arately (34) developed higher levels of caries activity than those caged with crossbreeds (35): carious teeth, 11.1 and 10.8; carious lesions, 36.9 and 32.7; carious areas, 62.3 and 53.5, respectively. Conversely, the crossbred animals caged separately (30) developed lower levels of activity than those in mixed groups (30): carious teeth, 8.5 and 9.2; carious lesions, 17.8 and 19.0; and carious areas, 28.4 and 30.1, respectively. These results suggest that the O-M's are more capable than the crossbreeds of supporting the "caries conducive flora" provided by the mother. Even though every animal was exposed to the same microbiota during the nursing period, that which was retained during the test period was only that which the animals within a given cage could support on the diet provided. Additional studies are under way to further identify heritable factors which may contribute to these results.

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25 June 1965

## Hemadsorption-Negative Plaque Test: New Assay for Rubella Virus Revealing a Unique Interference

Abstract. *A simple and rapid plaque procedure has been developed for detecting and accurately assaying rubella virus in a noncytopathic virus–cell relationship. Plaque-formation is based on the development, in individual cells infected with rubella virus, of a unique type of intrinsic interference to infection with Newcastle disease virus. Rubella virus–infected cells challenged with Newcastle disease virus and tested for hemadsorption 15 hours later stand out as hemadsorption-negative areas. Individual living cells infected with rubella virus can be resolved under conditions allowing standard cloning procedures. In principle, the hemadsorption-negative plaque test can be used to search for a new class of noncytopathic, nonhemadsorbing viruses—those that induce an intrinsic interference to infection by any hemadsorbing virus.*

Research on the biochemistry, genetics, and teratogenic effects of rubella virus has been seriously limited by the lack of a test to score individual infectious particles and single infected living cells. We have devised a simple plaque assay for rubella virus as a noncytopathic virus under conditions that do not adversely affect viability of individual infected cells and, hence, allow isolation of virus or cell by standard cloning techniques. Detection of rubella virus depends on the development in infected cells of a unique interference to superinfection by Newcastle disease virus (NDV), a hemadsorbing, cytopathic myxovirus (*1*). In the test, rubella virus–infected cells (NDV-resistant) stand out as hemadsorption-negative plaques against a background of hemadsorption-positive (NDV-susceptible) cells.

The hemadsorption-negative plaque test is carried out as follows. Susceptible host cells (*2*) are grown to confluency in 60-mm petri dishes (Falcon Plastics No. 3002) containing 5 ml of attachment solution (*3*) supplemented with 3 percent fetal bovine serum (standard growth medium). This medium is removed at the time of infection, and rubella virus (*4*) contained in 0.3 ml of attachment solution is adsorbed for 60 to 120 minutes at 35°C. The plates are then rinsed once, flooded with 5 ml of standard growth medium, and incubated undisturbed at 35°C in an atmosphere of continuously flowing $CO_2$ and air. To reveal the rubella plaque areas at the end of the incubation period, usually 3 to 4 days, medium is removed from the infected monolayers and NDV is added at an attachment multiplicity, that is, a virus: cell ratio of 10 to 20 plaque-forming or hemadsorption-producing particles (*5*). Routinely, 0.1 ml of NDV stock, harvested as allantoic fluid 48 hours after infection of 9-day-old chick eggs, and 0.2 ml of attachment solution are applied, per plate, for 60 minutes at 37°C. Unattached virus is then removed, and the monolayer is rinsed once with 2 ml of attachment solution. To preclude spurious binding of red blood cells due to the presence of residual input virus on the surface of otherwise hemadsorption-negative (HAD−) cells, the rinsed monolayer is exposed to 0.5 ml of antiserum to NDV at a concentration which neutralizes over 99 percent of the plaque-forming particle activity of NDV stock preparations within 15 minutes at 37°C, usually a 1:50 dilution of serum from immunized rabbits (*6*). Antiserum is removed after 20 minutes; the monolayer is washed once with 2 ml of attachment solution, flooded with 5 ml of this solution, and incubated for $15 \pm 2$ hours at 37°C to develop the hemadsorption-positive (HAD+) state in all cells susceptible to NDV at the time of challenge. The single-cell hemadsorption test is completed as described (*5*), and care is taken to minimize elution of red blood cells and loss due to mechanical trauma in the washing procedure. Briefly, the medium is removed and replaced with 3 ml of a suspension of bovine erythrocytes ($6 \times 10^7$/ml) in phosphate-buffered saline (*p*H 7). Red blood cell adsorption is carried out for 20 minutes at 4°C (cold room), and the plates are rinsed gently eight to ten times with copious amounts of cold, phosphate-buffered saline (*p*H 6) to remove unattached erythrocytes. Several milliliters of cold saline are left on the plate after the final rinsing. The hemadsorption-negative plaques stand out as dark areas when examined by a dark field–like illumination obtained by viewing the underside of plates held up to a light source from the ceiling. These plaques are stable in cold saline (*p*H 6) for 1 day, or they may be fixed with $OsO_4$ (*7*) and sealed in Gurr's water-mounting solution for a permanent record (Fig. 1).

As early as 20 hours after infection with rubella virus, challenge with NDV and application of the single-cell hemadsorption test reveal discrete foci which contain one or a few hemadsorption-negative cells. Figure 2 illustrates an area from a plate challenged with NDV 20 hours after infection with rubella virus. These small, nonhemadsorbing areas spread to encompass scores of cells as seen in Fig. 3, and in early infection often contain a few individual hemadsorbing cells that are also illustrated. Further incubation eventually

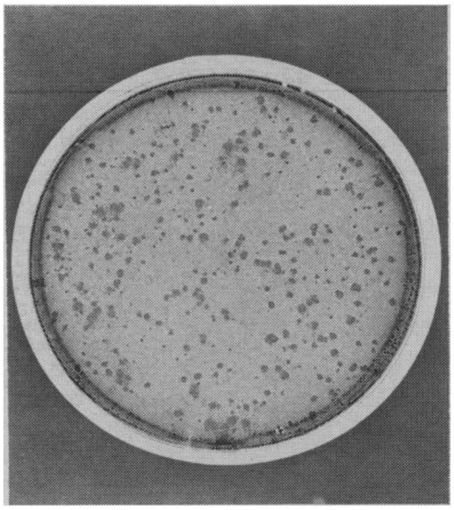

Fig. 1. Hemadsorption-negative plaques of rubella virus. Monolayers of African green monkey kidney cells were inoculated with rubella virus, incubated for 3 days, challenged with Newcastle disease virus (NDV), incubated for an additional 15 hours, and subjected to the single-cell hemadsorption test. The nonhemadsorbing (NDV-refractory) cells stand out as dark areas against a light background of bovine erythrocytes which cover the hemadsorption-positive (NDV-susceptible) host cells. Actual size.

produces monolayers in which every cell is hemadsorption-negative.

The rubella-infected cells that constitute a hemadsorption-negative area, whether it consists of a single cell or a confluent monolayer, are completely refractory to NDV. Thus, a 48- to 72-hour incubation period further differentiates the susceptible HAD+ cells, which undergo total destruction, from the refractory HAD− cells. The hemadsorption-negative cells survive and continue to divide to form colonies.

The number of HAD− plaque areas on a plate increases linearly with the concentration of rubella virus, as documented in Fig. 4, and constitutes evidence that each plaque area is initiated by a single infectious unit of virus (*8*). The exponential rate of inactivation of rubella virus by ultraviolet light (*9*) offers support to the hypothesis that this infectious unit is most likely a single virus particle and that significant clumping of infectious particles does not occur in stock preparations.

The maximum number of HAD− plaque-forming units is registered after an adsorption period at 35°C of from 2 to 2½ hours. During the 1-hour adsorption period routinely employed for practical reasons, 60 percent of the total input virus binds to the cell monolayer.

Stock preparations of rubella virus were assayed simultaneously, by the same method described earlier, for their content of HAD− plaque-forming particles and for 50-percent interfering doses ($InD_{50}$) as described by Parkman *et al.* (*10*). In one such assay, HAD− plaque counts on replicate plates were recorded as 135, 152, 116, 141, 125, 145, 133, and 128, with an average and standard deviation of $134 \pm 12$. This preparation of rubella virus stock was measured as $6.7 \times 10^4$ HAD− plaque-forming particles per milliliter. The interference-inducing particle titer was $6 \times 10^4$ per milliliter when $InD_{50}$ units were converted to interference-inducing particles by the Poisson formula, where 1 $InD_{50}$ = 0.693 interference-inducing particle. These results show that there is nearly a 1 : 1 ratio between the number of plaque-forming and interference-inducing particles, an indication that both tests most likely measure the same basic infectious property of the virus. However, the plaque technique has the important practical advantage, pointed out earlier by Dulbecco (*8*), that the accuracy obtained with a single plaque plate is equalled only by using 100 or more tubes of cells, as in the $InD_{50}$ test.

One of the most intriguing aspects of the HAD− plaque system is the unique intrinsic type of interference to NDV that is manifested by the rubella virus–infected cells. This was revealed when viruses of several representative types were used to challenge monolayers containing cells, all of which were refractory to NDV; that is, 100-percent hemadsorption-negative. Medium was discarded from the hemadsorption-negative monolayers and, after adsorption of enough challenge virus to infect essentially all cells at the start, replaced by fresh medium. Only strains of NDV (California, Beaudette, and Massachusetts-HiK) fail to infect rubella virus–treated cells; thus, 24 hours after challenge, cell monolayers show no cytopathic effects, are completely hemadsorption-negative, and, hence, are indistinguishable from unchallenged control cells. In contrast, Echo-11, polio-1, vaccinia, and influenza B viruses produce gross cytopathic changes in essentially all cells of the monolayer within 24 hours. A noncytopathic, hemadsorbing simian virus that we had isolated as a contaminant was also able to infect all of the hemadsorption-negative cells, as evidenced by their extensive hemadsorption-positive response 24 hours after infection.

The inclusion of Echo-11 as a cytopathic virus presents an apparent anomaly since this enterovirus is used as a standard challenge virus in the usual interference test (*10*). This anomaly is resolved when the experimental conditions of the two tests are examined. The primary difference lies in the amount of the challenge virus used to test for the refractory state. In the standard interference test, only one cell in $10^3$ to $10^4$ cells is infected at the start, whereas the dose of challenge virus used in the HAD− plaque-type test is such that essentially every cell is infected initially. Presumably, conditions during the early days of the 7- to 10-day interference test provide the maximum opportunity to accumulate a critical amount of any extrinsic interfering factor before the relatively few particles of challenge virus reach a large enough number to register a measurable cytopathic effect. We found that, after removal of the original medium, Echo-11 virus, in an amount sufficient to produce extensive cytopathic effects within 24 hours when the original medium was replaced by fresh medium after challenge, had little or no effect on cells when the original medium was introduced after adsorp-

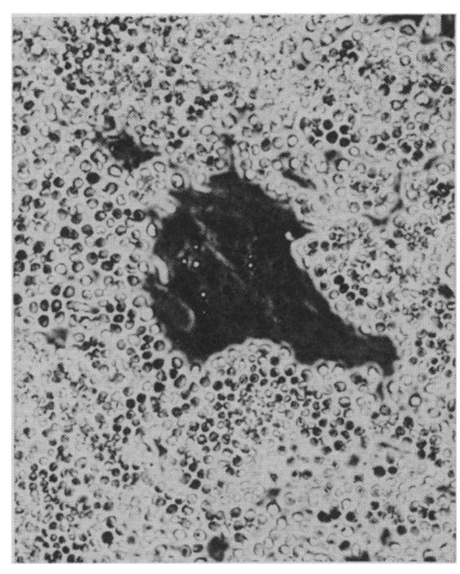

Fig. 2. Hemadsorption-negative plaque induced by rubella virus. Green monkey kidney cells were challenged with Newcastle disease virus (NDV) 20 hours after infection with rubella virus. Three hemadsorption-negative cells are illustrated, surrounded by a confluent background of NDV-susceptible cells, not visible beneath the layer of adsorbed, bovine red blood cells. Phase-contrast, living cells. Diameter of red blood cells, 6 $\mu$.

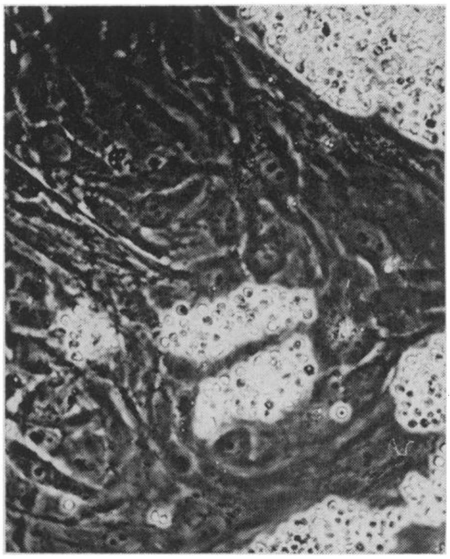

Fig. 3. Portion of a hemadsorption-negative plaque induced by rubella virus. Conditions as in Fig. 2, except that the cells were challenged with Newcastle disease virus 72 hours after infection with rubella virus. The few remaining individual cells that do adsorb bovine erythrocytes stand out against the background of cells that do not.

tion of the challenge virus. Further evidence for distinguishing between extrinsic and intrinsic states of interference is provided by the fact that plates infected with rubella virus containing as few as 25 percent of their cells refractory to high multiplicities of NDV were scored as totally refractory to Echo-11 virus in the standard interference test only if the medium was not changed prior to challenge. These results point out that interference to Echo-11 in the standard test is mediated by an extrinsic factor, whereas resistance to NDV has an intrinsic basis possibly of a fundamentally different type from that responsible for interference to Echo-11. We are presently engaged in defining the nature of this difference (*11*).

Although the mechanism of the NDV-refractory state induced by rubella virus is not understood, it is not based on any obvious relationship between rubella virus and the myxoviruses. For example, complete destruction of cellular receptors for myxovirus with neuraminidase has no effect on the initiation or development of the rubella-induced plaques; also, attachment and eclipse of NDV appears normal on monolayers containing 100-percent NDV-refractory cells.

Several aspects of the hemadsorption-negative plaque test raise basic questions concerning the biology of noncytopathic viruses, such as the relation of virus-production and plaque-formation in a liquid environment. Once the maximum rate of production of rubella virus is attained in monolayer culture, infected cells release virus at a relatively constant rate, averaging one $HAD^-$ plaque-forming unit/166 cells per hour during the 2-month test period; however, in view of the rapid rate of inactivation of rubella virus in our standard growth medium (half time is 63 minutes at 37°C), the actual formation of infectious virus is considerably higher. Infectious-center assays of monodisperse cells, obtained after treatment with trypsin from monolayers producing rubella virus at the maximum rate, show that only one cell in 150 was releasing sufficient virus to initiate formation of a nonhemadsorbing plaque. The low output of infectious particles suggests the reason for the success of the assay system since an effectual low yield of infectious virus is prerequisite for maintaining discrete plaques in a liquid environment. Formation of hemadsorption-negative plaques is prevented in the presence of high concentrations of antiserum to rubella (*12*), which points to the necessity of an extracellular phase in the continuous cycling of infectious virus needed to produce a plaque. Since no cytopathic effects were evident in infected monolayers, there is little question that the observed rate of production of rubella virus could be projected beyond the 2-month period tested to the limit of survival of these primary cells.

We have become acutely aware of the important role of the host cell in controlling the rate of spread of the rubella-induced refractory state to NDV. For example, the time required for an inoculum of $1 \times 10^4$ $HAD^-$ plaque-forming particles to produce a monolayer with 100 percent of the cells hemadsorption-negative may be as little as 2 days or as long as 7 to 10 days, depending on the lot of cells used and the temperature. This close interdependence of host cell and virus, coupled with an optimal temperature of 34° to 35°C for the development of plaques, is not usual in cytopathic virus–cell systems.

We do not know how many viruses will exhibit the unique intrinsic type of interference characteristic of the rubella virus–Newcastle disease virus system; however, we can report that Sindbis virus also produces hemadsorption-

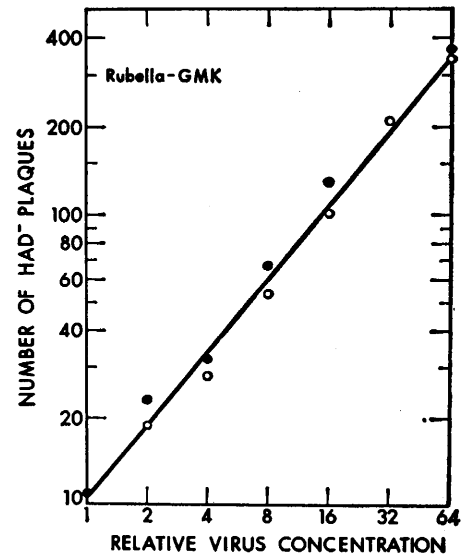


Fig. 4. Proportionality between the relative concentration of rubella virus and the number of hemadsorption-negative plaques observed. Open and closed circles represent the results of two different experiments.

negative plaques under these same experimental conditions. The existence of a whole spectrum of unknown, noncytopathic, nonhemadsorbing viruses might well be discovered by the hemadsorption-negative plaque technique.

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**References and Notes**

1. Host cells infected with myxoviruses incorporate into their plasma membrane a viral antigen, hemagglutinin, which specifically adsorbs erythrocytes from several species. This so-called hemadsorption test was discovered by Vogel and Shelokov [*Science* **126**, 358 (1957)] and can be used to detect cells infected with cytopathic or noncytopathic myxoviruses. This test has been refined to define and resolve the pattern of hemadsorption on single infected cells (*5*).
2. Primary African green monkey (*Cercopithecus*) cells obtained from Microbiological Associates or Baltimore Biological Laboratories were used as host cells throughout this study. However, $HAD^-$ plaques from rubella virus have been obtained on L-cells (G. Henle, cited by W. Henle in "Some aspects of persistent viral infections of cell cultures," a lecture given at the University of Maryland, November 1964), primary human embryonic kidney and lung (Microbiological Associates), human skin, and the GRKL strain of rabbit kidney supplied by Dr. S. Plotkin, Wistar Institute. In our hands, the GRKL strain showed no evidence of the degenerative effects reported for some strains in a preliminary communication by C. H. Taylor-Robinson *et al.*, *Lancet* **1964-I**, 1304 (1964).
3. Attachment solution is NCI medium plus 6 percent calf serum. NCI is the nutrient solution described by P. I. Marcus, S. J. Cieciura, T. T. Puck, *J. Exp. Med.* **104**, 615 (1956), to

which has been added i-inositol at 0.0010 g per liter, the glutamine increased to 0.200 g per liter, and the phenol red concentration reduced by a factor of 10. NCI medium is available from Grand Island Biological Co., Grand Island, N.Y.
4. Rubella virus as strain F-8 (supplied by Dr. Balsamo of the New York University Medical School) was used to develop stocks of virus. The original F-8 strain produced small HAD- plaques in 4 days, whereas passage of this strain on green monkey kidney cells in our laboratory seems to have selected for a large-plaque varient which we have designated F-8L and used as rubella stock throughout this study. We have also obtained small HAD- plaques upon direct isolation from the blood of a patient with an active case of rubella.
5. P. I. Marcus, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 351 (1962).
6. Experience with the single-cell hemadsorption technique (*5*) has demonstrated that infection with high multiplicities of myxovirus sometimes results in a small amount of red-blood-cell adsorption due to residual surface-bound virus. This "background" can be eliminated completely by exposing the NDV-infected cells to specific viral antiserum, thus coating the viral hemagglutinin. This step (addition of NDV-antiserum) is included routinely in the HAD- plaque test since it is not possible to predict a priori whether the background level of hemadsorption will be high or low for a given lot of cells.
E. Robbins, *J. Biophys. Biochem. Cytol.* **11**, 449 (1961).
R. Dulbecco, *Proc. Nat. Acad. Sci. U.S.* **38**, 747 (1952).
9. The 37-percent survival dose, D⁰, for rubella virus, defined as the dose of ultraviolet light required to reduce the number of HAD- plaque-forming particles to $e^{-1}$ (0.37 survivors = 1 lethal hit) is 110 ergs mm$^{-2}$. Ultraviolet-light dosimetry was based on the $D^0$ values obtained from the exponential inactivation curves of T2 phage and Newcastle disease virus irradiated in the same medium. T2 phage qualifies as a biological actinometer of known uniformity (*13*). For comparison, $D^0$ values for T2 phage and NDV are 25 and 42.5 ergs mm$^{-2}$, respectively.
10. P. D. Parkman, E. L. Buescher, M. S. Artenstein, J. M. McCown, F. K. Mundon, A. D. Druzd, *J. Immunol.* **93**, 595 (1964).
11. Because of the preliminary nature of this aspect of our investigation, we have deferred consideration of the possible relationship of these two types of interference to classical interferons.
12. Antiserum to rubella virus was obtained as serum from convalescent individuals who had been diagnosed as having German measles. A 1 : 50 dilution of serum neutralized more than 90 percent of the HAD- plaque-forming particle activity within 15 minutes at 37°C.
13. S. E. Luria, *General Virology* (Wiley, New York, 1953), p. 153; P. I. Marcus and T. T. Puck, *Virology* **6**, 405 (19 H. H. Lee and T. T. Puck, *Radiation Res.* **12**, 340 (1960).
14. Part of this work was presented at the 65th annual meeting of the Amer. Soc. for Microbiol., April 1965, *Bacteriol. Proc.*, abstr. V8 (1965), p. 98. Aided by grants AI-03619-05VR and GM-12646-01A1 from NIH. One of us (P.I.M.) is a research career development awardee of the National Institute of Allergy and Infectious Diseases (2-K3-GM-15, 461-05).

18 May 1965

## Serum Prealbumin: Polymorphism in Man

Abstract. *Serums from 390 Norwegian blood donors and 31 members of two families were studied by starch gel electrophoresis. Five different prealbumin phenotypes were found, indicating a genetic theory of three codominant alleles for which the terms* $Pr^F$, $Pr^M$, *and* $Pr^S$ *are proposed.*

When human serum is subjected to zone electrophoresis in starch gel, two (*1*) or three (*2*) protein bands, the prealbumins, migrate in front of the albumins. We have examined prealbumins in serums from 390 Norwegian blood donors and from 31 members of two families, using a method based upon Poulik's (*3*) horizontal discontinuous system (gel buffer *p*H 4.95). (Details of our technical procedures are in preparation.)

Our studies revealed a rather high number of prealbumin bands. However, the number of bands depends upon the technique, and only the prealbumins that were clearly recognized by our routinely used method are considered here. These prealbumins (Pr) appeared as three to four bands of varying strength and they were found in five different patterns (Fig. 1). In our hands the prealbumins in serums from most individuals appeared as a three-band pattern: one weak band in front of two relatively heavy bands. This pattern is designated MM (M = medium). Another three-band pattern, which is slower, is called SS. A phenotype which seems to be a combination of the medium three-band pattern and a hypothetical fast-moving phenotype is called FM. The last two phenotypes are called FS and MS. In combination phenotypes, the bands which do not overlap show about half the strength and staining intensity of the corresponding bands in the three-band patterns. The distance between the two main bands of each three-band pattern is largest for SS, intermediate for MM, and smallest for "FF" (as judged from the F bands in the FS phenotype).

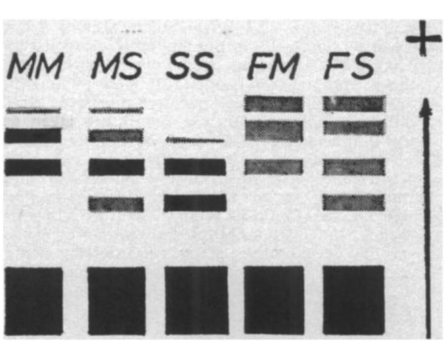


Fig. 1. Drawing of the five prealbumin phenotypes in front of the albumins.

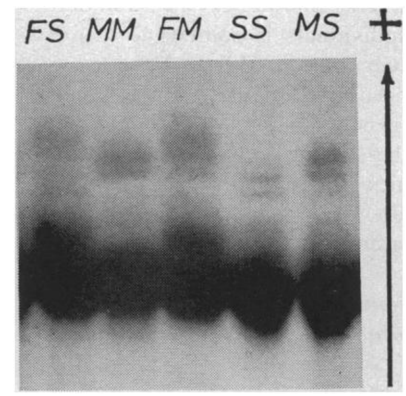


Fig. 2. Photograph of part of a stained gel where the five prealbumin phenotypes are present in front of the albumins.

Figure 2 is a photograph of part of an amido black–stained gel where samples representing the five phenotypes have been examined. Results from investigation of the families of two blood donors are shown in Fig. 3.

These findings indicate a genetic theory of three codominant alleles for which we propose the terms $Pr^F$, $Pr^M$, and $Pr^S$, corresponding to the fast, medium, and slow migration rates of the respective allele products. In 390 randomly selected blood donors, the phenotype MM was found in 374 individuals, while MS was found in 9, FM in 4, SS in 2, and FS in 1. These figures and the family studies fit the genetic theory advanced, the $Pr^M$ being the most common allele and $Pr^F$ and $Pr^S$ rare. The FF phenotype was not found, which is according to expecta-

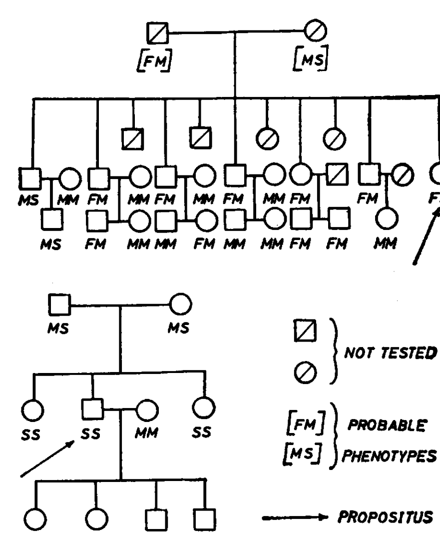


Fig. 3. Prealbumin phenotypes in members of two families.