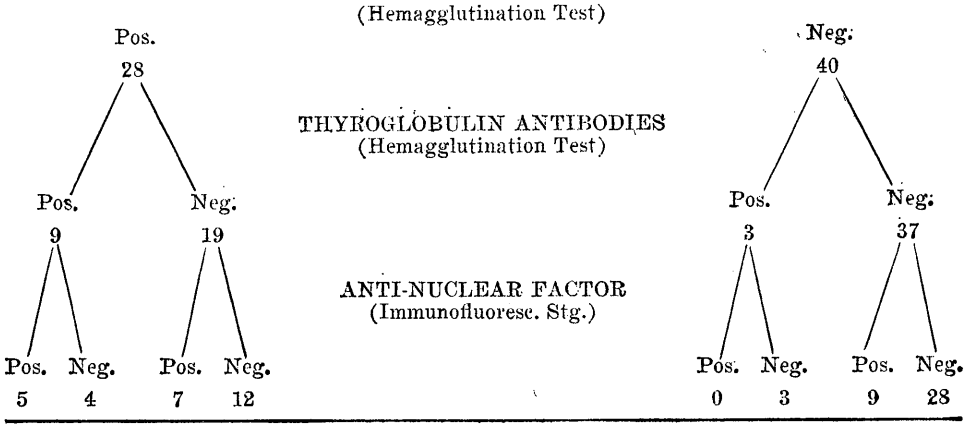
TABLE II. Results of Serological Examination of 68 Patients Clinically Diagnosed as Suffering from Myasthenia Gravis.



appear to be essentially the same as those obtained with fresh tanned cells. However, the formalinized and coated tanned cells can be kept in the refrigerator for 1 to 2 weeks or in a frozen state in the deep-freeze for as long as 6 months. Thyroid antibodies and antinuclear antibodies seem to occur more frequently in the sera of patients suffering from myasthenia gravis containing muscle autoantibodies than in groups of those patients without muscle antibodies.

The authors wish to acknowledge the invaluable help provided by Dr. Frank Howard of the Mayo Clinic, who supplied most of the sera included in these studies.

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Measurement of Hemolysin Transfer Using Untreated Red Blood Cells. (31409)

HAZEL P. GUMP AND LAURENCE R. DRAPER (Introduced by Maurice Landy)
Laboratory of Physiology, National Cancer Institute, National Institutes of Health, USPHS, Bethesda, Md.

Taliaferro *et al*(1) determined the avidity of rabbit anti-sheep homolysin in terms of its intercellular transfer to which avidity is inversely related. They measured the proportion of hemolysin which transferred from a population of sensitized sheep erythrocytes

to an unsensitized second population of erythrocytes whose hemoglobin was labeled with Cr^{51}. The extent of transfer was derived from the degree of secondary cell lysis, determined by radioassay, after incubation with complement. Goodman and Masaitis(2) employed a similar method using unlysable formalinized erythrocytes as one of the two populations of cells. We present a modification of the method of Taliaferro *et al* utilizing untreated cells for both populations. The present method is less tedious and the possibility of differences between the primary and secondary cells with respect to their affinity for the antibody is avoided.

Materials and methods. Hemolytic sera of various avidities were obtained from New Zealand rabbits before and during primary and secondary responses to intravenous injections of 2×10^9 sheep RBC/kg. All sera were inactivated for 30 minutes at 56 C and stored at —20 C. Veronal buffered saline (VB) at pH 7.3 was used to dilute serum and complement and as the suspending medium for the erythrocytes(3). VB containing 0.1% bovine serum albumin was used for antiserum dilutions greater than 1/1000. Sheep erythrocytes (RBC), collected in sterile Alsever's solution, were washed with, and resuspended in, VB. The suspension was standarized so that 1 ml lysed with 4 ml of water gave a reading of 150 (O.D. = .3) on a Klett-Summerson colorimeter (#54 filter). One ml of a standard suspension (= 1 unit of RBC) contained about 2×10^8 cells. The Cr^{51}-labeled erythrocytes (RAC) were prepared by mixing 4 ml of washed RBC in 2 ml VB with 0.2 to 0.4 mc sodium radiochromate (Abbott Laboratories, North Chicago, Ill.) isotonic in 2.5 ml VB. The mixture was agitated gently while incubated at 37 C for 4 hours. The labeled cells were then thoroughly washed and standardized as described above. Guinea pig complement (C′) (Carworth Farms, New City, N. Y.) was absorbed at 0 C with ¼ volume of packed sheep erythrocytes and then titrated according to Taliaferro and Taliaferro(4). The use of 12 $C'H_{50}$ units per tube in the hemolysin and transfer tests was considered an adequate, although not a truly unlimiting(5), excess.

Hemolysin titrations. The method of Taliaferro and Taliaferro(4) was used except that the temperature and time of RBC or RAC sensitization with hemolysin were 37 C and 30 minutes, respectively. Titers are expressed as 50% hemolytic units per ml of serum where 1 unit is defined as that volume which will lyse half of 2 units of RBC (or RAC) in 30 minutes at 37 C in the presence of 12 units of C′. Hemolysis was measured colorimetrically in all tests and by radioassay of 2 ml portions of the supernatants in those tests where RAC were used.

Transfer test. The protocol, including the adaptations for the comparative studies using the 4 combinations of RBC and RAC as primary and secondary cells, is summarized in Table I. When possible, the range in hemolysin units used to sensitize the primary cells was such that after transfer secondary cell immune lysis extended from 25 to 75%. The range needed was estimated from rough preliminary tests. The final transfer and hemolysin tests for a given serum were run on the same day using the same stock antiserum dilution, red cell suspension and C′ solution to reduce variation attributable to differences between different preparations of reagents. The endpoint of the test was 50% lysis of the secondary cell population. Since one hemolysin unit lyses half of 2 units of cells (in the titration), the transfer test endpoint was taken to represent the effective transfer of 0.5 hemolysin unit to the secondary cells. Avidity was expressed as either the number of hemolysin units needed to achieve this endpoint or as the percent hemolysin transferred: 100×0.5/hemolysin units required. The maximum transfer in these terms would be 50% (1 hemolysin unit distributed equally between the 2 cell populations). The degree of secondary cell immune lysis, y, in each tube was calculated from the colorimetric readings, K, using the expression,

$$y = \frac{K_S - K_B - [K_{1u} - .5(K_B - K_{C'})]}{K_{1u} - .5(K_B - K_{C'})}$$

where K_S = test supernatant, K_B = blank (which includes the nonspecific lysis among

TABLE I. Outline of Protocol and Calculations for Assay of Hemolysin Intercellular Transfer.

	Exp tubes, 1–7*	Standard (1 unit of cells)	Blank	C′ Color.
Diluted antiserum, ml	1.0	—	—	—
Diluent, ml	—	—	1.0	3.0
Water, ml	—	4.0	—	—
Primary cells, ml†	1.0	1.0	1.0	—
Sensitization period: 30 min at 37 C				
Secondary cells, ml†	1.0	—	1.0	—
Transfer period: 30 min at 37 C				
Complement, ml (6 u/ml)	2.0	—	2.0	2.0
Hemolysis period: 30 min at 37 C; centrifuge 900 × *g* for 15 min				
Colorimeter reading, supernatant	K_S	K_{1u}	K_B	$K_{C'}$
Radioassay (cpm), 2 ml of supernatant	C_S	C_{1u}	C_B	—

Calculation of degree of secondary cell lysis, y

Test system	Colorimetric	Radioassay
RBC → RBC	$\frac{K_S - K_B}{K_{1u} - .5\,(K_B - K_{C'})} - 1.0$	—
RAC → RAC	*Idem*	$\frac{C_S - C_B}{C_{1u} - .5\,C_B} - 1.0$
RAC → RBC	″	$\frac{C_S - C_B}{C_{1u} - C_B}$ ‡
RBC → RAC	″	*Idem*

* The series of experimental tubes, 1–7, contained a gradient of hemolysin units, in 1 ml solution, prepared from a suitable stock antiserum dilution. The interval in hemolysin units between tubes normally approximated 0.1 log.

† 1 unit of either RBC or RAC depending on the test system.

‡ In the RAC → RBC test, the radioassay data yield the degree of primary cell immune lysis.

both cell populations and the color of the added C′), K_{1u} = 1 ml of standard cell suspension lysed completely with 4 ml water, $K_{C'}$ = color of 12 u C′ in 5 ml VB. Thus .5 $(K_B - K_{C'})$ = nonspecific lysis of 1 unit of cells, and $[K_{1u} - .5(K_B - K_{C'})]$ = the complete *immune* lysis of 1 unit of cells. An equivalent simplified expression is given in Table I. The value, y, was derived similarly from radioassay data in tests involving RAC except that no correction for C′ was necessary (Table I). The log of the hemolysin units at the endpoint was interpolated graphically using the von Krogh equation(1).

Results. Since in transfer tests on most sera the number of hemolysin units added to the primary cells exceeded 2, it was expected that even after the transfer of sufficient antibody to lyse 25-75% of the secondary cells (<1 unit) enough hemolysin would remain bound to the primary cells to lyse them. Transfer experiments on various sera using RAC as primary cells and RBC as secondary cells (RAC → RBC tests), in which the degree of primary lysis was determined by radioassay, verified that primary cell lysis was nearly complete in most cases (Fig. 1). Thus in the calculation of the degree of secondary cell lysis from colorimetric readings, shown above, we assumed complete immune lysis of the primary cells. It should be noted, however, that with the least avid sera in which only a few hemolysin units were required in the transfer test, the discrepancy between the actual degree of primary cell lysis and the 100% assumed was often marked in some of the tubes.

Experiments were performed to test the effects of the removal of the supernatant after centrifuging the sensitized primary cells, a step included in the method of Taliaferro *et al*(1). Whereas centrifugation alone had no effect, small but significant decreases in the degree of secondary cell lysis occurred when the fluid phase was removed and replaced with fresh diluent. Hence in the present

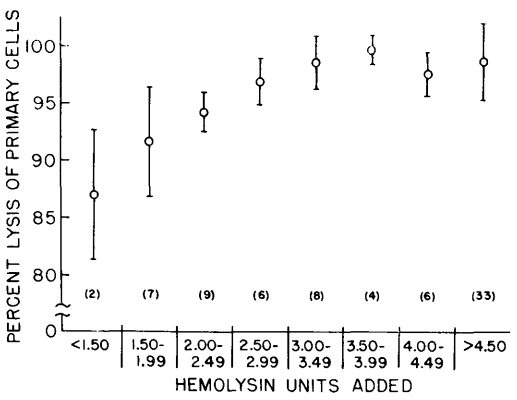

FIG. 1. Percent lysis of primary cells, ± S.D., in transfer tests as a function of number of hemolysin units added. Numbers in parentheses give number of values included in each mean. Data compiled from RAC → RBC tests of various antisera of differing avidities. Note marked incomplete lysis occurring most often below 2.5 units of added hemolysin.

method this step was omitted to avoid discarding dissociable, but lytically effective, hemolysin.

The kinetics of the hemolysin transfer test have been studied extensively(1). In one of our experiments using the colorimetric procedure (RBC → RBC) with an avid and a nonavid antiserum, the transfer values observed after a 30 minute transfer period had attained about ¾ of the values found after a 120 minute transfer period. These findings agree well with those reported by Taliaferro *et al*(1) and indicate that the 30 minute transfer period is adequate to show the major part of the transfer. In another kinetic experiment 1 unit of hemolysin from either an avid or nonavid, normal serum was used in paired RAC → RBC and RBC → RAC systems in which the transfer period was varied from 0 to 240 minutes (30 minutes for primary cell sensitization and 30 minutes for hemolysis). By using this amount of hemolysin which was insufficient to lyse all the cells in 30 minutes, the transfer of antibody from primary to secondary cells would be expected to result in both a decrease in primary cell lysis and an increase in secondary cell lysis as determined by radioassay. Fig. 2 shows that most of the transfer took place within the first hour after adding the secondary cells (30 minute transfer and hemolysis periods). The 13% secondary cell lysis after 0 transfer time with the nonavid serum reflects the transfer which occurred during the hemolysis period. With the nonavid, normal serum, colorimetric data showed that total hemolysis, primary and secondary cell lysis combined, decreased gradually as either the sensitization or transfer times were extended beyond 30 minutes to 4 hours. The factors which may be responsible for this loss of activity are being investigated. Changes in susceptibility of cells to nonspecific lysis were not a factor in either experiment since the blank values remained constant.

Simultaneous hemolysin and transfer tests were performed on various sera using all combinations of RBC and RAC to test whether the two cell types reacted identically and to compare the proposed transfer test method with a similar test using RAC as secondary cells.

Usually 3 hemolysin titrations were run on each serum using (1) 2 units of RBC, (2) 2 units of RAC and (3) 1 unit each of RBC and RAC in each tube. The titers for a given serum derived from colorimetric, and, where possible, from radioassay measurements

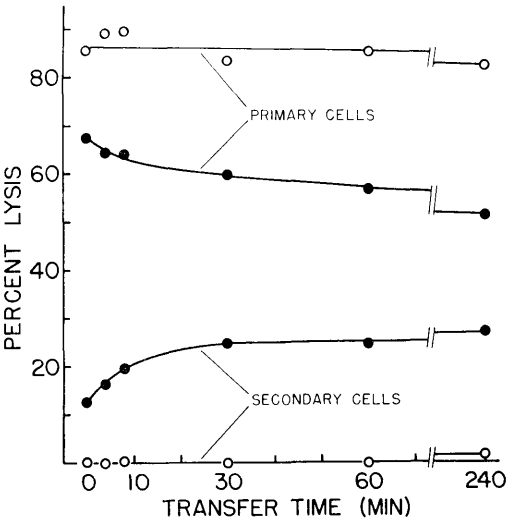

FIG. 2. Percent lysis of primary and secondary cell populations after various transfer times in a system in which only 1 hemolysin unit of a nonavid, normal serum, ●, or an avid, primary response serum, ○, was absorbed initially on the primary cells. Sensitization and hemolysis periods were 30 min each.

TABLE II. The Mean Hemolysin Titer and Percent Antibody Transferred Using Various Combinations of Cr^{51}-Labeled (RAC) and Unlabeled (RBC) Sheep Red Cells.

Serum	Mean titer (50% units/ml)	% Antibody transferred: Colorimetric RBC → RBC	Colorimetric RAC → RAC	Colorimetric RAC → RBC	Colorimetric RBC → RAC	Radioassay RAC → RAC	Radioassay RBC → RAC
Normal*	27	18.4	18.7	15.7	22.2	19.9	23.9
"	27	22.6	23.7	19.5	27.8	26.3	31.6
"	79	19.0	19.0	15.5	21.8	20.6	25.4
Primary (4th day)	25			24.8	34.0		36.6
" "	31			22.8	27.3		27.3
" "	240	9.5	8.5	9.0	10.0	11.0	11.0
" "	380	8.5	7.8	7.8	8.8	10.0	10.5
" "	630	8.3	8.3	8.1	8.9	9.4	10.1
" "	680			9.2	10.7		9.8
" (10th day)	7750	2.9	2.3	2.7	2.6	2.9	2.9
" "	16500			2.1	2.5		2.2
Secondary (25th day)	22900	23.1	24.6	23.2	24.9	24.8	25.9

* Serum from unimmunized rabbits containing naturally-occurring hemolysin, usually nonavid.

agreed closely. Thus the average titer for each serum was used to calculate the number of hemolysin units that had been added to each tube of the corresponding transfer tests.

The colorimetric transfer tests using untreated red cells for both populations (RBC → RBC) yielded results similar to, but usually lower than, the radioassay method using labeled cells for the secondary population (RBC → RAC) (Table II, Col. 3 and 8). Two other observations shed light on the reasons for this discrepancy. First, for 11 of the 12 sera tested, the colorimetrically-determined percent antibody transfer value from the RAC → RBC test was lower than that from the corresponding RBC → RAC test (Col. 5 *vs.* 6) which suggests that these RAC had a greater affinity for hemolysin than RBC. An increased susceptibility of RAC to lysis seems unlikely (compare Col. 3 and 4). Secondly, the values derived from radioassay data were most frequently higher than the corresponding values calculated from colorimetric readings (Col. 7 *vs.* 4; 8 *vs.* 6). This latter result may have been due, in part, to inhomogeneous Cr^{51}-labeling with a preferential lysis of the more heavily labeled cells.

Reproducibility. Forty-two pairs of hemolysin and transfer tests performed on a pooled primary serum over a 7 month period gave remarkably consistent results. The mean log titer was 4.14 ± .01 (S. E.) and the mean transfer value, in terms of the hemolysin units needed to achieve the endpoint, was 9.8 ± 0.2 (5.1% transfer). Each transfer value was calculated using the titer obtained from the hemolysin titration performed on the same day as the transfer test.

Discussion. The colorimetric method presented here for measuring intererythrocyte hemolysin transfer in which only unlabeled red cells are used, does not alter the essential principles underlying the analysis set forth by Taliaferro *et al*(1). However because we elected to express our results in terms of antibody transfer to secondary cells only, our values would be half those calculated by the previous method(1) in which it was assumed that transfer to the secondary cells was matched by transfer among primary cells.

Although the colorimetric and radioassay methods gave similar results, the comparative tests indicated that the Cr^{51}-labeled cells we prepared had a slightly greater affinity for hemolysin than unlabeled cells. The colorimetric method was especially effective in testing avid and moderately avid antisera but was somewhat less precise in testing unusually nonavid sera because primary cell lysis was incomplete in some of the tubes of the test. This disadvantage was partially overcome by obtaining 5 to 7 points in the 40 to 75% range of secondary cell lysis and by giving more weight, when plotting, to those points

representing the greater degrees of lysis. It is also possible to employ a correction for unlysed primary cells derived from data such as given in Fig. 1.

Whereas Taliaferro *et al*(1) reported no difference between the slopes of the hemolysin and transfer test von Krogh plots for a given serum, a significant difference was frequently observed in the present studies.

Although a transfer test is useful for indicating qualitative differences between hemolytic antisera, results must be interpreted cautiously. Two antisera with comparable titers and transfer values are not necessarily identical because the proportion and avidity of each hemolysin species which may comprise an antiserum are unknown. Moreover, since fixed transfer and hemolysis times are used in this procedure, the results are dependent on the rates of transfer and hemolysis.

It is reasonable to assume that a similar approach could be used to study antibody transfer in other immune cytolytic systems in which it would be preferable to avoid labeling the target cells.

Summary. A modified method is described for measurement of the avidity of rabbit anti-sheep hemolysin in terms of the proportional transfer of the antibody from one population of erythrocytes to another. It is simple, involves colorimetric measurements only, eliminates the need for pretreatment (*e.g.*, Cr^{51}-labeling) of one of the 2 cell populations, yet yields results comparable to those obtained using an isotopic label.

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Delayed Implantation in Gonadotropin-Treated Immature Rats.* (31410)

JUNG-TSUNG WU AND ROLAND K. MEYER
Department of Zoology, University of Wisconsin

In the study of implantation processes in the rat, a convenient means of obtaining as many blastocysts as possible from a delayed-implantation rat became of importance to us. The fact that the immature rat can be induced to superovulate with pregnant mare's serum (PMS)(1) and, if mated, shows an unusually large number of implantation sites ("superfecundity")(2) encouraged us to produce delayed implantation by ovariectomy and progesterone treatment(3) in the PMS-treated and mated immature rat. Recently, Strauss and Meyer(4) and Strauss(5) in this laboratory developed a regimen for producing ovulation, and high incidence of mating and pregnancy in the prepuberal rat using PMS. We used this basic method to induce superovulation and mating in immature rats, and to obtain large numbers of preimplantation blastocysts and blastocysts which had been prevented from implanting. This was accomplished by injecting human chorionic gonadotropin (HCG) in addition to PMS. The data obtained are the subject of this report.

* Supported by a grant from the Ford Foundation.

Methods. Immature female rats, 26 days old, weighing 65 to 70 g, were obtained from the Holtzman Co., Madison, Wis. They were maintained *ad libitum* on Rockland rat chow and tap water. The room temperature was kept between 74 and 80°F. The light was