number of transfers with increasing concentrations of the drug in HeLa cell cultures, became thousands of times more resistant to guanidine than the original virus. After further transfers the virus became guanidine-dependent, growing only in the presence of high concentrations of guanidine (Table 1) (*4*).

This unique dependency of poliovirus for exogenous guanidine has been confirmed recently by Lwoff (*5*). By repeated subculturing (*4*) we have developed guanidine dependency in a number of poliovirus strains, including the Brunenders, Mahoney, and Sabin strains (Table 2).

Since the concentration of guanidine required for growth by a guanidine-dependent poliovirus is much higher than that present in mammalian cells and fluids, we considered the possibility that the guanidine-dependent poliovirus might be nonpathogenic for mammals. Therefore we infected cercopithecus monkeys (*6*) with a virulent Mahoney strain of poliovirus type 1, or with the same strain made guanidine-dependent.

The results (Table 3) demonstrate the absence of paralysis in monkeys injected intramuscularly with the guanidine-dependent virus. Intracerebral inoculation gave similar results.

The immunizing effect of injection of guanidine-dependent virus is reflected in the development of serum antibodies and protection against subsequent challenge with virulent Mahoney virus. Two monkeys injected intramuscularly once, and two injected twice (28 days apart) with the guanidine-dependent virus (3×10^8 CPU) were then injected (challenged) intramuscularly, 19 days after the last inoculation, with five to ten paralyzing doses of the original, virulent strain. These four monkeys showed no signs of paralysis 1 year from the time of the viral infection. At the same time two control monkeys, which had not received the guanidine-dependent virus, were injected intramuscularly with comparable doses of the untreated strain. Both animals developed paralysis, one 4 days and the other 11 days after inoculation.

This finding prompted us to study the Sabin poliovirus strains 1, 2, and 3. These strains can also be made highly guanidine-dependent in vitro (Table

Table 2. Guanidine dependence induced in various poliovirus strains, propagated in HeLa cells. The results are expressed in cytopathic units which were calculated by the end-point method.

Modified poliovirus strain	Guanidine HCl in culture medium (μg/ml)		
	0	200	1000
Polio 1 Bruenders			
Control	10^8	10^3	10^3
108G*	10^3	10^3	10^6
Polio 1 Mahoney			
Control	10^8	10^3	10^2
25G†	10^4	10^5	10^8
Polio 1 Sabin Lsc 2 ab‡			
Control	5×10^6	10^8	10^2
40G§	10^3	10^6	10^7
Polio 2 Sabin P 712, Ch, 2 ab‡			
Control	10^6	10^2	10^2
40G§	10^3	3×10^5	10^7
Polio 3 Sabin Leon 12 ab‡			
Control	10^6	10^3	10^2
40G§	2×10^3	10^5	3×10^7

* 108G is 52G propagated 56 times with guanidine HCl at 1000 μg/ml (see Table 1). † Polio 1 Mahoney propagated twice at each of the following concentrations of guanidine HCl: 10 μg/ml, 20 μg/ml, 40 μg/ml, 100 μg/ml, 200 μg/ml, 1000 μg/ml; and 13 times at 2000 μg/ml. ‡ Virus strains obtained by the Instituto Superiore di Sanità. § Polio Sabin propagated once with guanidine HCl at 10 μg/ml and once at 20 μg/ml; twice at concentrations of 40 μg/ml, 100 μg/ml, 200 μg/ml, and 500 μg/ml; and 30 times at 1000 μg/ml.

Table 3. Lack of neurovirulence of guanidine-dependent (g.d.) poliovirus 1 Mahoney injected into 25 monkeys (*Cercopithecus aethiops*). The dosages, expressed in cytopathic units (CPU), were calculated by the end-point method. Numbers in parentheses indicate the number of monkeys injected with each dose; i.m., intramuscular injection; i.c., intracerebral injection.

Treatment of strain injected	Onset of paralysis (days after last injection)	Virus isolated from spinal cord*	Reciprocal of serum antibody titer†	
			27–28 days after 1st injection	19 days after 2nd injection
3×10^6 to 3×10^8 CPU, i.m. (7)				
No	4 to 11	+		
3×10^5 CPU, i.m. (1)				
No	None‡			
3×10^8 CPU, i.m. (5)				
25G, g.d.	None‡		5 to 25	
Two, 28 days apart, of 3×10^8 CPU, i.m. (2)				
25G, g.d.	None‡		125; 625	3,125; 15,625
10^3 to 5×10^4 CPU, i.c. (4)				
No	8 to 9	+		
Dose: 10^2 CPU, i.c. (1)				
No	None‡			
4×10^5 to 4×10^6 CPU, i.c. (4)				
25G, g.d.	None‡		5	
4×10^6 CPU, i.c. (1)				
25G, g.d.	12	+		

* Monkeys with paralysis were killed 1 to 4 days after paralysis developed. † Antibody tests, performed on the preinoculation specimens, gave no titer. ‡ No paralysis after 1 year's observation.