ELECTROPHORETIC MOBILITY AND AGGLUTINABILITY OF RED BLOOD CELLS: A "NEW" POLYMORPHISM IN MICE*

By PABLO RUBINSTEIN, NGUKOY LIU, EDWIN W. STREUN, AND FRANCINE DECARY‡

(From the Serology and Genetics Division, The New York Blood Center, New York 10021, and the Department of Pathology, Mt. Sinai School of Medicine, City University of New York, New York 10029)

(Received for publication 26 October 1973)

It has been recognized since 1954 (1) that the red cells of mice of some strains, notably A, can be specifically agglutinated more readily by anti-*H-2* sera than those of others. This difference has been shown to apply to several *H-2* antigens (2) and has been ascribed to a different cell concentration of antigenic determinants (3). Experience shows, however, that nonspecific agglutinability is also different. Thus, it is much more frequent to observe considerable rouleaux formation in the controls of reactions of A-strain erythrocytes than in those of C57BL/6 and its sublines. In studies using AutoAnalyzers (Technicon Corporation, Tarrytown, N.Y.) to measure hemagglutination induced by *H-2*-anti-*H-2* reactions it has been necessary to carry out the procedure in media that enhance agglutination to different degrees, since conditions adequate for A red cells do not support significant agglutination of C57BL/6 erythrocytes. Conversely, those under which C57BL/6 red cells are efficiently agglutinated lead to complete nonspecific clumping of A-strain erythrocytes (Contreras and Rubinstein, manuscript in preparation, Decary and Rubinstein, unpublished observations). These observations suggest that the agglutinability of mouse erythrocytes may vary for reasons unrelated to the concentration of antigen. It remains possible, however, that variation in antigen density at the surface may have two independent effects on agglutination: a direct influence over antigen-antibody reactions, and a nonspecific one, favoring rouleaux formation, perhaps through a modification of electrical charges at the membrane.

The need to obtain quantitative evaluation of *H-2* antibody has made it necessary to study this problem systematically. We report here on data showing that agglutinability is controlled by a single autosomal locus that is not linked to *H-2*, to the genes for coat color, nor to albinism.

Materials and Methods

Mice.—Animals of all the inbred strains used and (A/JxC57BL/6)F_1 (B6AF_1) hybrids were obtained from the Jackson Laboratory, Bar Harbor, Me. A SW animals were kindly provided by Dr. G. D. Snell. Backcrosses of B6AF1 to both parental strains and the respective F_2 generation were raised in our facilities. All mice were fed regular chow and had water ad libitum. Tests were done on mice 3–6 mo old.

* Supported by Grant HL09011 of the National Institutes of Health.

‡ Research Fellow of the Canadian Medical Research Council.

Bleeding.—Blood from the central artery of the tail was introduced into 50 vol of normal saline; carefully mixed, and washed three times in normal saline.

Red Cell Electrophoresis.—The washed, packed red cells were suspened to 0.2% in saline-glycine solution (2 g NaCl + 9 g glycine, distilled water 1:1; adjusted to pH 7.4 with 1 N NaOH) and introduced in the cylindrical chamber of a Zeiss cytopherometer [(Carl Zeiss, Inc., New York) kindly made available by Professors S. Kochwa and R. E. Rosenfield] fitted with platinum electrodes. The displacement of 50 individual cells in each sample was measured in both directions and recorded with a timer-printer. Means and standard deviations were calculated and electrophoretic mobility (EM)[1] computed according to:

$$EM = \frac{L/t}{A \times \Omega/h \times d}\left[\frac{cm^2}{volts \times sec}\right]$$

where: L, distance traveled (in centimeters); t, time measured (in seconds); A, current (in amperes); Ω, specific resistance of the buffer (in Ω per centimeters); h, height of the electrophoresis chamber; and d, depth of the electrophoresis chamber. Specific resistance was determined daily with a conductivity bridge (Model RC 16B2, Industrial Instruments, Cedar Grove, N.J.). A circulating water pump (Chas. F. Haake, Seaford, N.Y.) was used to ensure constant temperature.

Except for the use of platinum electrodes and glycine/saline medium, the method was exactly as described by several authors in reference 4. A buffer of lower ionic strength was used in order to achieve higher speeds of migration and minimize the brief interruptions in current flow due to electrolysis at higher ionic strengths.

Nonspecific Agglutination System.—Agglutinability (AGG) was determined with the Auto-Analyzer system depicted in Fig. 1. Briefly, a mixture of red cells (5% in normal saline) and Polybrene[2] was added to an air-segmented stream of polyvinyl pyrrolidone (PVP) and mixed during 12 min at 37°C. The agglutinated erythrocytes were then removed with T fittings. The remaining cells were lysed and their optical density (OD) was determined at 420 nm with a 15 mm continuous-flow cuvette. The resulting peaks were recorded on OD ruled paper. Each test included duplicate runs with two dilutions of Polybrene. Results are expressed as the sum of the average %Δ OD obtained with each dilution. %Δ OD = OD test × 100/OD control. Controls performed by substituting saline for Polybrene provided baselines for each cell preparation. The optimal concentrations of polyvinylpyrrolidone (PVP K-90; GAF) and Polybrene, chosen with block titration experiments, were 2% PVP and Polybrene $\frac{1}{12,500}$ and $\frac{1}{25,000}$ (w/v), respectively, in normal saline.

H-2 Typing.—*H-2* typing was performed with Kaliss's PVP method (6) as modified by us (7). Briefly, antisera were serially diluted in PVP K-90 and bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), maintaining the final concentration of PVP at 0.6%, bovine serum albumin (BSA) at 5%, pH 7.3 and a molarity of 310 mosmol. Red cells suspended in 5% BSA were added, incubated 1 h at 37°C and examined under low power magnification. Four antisera were used: two anti-*H-2.2* (one kindly provided by Dr. N. Kaliss), one anti-*H-2.4*, and one anti-*H-2.11*. These reagents, prepared by suitable absorption from sera that contained other specificities, were shown to react only with products of $H\text{-}2^b$ and $H\text{-}2^a$ respectively, in tests with adequate panels for the crosses studied.

---

[1] *Abbreviations used in this paper:* AGG, agglutinability; EM, electrophoretic mobility; and PVP, polyvinylpyrrolidone.

[2] Polybrene (Aldrich Chemical Co., Inc., Milwaukee, Wis.) induces nonspecific agglutination of red cells (in saline solution) that has been found to be dependent on the sialic acid content of the erythrocytes in humans (Dr. P. Lalezari, personal communication). Sialic acid is the major factor in determining EM of red cells. (5)

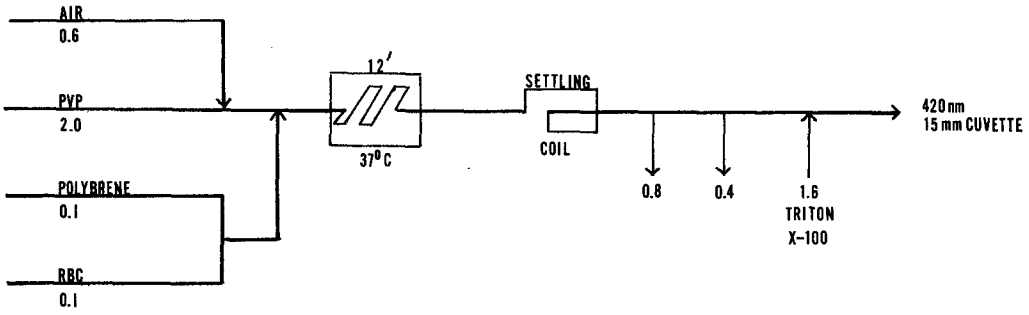

FIG. 1. Flow diagram of the agglutination system. Numbers are volumes (in milliliters) per minute.

RESULTS

Electrophoretic Mobility.—Table I shows means and standard deviations of EM values for the inbred strains studied. Means and standard deviations determined for different individuals of the same inbred strain agreed within 3%, with the exception of C58, the standard deviation of which was much higher. The values found for "type" strains were: A/J = 2.33 ± 0.03; and C57/BL6(B6) = 2.20 ± 0.028 [10^{-4} (cm^2 $s^{-1}v^{-1}$)].

Agglutinability.—Σ%ΔOD values for the strains studied are given in Table I. All measurements were repeated, and average Σ%Δ OD values for different individuals of each strain agreed within 5%. The mean values found for type strains were: A/J = 72 ± 8.1; and B6 = 40 ± 7.2. The correlation between EM and AGG was established by the study of F_2 hybrid mice between A/J and B6 as shown in Fig. 2. The coefficient of correlation, $\rho = 0.93$.

Genetic Studies.—B6AF_1 hybrids were mated to either A/J, B6, or B6AF_1 animals. The resulting backcross and F_2 offspring were tested on two different occasions by the agglutination method and the results are shown in Table II. 47 F_2 individuals were also tested by the electrophoretic method. The result is shown in Fig. 2. The very significant linear correlation obtained permitted us to proceed without testing the EM of all the hybrids.

Due to the relatively large scatter of values of AGG in the homozygotes and F_1 hybrids, the corresponding means and SD are used to define each phenotype class in the F_2 and backcross hybrids. Overlap was not encountered between the parental classes but the range of F_1 values overlapped both homozygotes. Thus, the genotype of mice with AGG values in these areas cannot be ascertained. A histogram of AGG values in the F_2 generation and those found in A/J, B6, and B6AF_1 mice are given in Fig. 3. The χ^2 values calculated with regard to expectations from a one-gene, two codominant allele hypothesis are: for the backcross to A/J = 0.14 ($P \cong 0.70$); for the backcross to B6 = 0.22 ($0.5 > P > 0.3$); and for the F_2 generation 2.15 ($0,2 > P > 0.1$).

Lack of Linkage to H-2 and Coat Color.—Backcross and F_2 animals were

TABLE 1

Electrophoretic Mobility and Agglutinability of Red Cells in Some Inbred Strains of Mice

Strain	EM*			AGG‡			EM – AGG phenotype
	N§	Mean	SD	N§	Mean	SD	
C57 BL6/J	32	2.21	0.030	49	40.1	7.2	L‖
C57 BL10/J	12	2.19	0.033	28	36.6	8.0	L
C57 BL/Ks	16	2.20	0.035	10	43.2	7.5	L
C57 BR/cdJ	8	2.20	0.039	10	47.1	7.6	L
C57 L/J	5	2.22	0.042	10	49.8	7.3	L
C58/J	12	2.26	0.112	20	49.1	16.1	L
B10A/J	15	2.19	0.043	19	42.8	7.9	L
Balb/cJ	5	2.21	0.038	10	41.5	6.9	L
ST/bJ	4	2.20	0.049		N.D.¶		L
SWR/J	5	2.19	0.035	10	43.1	9.6	L
RF/J	5	2.20	0.038	10	45.2	9.0	L
A/J	26	2.33	0.030	48	72.4	8.1	H‖
ASW	6	2.32	0.040	10	74.6	9.0	H
DBA/1J	6	2.34	0.039	10	73.4	8.1	H
DBA/2J	6	2.33	0.035	10	68.8	9.3	H
DBA/2DEJ	3	2.30	0.034	10	77.9	8.6	H
AKR/J	19	2.33	0.033	47	72.9	8.7	H
CBA/J	5	2.30	0.029	10	67.2	7.9	H
C3HeB/FeJ	5	2.30	0.033	10	73.1	9.8	H
C3H/HeJ	5	2.31	0.036	10	75.3	12.2	H
RIII/J	5	2.31	0.036	10	70.8	9.7	H
129/J	5	2.34	0.031	10	72.6	10.9	H
SjL/J	5	2.34	0.032	10	69.7	13.0	H
PL/J	4	2.33	0.028		N.D.¶		H
BDP.J	5	2.33	0.041	9	70.8	10.0	H

* Measured as $(cm^2v^{-1}s^{-1})\ 10^4$, and expressed as average of the mean values for the mice tested ± the average of their standard deviations.

‡ Measured as $\Sigma\%\Delta$ OD and expressed as in * above.

§ N, number of mice tested.

‖ L, low; and H, high.

¶ N.D., not done.

tested with the four anti-*H-2* reagents. The (improbable) linkage of *H-2* with EM is depicted in Table III, as is that of coat color and albinism.

B10A mice had the EM of the congenic lines C57BL/10 (B10) and B10.D2 rather than that of A/J (the donor of its *H-2* gene) also showing the independence of EM from *H-2*.

DISCUSSION

AGG, the property of red cells that enables them to be aggregated more or less readily by nonspecific changes in their suspending medium, has been assessed and measured. The AutoAnalyzer system employed to measure AGG is simple and reliable and provides reproducible numerical estimates of the pro-

TABLE II

Genetic Studies of Mouse Red Cell Agglutinability

(*a*) $\Sigma\%\Delta$ OD Values in the Parental Classes

	No. tested	Mean	SD	Range	EM – AGG phenotype*
B6	49	40.1	7.2	31–49	L
A/J	48	72.4	8.1	64–83	H
F1	20	55.1	9.8	45–66	HL

(*b*) Agglutinability of Erythrocytes in the Offspring from Different Crosses

	Phenotypes*			
	L	HL	H	Total
Backcross to B6	15	12	0	27
Backcross to A/J	0	30	33	63
F2	34	79	29	142

* Phenotype designations H (high), L (low), and HL (high-low) refer to both AGG and EM.

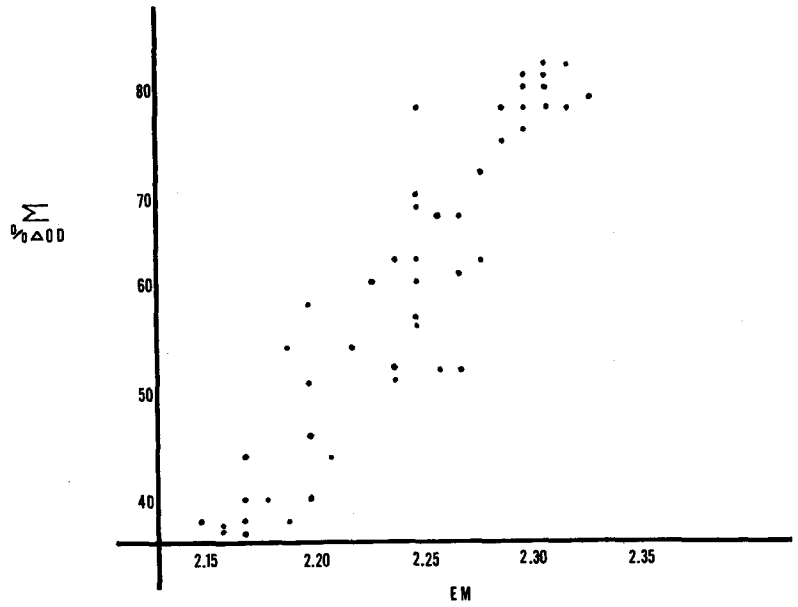

FIG. 2. Correlation between EM [in $(cm^2v^{-1}s^{-1})10^{-4}$] and AGG.

portion of erythrocytes that are agglutinated under standardized conditions. These conditions include a raised dielectric constant and a partial neutralization of the electric charge at the cell membrane. The first is accomplished with PVP, a large anisometric molecule, and the second, with Polybrene, a molecule

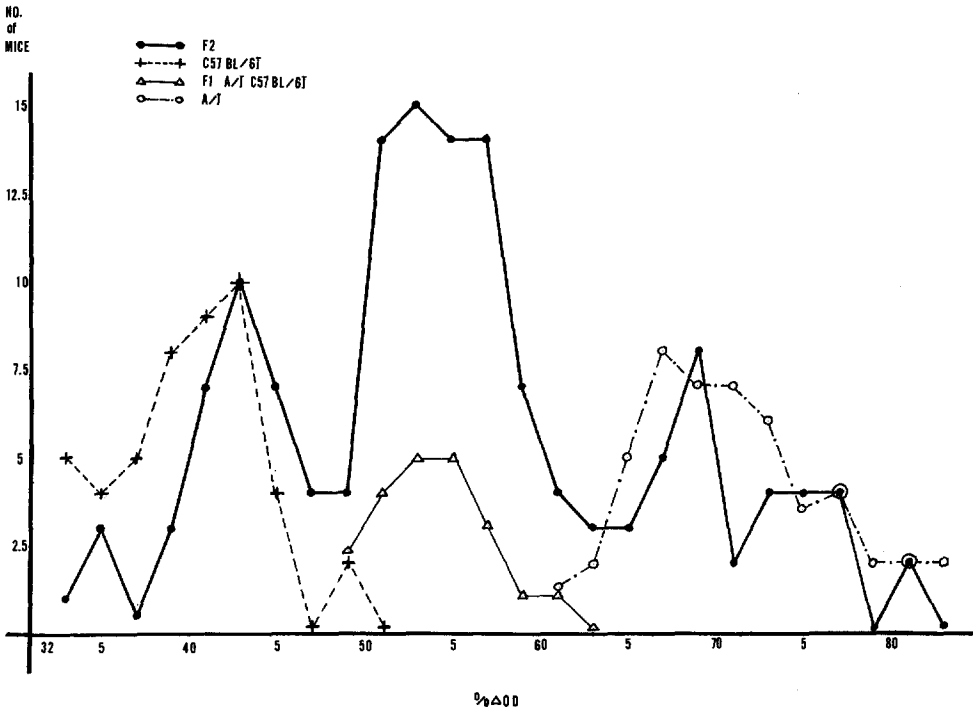

FIG. 3. Distribution of values of AGG in homozygote, F_1, and F_2 hybrid mice.

endowed with a strong positive charge. Together, they achieve a reduction of erythrocytic Z-potential below critical levels. The degree of agglutination that follows depends on the native Z-potential of the target red cells, which under identical conditions, is determined by the net electric charge at the cell membrane (8). EM is a direct consequence of this charge.

Erythrocytes from several inbred strains have been tested for both AGG and EM and two groups of strains emerge, one with high AGG and fast EM (type strain: A/J), and the other, with low AGG and slow EM (type strain: B6). That the association between AGG and EM is probably real is shown by studies on F_2 hybrids between strains A/J and B6, in which the coefficient of correlation was extremely high. In these studies, variation was caused by the segregation of the genes responsible plus a considerably wide range of expressivity of these genes in different individuals.

Alternatively, EM and AGG values could be determined by the interaction of multiple genes, thus accounting for the overlap encountered in the values of hetero- and homozygotes. However, the overall agreement between the distribution of EM phenotypes in the different crosses and the expectations from a simple diallelic situation is sufficiently good to assume that one gene is of either unique or overriding importance in determining the surface electric charge of mouse erythrocytes.

TABLE III

Association of AGG with H-2, Sex, and Coat Color in B6 × A/J Crosses

(*A*) Backcross to A/J

	Phenotype*		
H-2^b	HL	H	
−	12	19	31
+	18	14	32
	30	33	63

$X^2 = 1.86$

$0.20 > P > 0.10$

sex	HL	H	
♂	17	14	31
♀	13	19	32
	30	33	63

$X^2 = 1\text{–}24$

$0.30 > P > 0.20$

Color	HL	H	
Black	9	7	16
Brown	8	8	16
White	13	18	31
	30	33	63

$X^2 = 0.95$ ($df = 2$)

$0.70 > P > 0.50$

(*B*) Backcross to B6

H-2^a	L	HL	
+	7	8	15
−	8	4	12
	15	12	27

$X^2 = 1.06$

$P = 0.30$

sex	L	HL	
♂	4	6	11
♀	11	6	16
	15	12	27

$X^2 = 0.78$

$0.50 > P > 0.30$

(*C*) F_2

H-2	L	HL	H	
a + *b*−	12	16	8	36
2 + *b*+	15	43	17	75
a − *b*+	7	20	4	31
	34	79	29	142

$X^2 = 4.28$ (4 df)

$P \leqq 0.5$

sex	L	HL	H	
♀	19	35	13	67
♂	15	44	16	75
	34	79	29	142

$X^2 = 1.3$ (2 df)

$P \cong 0.5$

Color	L	HL	H	
Black	17	27	14	58
Brown	9	22	3	34
White	8	30	12	50
	34	79	29	142

$X^2 = 6.1$ (4df)

$02 > P > 0.1$

* Designations L (low), H (high), and HL (high-low) refer to AGG phenotypes.

EM and AGG are thus apparently controlled by an autosomal gene with two codominant alleles in the inbred strains studied. The character segregated independently of *H-2*, of coat color, and of the gene for albinism. Thus, association to linkage groups I, VIII, or IX was not detected. Strains derived from a single original stock possess the same EM phenotype with the exception of AKR and RF.

Few studies of the EM of red cells of nonhuman species have been reported. It has been claimed (9, 10) that the electrophoretic behavior of normal mammalian erythrocytes is constant for a given species, and the EM of mouse RBC described as 2.7 [10^{-4} (cm^2 s^{-1} v^{-1})] (11) using a buffer somewhat similar to ours. This figure was approximately the same as that found for human red cells. Similar mobilities for mouse and human erythrocytes have also been reported by Arnold (12) and Rottino and Angers (13), although different buffers account for significantly different absolute measurements, as expected. However, Abramson (14) obtained a slightly higher figure, 1.40 [10^{-4} (cm^2 v^{-1} s^{-1})] for the mouse, as opposed to 1.31 for humans. We measured a similar difference (not shown).

There seem to be no previous reports of interstrain variability of EM in mice. In humans, on the other hand, differences in EM of an inherited nature have been described as associated with antigens M^g and M^k of the MNSs system (15) and En, an apparently independent locus (15, 16). These inherited variants expressed an intermediate mobility in the heterozygote, as do F_1 mice. Moreover, the effects of this lower EM are also appreciated in that affected red cells become agglutinable by incomplete (IgG) anti-Rh antibodies (15, 16) and inagglutinable in the Polybrene test, (P. Lalezari; L. Marsh, personal communications.) The lower AGG is due, in humans, to a decreased quantity of sialic acid at the cell membrane (15) but this information is still lacking in mice. If variation in EM and AGG is a consequence of differential quantities of sialic acid at the respective cell surfaces, the genes responsible probably act by regulating either the quantity of sialyltransferase or its K_m value. Similar regulation of the quantity of terminal *N*-acetyl-galactosamine, that underlies the $A_1 - A_2$ distinction in humans, has been traced to the K_m value of the corresponding transferase (17). In view of the importance of sialic acid in cell biology (18–20) especially in its possible role as receptor site for several tumor-inducing viruses (21, 22), experiments are in progress to determine the chemical nature of the phenotypic variation described, and to identify the linkage relationships of the corresponding gene.

SUMMARY

A quantitative method has been developed to determine agglutinability of mouse red blood cells. Tests with different inbred strains of mice revealed only two phenotypes. The same inbred strains were tested with the cytopherometer to determine the electrophoretic mobility of the corresponding red cells. Again, two phenotypes were uncovered, and faster mobility was found in the red cells

that had higher agglutinability. The genetic control of this character is autosomal and codominant, and segregates independently of *H-2* and coat color.

The advice and criticism of Doctors F. H. Allen, S. Kochwa, R. Leonardi, and R. E. Rosenfield are gratefully acknowledged.

REFERENCES

1. Gorer, P. A., and L. B. Mikulska. 1954. The antibody response to tumor inoculation. Improved methods of antibody detection. *Cancer Res.* **14:**651.
2. Stimpfling, J. H., and G. D. Snell. 1962. Histocompatibility genes and other immunogenetic problems. *In* Proceedings of the International Symposium on Tissue Transplantation. A. Cristofannini and G. Hoecker, editors. Universidad de Chile, Santiago. 37.
3. Herberman, R., and Ch. A. Stetson. 1965. The expression of histocompatibility antigens on cellular and subcellular membranes. *J. Exp. Med.* **212:**533.
4. Ambrose, E. J., editor. 1965. Cell Electrophoresis, Little, Brown and Co., Boston.
5. Eylar, E. H., M. A. Madoff, O. V. Brody, and J. L. Oncley. 1962. The contribution of sialic acid to the surface charge of the erythrocyte. *J. Biol. Chem.* **237:** 1992.
6. Kaliss, N. 1973. An improved polyvinylpyrrolidone (PVP) method for titering mouse hemagglutinins. *Transplantation.* **15:**251.
7. Decary F., and P. Rubinstein. 1973. In reference to Kaliss. *Transplantation.* **25:** 630.
8. Pollack, W., H. J. Hager, R. P. Reckel, H. O. Singher, and D. A. Toren. 1965. A study of the forces involved in the second stage of hemagglutination. *Transfusion (Phila)* **5:**158.
9. Ruhenstroth-Bauer, G. 1965. The normal and pathological hemocytopherogram of man. *In* Cell Electrophoresis. E. J. Ambrose, editor. Little, Brown and Co., Boston, 66–72.
10. Abramson, H. A., L. S. Mojer, and M. H. Gorin. 1942. Electrophoresis of Proteins and the Chemistry of Cell Surface. Reinhold Publishing Corp., New York.
11. Doljanski, F., and S. Eisenberg. 1965. The action of neuraminidase on the electrophoretic mobility of liver cells. *In* Cell Electrophoresis. E. J. Ambrose, editor. Little, Brown and Co., Boston. 78–84.
12. Arnold, R. 1965. Pathological hemocytopherograms of rats and mice. *In* Cell Electrophoresis. E. J. Ambrose, editor. Little, Brown and Co., Boston. 36–41.
13. Rottino, A., and J. W. Angers. 1962. Erythrocyte microelectrophoretic mobility in AK mice with spontaneous leukemia. *Proc. Am. Assoc. Cancer Res.* 3 (Abst. 228).
14. Abramson, H. A. 1934. Electrokinetic Phenomena. New York Chemical Catalogue Co., New York.
15. Nordling, S., R. Sanger, J. Gavin, V. Furuhjelm, G. Myllyla, and M. N. Metaxas. 1967. M^g and M^k: Some serological and physicochemical observations. *Vox Sang.* **17:**300.
16. Furuhjelm, V., G. Myllyla, H. R. Nevanlinna, S. Nordling, A. Pirkola, J. Gavin, A. Gooch, R. Sanger, and P. Tippet. 1967. The red cell phenotype En^a (−) and anti-En^a. Serological and physicochemical aspects. *Vox Sang.* **17:**256.
17. Schacter, H., M. A. Michaels, M. C. Crookston, C. A. Tilley, and J. H. Crookston.

1971. A quantitative difference in the activity of blood group A-specific n-acetylgalactosaminyl transferase in serum from A_1 and A_2 human subjects. *Biochem. Biophys. Res. Commun.* **45**:1011.

18. Gottschalk, A. 1960. The Chemistry and Biology of Sialic Acids and Related Substances. Cambridge University Press, London.
19. Weiss, L. 1967. The cell periphery, metastasis and other contact phenomena. *In* Frontiers of Biology. A. Neuberger and E. L. Tatum, editors. North Holland, Amsterdam. **7**.
20. Schauer, R. 1973. Chemistry and biology of the acylneuraminic acids. *Angew. Chem. Int. Ed. Engl.* **12**:127.
21. Grimes, W. J. 1973. Glycosyltransferase and sialic acid levels of normal and transformed cells. *Biochemistry.* **12**:990.
22. Howe, C., and L. T. Lee. 1972. Virus-erythrocyte interactions. *Adv. Virus Res.* **17**:1.