# Quantitative Immunochemistry and the Evolution of Primate Albumins: Micro-Complement Fixation

Abstract. *Quantitative micro-complement fixation was used to compare human serum albumin with the serum albumins of apes, monkeys, and prosimians. The results are consistent with those obtained by other immunological techniques, and they are consistent with the accepted phylogenetic position of these groups. The method requires much less antigen and antibody is more sensitive to small differences in albumin structure. A large scale survey of species differences in protein structure is possible with less than a milliliter of antiserum.*

Even without recourse to analyses of amino acid sequences data can be obtained by immunological methods concerning the degree of structural relatedness between the homologous protein molecules of different species. Direct evidence for a correlation between immunological cross-reactivity and structural relatedness is available from studies of hemoglobins (*1*, *2*) and cytochromes *c* (*3*) of known amino acid sequence. Indirect evidence is provided by the correspondence between cross-reactivity and phylogenetic relatedness demonstrated by Nuttall (*4*) and others (*5*, *6*) for a variety of proteins.

Several immunological techniques have been used for such studies; these are quantitative precipitation, immunodiffusion, turbidimetry, and immunoelectrophoresis. The serum albumins of many primate species have been compared to human serum albumin by all of these methods (*5*, p. 80; *6*, *7–9*). We now report the comparison of a series of primate serum albumins by the sensitive and economical method known as quantitative micro-complement fixation (MC′F).

The MC′F technique, developed by Wasserman and Levine (*10*), has been used for the study of the immunological reactions of small quantities of purified macromolecules, particularly nucleic acids and proteins (*11*). The concentrations of reactants (antigens and antibodies) used are 10 to 100 times lower than those required for the conventional quantitative complement fixation procedure (*12*), which we term macro-complement fixation, and some 1000 times lower than for the quantitative precipitin procedure (*13*). MC′F is sensitive to small variations in the structure of protein antigens and makes a distinction between hemoglobins A, S, and C, which differ by two amino acid residues out of 574 (*1*). The method also gives enhanced discrimination with several other antigen-antibody systems (*2*, *13*), including the serum albumin immune system. Thus chimpanzee serum albumin is readily distinguishable from human serum albumin with this technique, although conventional immunological methods make this distinction only with difficulty (*2*).

Most of our experimental work was done with 11 antiserums prepared (in several laboratories) by immunization of rabbits with purified human serum albumin (HSA). In our laboratory at Berkeley, each of two male rabbits (New Zealand white) received a primary injection of an emulsion containing 0.5 ml of Freund's complete adjuvant (Difco) and 10 mg of HSA in 0.5 ml of 0.9 percent NaCl. The injections were distributed among four intradermal back and two toe-pad sites. The rabbits then received intravenous injections (2.5 mg of HSA in 0.25 ml of saline per rabbit per injection) at 4 weeks, 6 weeks, and 8 weeks. The rabbits were bled 8 or 9 days after each injection. One of the rabbits was rested for 3 months and then received two further 2.5-mg intravenous injections of HSA 1 week apart. A final bleeding was made 8 days after the last injection. As no bleedings were pooled, this provided seven antiserums from the two Berkeley rabbits. The eighth antiserum, obtained from L. Levine, was prepared as follows. One rabbit was injected with increasing doses of alum-precipitated HSA on alternate days for 5 weeks, the total being 60 mg of HSA. The rabbit was bled 6 days after the last injection. This is the antiserum that was used to obtain the results reported in reference (*2*). The ninth antiserum was purchased from Nutritional Biochemicals Corporation (antiserum to human serum albumin, lot number 6390). The remaining two antiserums were supplied by C. A. Williams who has described their preparation (*8*). Briefly, his rabbits received two courses of HSA injections before being bled. Bleedings from five rabbits were pooled to give pool II; pool III resulted from combining bleedings from 12 rabbits.

The HSA used for immunization of the Berkeley rabbits (antiserums $5_2$, $5_3$, $5_4$, $6_2$, $6_3$, $6_4$, $6_F$) (*14*) was purified from the serum of a single individual (V.M.S) by the heat-caprylate method (*15*) and tested for homogeneity by starch-gel electrophoresis and analytical ultracentrifugation. In a single run at a protein concentration of 10 mg/ml, the HSA behaved as a single component with a sedimentation coefficient ($S_{20,w}$) of 4.1. Electrophoresis at *p*H 8.7 in tris-borate buffer (*16*) resulted in a single major component, as judged by the amido black stain. A less anodic protein was detectable when large amounts of HSA were applied to the gel. The component had the same mobility as an $\alpha_1$-globulin and its concentration was estimated as 0.5 percent that of the albumin. For immunization of Levine's rabbit, commercial HSA was crystallized three successive times as the mercury dimer (*17*). Mercaptalbumin monomer was prepared by dialysis of the dimer against an excess of cysteine, which removes the mercury, and then against several changes of water. Williams' rabbits were immunized with commercial crystalline HSA (Pentex).

The serum albumins for the MC′F cross-reaction tests were contained in samples of whole serum or plasma from various primate species. The samples were obtained from several sources (*18*) and stored at −10°C. They were assumed to contain 40 mg of albumin per milliliter. For some species, albumin was purified by the heat-caprylate method before it was used for MC′F. The concentrations of purified albumin were estimated from absorbance measurements at 280 m$\mu$ with a Zeiss spectrophotometer, crystalline HSA (Pentex) being used as a standard.

The MC′F experiments were performed with 7-ml reaction volumes (*10*). All reagents were diluted in a buffer (*p*H 7.45) containing 0.14*M* NaCl, 0.01*M* tris hydrochloride, $5 \times 10^{-4}M$ $MgSO_4$, $1.5 \times 10^{-4}M$ $CaCl_2$. The reaction time at 5°C was standardized at 18 hours. Immunodiffusion tests were performed as recommended (*19*), except that smaller and more closely spaced wells were used (*20*).

The antiserums were first tested for homogeneity. Williams has already discussed the purity of his antiserums (*8*).

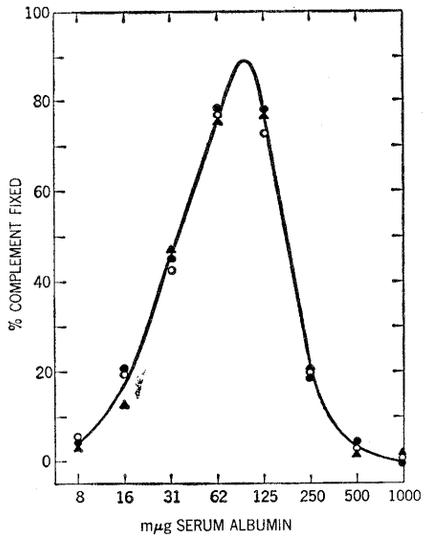



Fig. 1. Reactivity in the MC'F procedure of high-liter, final-course antiserum $6_F$ with (●) human serum, (○) crystalline HSA (Pentex), (▲) HSA (heat caprylate preparation).

He obtained immunoelectrophoretic evidence that antiserum pools II and III contain small amounts of antibody directed to an $\alpha_1$-globulin. This impurity plus smaller amounts of a second contaminant was also demonstrated in the Berkeley antiserums by immunodiffusion and immunoelectrophoresis. However, these impurities had no effect on MC'F reactions (Fig. 1). Curves of identical peak heights, shape, and position were obtained regardless of the state of purity of HSA (*21*). Thus the MC'F procedure effectively isolates a single antigen-antibody system for comparative study.

Figure 1 demonstrates another advantage of the MC'F technique. The peak of the complement fixation curve occurs at an albumin concentration of 0.08 μg/ml and an antiserum concentration of 1:11000. The generation of such a curve requires only $3 \times 10^{-5}$ ml of serum and $1 \times 10^{-3}$ ml of antiserum. Therefore thousands of cross-reaction experiments can be set up with an antiserum derived from a single bleeding of a single rabbit. The technique is also extremely conservative of samples of rare serums.

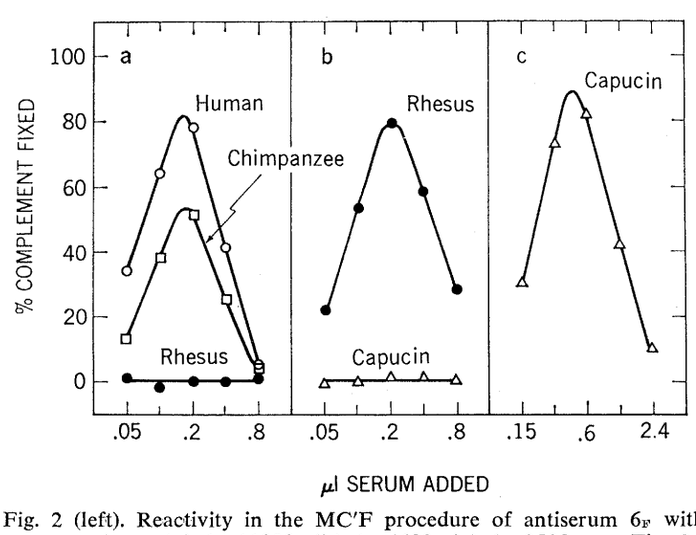
Antiserum number $6_F$ was then tested for reactivity with serum albumin from chimpanzee (*Pan troglodytes*), rhesus monkey (*Macaca mulatta*), and capucin monkey (*Cebus capucinus*). These species form an approximate evolutionary series, the chimpanzee being the closest relative of man and the capucin monkey the most distant. At a concentration of 1:11000 (Fig. 2a), chimpanzee serum albumin gave a detectable reaction but the monkey albumin did not. The chimpanzee curve has a peak whose height is 67 percent as great as that given by HSA. Other immunological methods fail to make such a large quantitative distinction between chimpanzee and human serum albumins. Rhesus serum albumin reacted well when the antiserum concentration was raised by a factor of 2.5 (Fig. 2b), and capucin serum albumin gave a complement fixation peak comparable to the homologous one when the concentration of antiserum was raised by a factor of 4.5 (Fig. 2c).

For any one species of serum albumin, the peak height of the complement fixation curve bears a linear relationship to the log of the antiserum concentration over a range from 20 to 90 percent fixation (Fig. 3). The slope of the line is identical for human, rhesus, and capucin albumins (*22*).

Table 1. Variability in specificity of antiserums directed to HSA.

| Species of albumin | Antiserums tested (No.) | I.D. | |
|---|---|---|---|
| | | Mean | S.D. |
| Chimpanzee | 11 | 1.18 | 0.03 |
| Rhesus | 10 | 2.40 | 0.24 |
| Capucin | 10 | 6.1 | 0.9 |

The fact that the slopes are alike provides a basis for measuring the immunological distance between HSA and other albumins. Although in many immunological methods a fixed antiserum concentration is employed and cross-reactions are expressed as a percentage of the homologous reaction, this is not appropriate for the MC'F method. As noted, rhesus albumin gave no cross-reaction at the antiserum concentration used to demonstrate the homologous reaction. The rhesus cross-reaction was only detected when the antiserum concentration was raised. As a measure of cross-reactivity (or immunological distance), we use the factor by which the antiserum concentration must be raised in order that a particular serum albumin give a peak height equal to that given by the homologous one (HSA in this case). This number we call the index of dissimilarity (*2*) or immunological distance (I.D.). The I.D. is in-



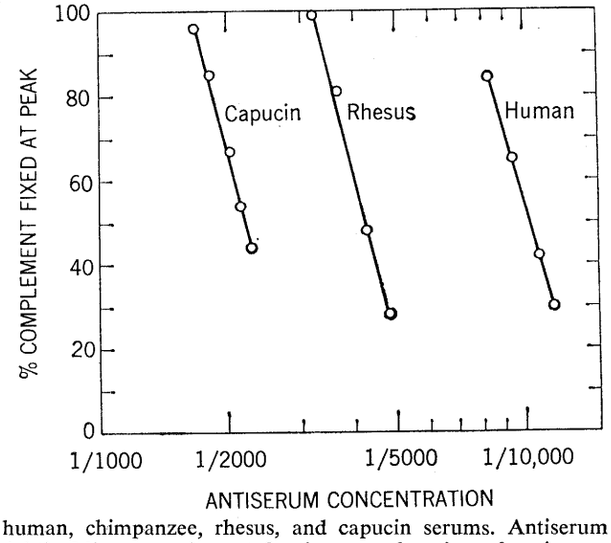




Fig. 2 (left). Reactivity in the MC'F procedure of antiserum $6_F$ with human, chimpanzee, rhesus, and capucin serums. Antiserum concentrations: (a) 1 : 11000, (b) 1 : 4600, (c) 1 : 2500. Fig. 3 (right). Micro-complement fixation as a function of antiserum concentration. The antiserum was $6_F$ (directed to HSA) and the antigens were the albumins present in human, rhesus, and capucin serums. Serial dilutions of each serum were tested against several concentrations of antiserum. Each point represents the peak height of a complement fixation curve for a particular antiserum concentration.

dependent of the actual peak heights at which the comparison is made, because the slopes in Fig. 3 are identical for different albumins. With antiserum $6_F$, the indices of dissimilarity for the serum albumins discussed above are: human, 1.0 (by definition); chimpanzee, 1.17; rhesus, 2.38; capucin, 4.64. The experimental error to which these determinations are subject is no more than 2 percent.

Other antiserums have cross-reaction specificities similar to, but not identical with, that of antiserum $6_F$. For example, all 11 antiserums readily distinguished human from chimpanzee serum albumin; the indices of dissimilarity fall in the narrow range 1.12–1.23 (mean 1.18, standard deviation 0.03) (Table 1). Of the 11 antiserums, ten were also reacted with rhesus and capucin albumins. Table 1 shows that the mean indices are like those obtained with antiserum $6_F$, although variation between antiserums was greater for weaker cross-reactions, that is, higher indices. With capucin albumin, which gave the highest I.D., the standard deviation was 15 percent as great as the mean (*23*). However, very diverse antiserums were being tested. Some were first-course (4-week) antiserums of low titer (1:900) (*24*); others were from late bleedings of high titer (1:11000). Some were antiserum pools; others were single bleedings. The HSA for immunization had been purified by a number of methods, and varying immunization schedules were used. Antiserum variation is a relatively minor problem in MC′F testing, although serums from several rabbits are necessary for maximum accuracy and reliability, especially for studies of weak cross-reactions.

For the quantitative evaluation of the MC′F method of measuring structural resemblance between albumins, it is important to know how well reciprocal cross-reactions agree. A large number of antiserums to other primate albumins have been prepared and tested for reactivity with HSA (Table 2). The reaction of antiserums to rhesus albumin with HSA (I.D. = 2.20) is similar to that of antiserums to HSA with rhesus albumin (I.D. = 2.40). Comparable reciprocity has been obtained with all other pairs of albumins and antiserums tested.

We then tested serum samples from widely different primate species for reactivity with high-titer antiserums to HSA (Table 3). Every ape albumin reacted strongly with the antiserums,

Table 2. Reciprocity of albumin cross-reactions.

| Species of albumin | | I.D. (mean)* | |
|---|---|---|---|
| A | B | Antiserum to A vs. B | Antiserum to B vs. A |
| Man | Chimpanzee | 1.18 | 1.12 |
| Man | Rhesus | 2.40 | 2.20 |
| Man | Capucin | 6.0 | 5.4 |

* Based on experiments with 11 antiserums to human, 4 to chimpanzee, 8 to rhesus, and 2 to capucin albumin. The last 14 antiserums were prepared by injection of the appropriate species of serum albumin purified by the heat-caprylate method as for Berkeley rabbits 5 and 6.

the mean indices ranging from 1.12 to 1.30. As noted by others, the African apes (gorilla and chimpanzee) have albumins that react more strongly than those of the Asiatic apes (gibbon, siamang, orang). Six genera of Old World monkeys, with mean indices from 2.25 to 2.7, have albumins that are clearly more distantly related than those of the apes. The albumins of the New World monkeys (excluding *Aotes*) gave mean indices from 4.7 to 5.3 in accordance with the more remote taxonomic relationship of these monkeys to man. However, the night monkey (*Aotes*) gave indices overlapping those given by Old World monkey albumins. This surprising result, first noted by Goodman (*9*) and confirmed by Williams (*8*), is due to retarded albumin evolution in the *Aotes* lineage (*25*). The prosimians gave mean indices of 8.6 to 18. The most distant relatives of man tested were mammals such as cow and pig. Their albumins gave indices greater than 20. In summary the MC′F data are in qualitative agreement with the anatomical evidence, on the basis of which the apes, Old World monkeys, New World monkeys, prosimians, and nonprimates are placed in taxa which form a series of decreasing genetic relationship to man.

These data are also in qualitative agreement with those obtained with different immunological techniques. The order of placement of the species relative to man is the same whether one bases it on MC′F, quantitative precipitation, turbidimetric, immunodiffusion, or immunoelectrophoretic data. The MC′F data differ mainly by magnifying the differences between serum albumins.

Quantitative comparison of MC′F

Table 3. Reactivity in the MC′F procedure of serums from various species with three anti-antiserums directed to HSA.

| Species | Index of dissimilarity | | | |
|---|---|---|---|---|
| | Pool II | Antiserum $5_4$ | Antiserum $6_F$ | Mixture* |
| | Hominoidea (Man and apes) | | | |
| *Homo sapiens* † | 1.0 | 1.0 | 1.0 | 1.0 |
| *Pan troglodytes* | 1.12 | 1.19 | 1.17 | 1.14 |
| *Gorilla gorilla* | 1.12 | 1.19 | 1.13 | 1.09 |
| *Pongo pygmaeus* | 1.15 | 1.23 | 1.30 | 1.22 |
| *Hylobates lar* ‡ | 1.29 | 1.25 | 1.29 | 1.28 |
| *Symphalangus syndactylus* | 1.25 | 1.27 | 1.23 | 1.30 |
| | Cercopithecoidea (Old World monkeys) | | | |
| *Macaca mulatta* | 2.05 | 2.40 | 2.37 | 2.23 |
| *Papio papio* | 2.22 | 2.50 | 2.56 | 2.44 |
| *Cercocebus galeritus* | 2.00 | 2.48 | 2.53 | 2.30 |
| *Cercopithecus aethiops* | 2.46 | 2.65 | 2.66 | 2.59 |
| *Colobus polykomos* | 2.13 | 2.72 | 2.33 | 2.52 |
| *Presbytis entellus* | 2.33 | 2.93 | 2.75 | 2.65 |
| | Ceboidea (New World monkeys) | | | |
| *Aotes trivirgatus* | 2.5 | 3.3 | 2.3 | 2.65 |
| *Ateles geoffroyi* | 4.1 | 5.2 | 4.7 | 4.2 |
| *Saimiri sciurea* | 5.2 | 5.0 | 4.4 | 4.5 |
| *Cebus capucinus* | 6.3 | 4.9 | 4.7 | 5.0 |
| *Callicebus torquatus* | 4.2 | 6.3 | 4.4 | 4.2 |
| | Prosimii (Prosimians) | | | |
| *Tarsius spectrum* | 12 | 15.7 | 8.0 | 11.3 |
| *Galago crassicaudatus* | 10.0 | 9.7 | 5.6 | 8.6 |
| *Nycticebus coucang* | 11.7 | 14.0 | 7.8 | 11.2 |
| *Lemur fulvus* | 14 | 25 | 16.5 | 18 |
| *Tupaia glis* | 14.3 | 12 | 10.7 | 7.6 |
| | Nonprimates | | | |
| *Bos taurus* § | 23 | >30 | 25 | 32 |
| *Sus scrofa* § | 29 | >30 | 27 | >35 |

* The antiserum mixture was made by mixing the three individual antiserums in reciprocal proportion to their MC′F titers, that is, 8.8 parts of pool II (titer = 1/5000) + 6.3 parts of $5_4$ (titer = 1/7000) + 4.0 parts of $6_F$ (titer = 1/11000). † G. Sensabaugh has tested serum samples from 20 individuals of diverse human races and found no variation in I.D. ‡ Serum samples and purified albumins from six different gibbons have been tested with these and other antiserums directed to HSA. No individual variation was found in I.D. § Purified albumins were used in these cases to insure that we were measuring the cross-reaction with antibodies to HSA.

and precipitin data has been made with the pool II antiserum directed to HSA. Pool II was used by Hafleigh and Williams for their quantitative immunological survey of primate serum albumins (*8*). We have used this antiserum to test as many of the species Hafleigh and Williams used as we have on hand (Fig. 4). The agreement between their data and ours speaks highly of the comparability of the two techniques and the reliability of the MC'F procedure. Apart from the slight disagreement in the relative placement of the various prosimians, the only serious discrepancy concerns the reaction of the gibbon albumin.

According to the precipitin data, the gibbon (*Hylobates lar*) although a member of the *Hominoidea* (apes and man) has an albumin which is as distinct from that of man as that of rhesus monkey is. According to the MC'F data, gibbon albumin is far more closely related to HSA than rhesus albumin is (I.D. 1.30 vs. 2.05). In fact, at an antiserum concentration of 1:4000, which gives a peak fixation value of 75 percent with gibbon, no Old World monkey albumin gives any discernible reaction. All other antiserums directed to HSA or chimpanzee albumin confirm this distinction, the largest I.D. for gibbon yet obtained being 1.32, the smallest index for the Old World monkey being 2.00.

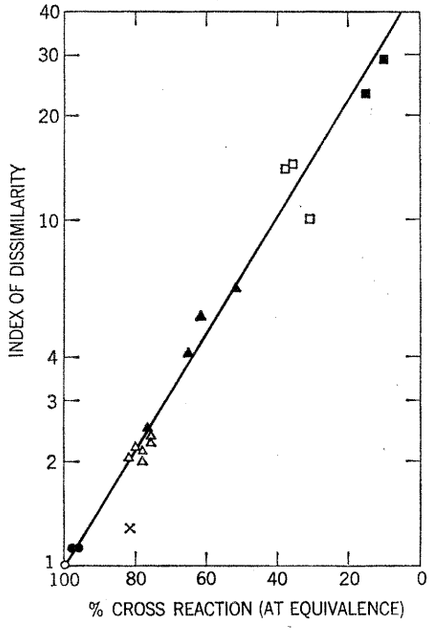



Fig. 4. Relation between percentage cross-reaction, measured by the precipitin technique (*8*), and index of dissimilarity, measured by the micro-complement fixation method (Table 3), with the use of pool II antiserum against HSA prepared by Hafleigh and Williams (*8*). Each point represents the result obtained with the serum albumin of a particular species (or genus); man (○), apes (●), gibbon (X), Old World monkeys (△), New World monkeys (▲), prosimians (□), nonprimates (■).

We have also used pool II antiserum in immunodiffusion experiments with purified human, rhesus, and gibbon albumins. No spur was obtained when human and gibbon were compared with each other; yet there was a distinct spur in the comparison of human albumin with rhesus albumin. Moreover, gibbon albumin showed only a slightly smaller spur when tested against rhesus albumin than human albumin did. Similar results have been obtained with all other antiserums to HSA confirming the original observations of Goodman (*9*) who used both rabbit and chicken antiserums to HSA. The immunodiffusion data are consistent with the MC'F data but not with the precipitin data for gibbon albumin (*26*). Except for the unexplained discrepancy with gibbon albumin the MC'F technique gives results in quantitative agreement with those obtained by the quantitative precipitin technique and does it with the use of about 1000 times less material (*27*).

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21. Antibodies to other serum proteins could be demonstrated in MC'F tests by use of an absorbed system. Antiserum $6_F$ was absorbed with HSA (Pentex crystalline) until no antibodies directed to HSA were detectable by immunodiffusion. The $\alpha_1$-globulin line was undiminished by this procedure. This absorbed antiserum was then reacted in the MC'F procedure with human serum. A peak was obtained at an antiserum concentration of 1:1100 and a serum concentration approximately 25-fold greater than that necessary at the peak of the albumin curve. It should be noted that the concentration of unabsorbed antiserum necessary for equivalent albumin fixation is 1 : 11000. The amount of antibodies directed to other serum proteins is estimated to be 10 percent.
22. Similar slopes have been obtained with other immune systems, such as rabbit antiserum to hemoglobin (*1*), and every other rabbit antiserum to protein studied in this laboratory.
23. As the time of immunization was increased for Berkeley rabbits 5 and 6, the indices for rhesus and capucin albumins tended to fall slightly. In addition the amount of albumin required for peak fixation also fell. Similar results have been obtained with antiserums directed to rhesus albumin.
24. We define the MC'F titer of an antiserum as that concentration required to fix 75 percent of the complement at the peak of the MC'F curve.
25. V. M. Sarich and A. C. Wilson, in preparation.
26. The phyletic position of the gibbon lineage is one of paramount importance in our understanding of hominoid evolution. We believe (unpublished experiment) that our measure of the immunological distance between man and gibbon is a realistic reflection of the actual phyletic distance separating these species and that all the *Hominoidea* share far more recent common ancestry than is generally supposed.
27. The MC'F and precipitin techniques give comparable results in inhibition experiments with antibodies to dextran [J. Gelzer and E. A. Kabat, *J. Exp. Med.* **119**, 983 (1964)].
28. Presented in part to the AAAS, 30 December 1965, Berkeley, California, and to the Amer. Assn. Physical Anthropologists, 8 April 1966, Berkeley. Supported in part by a predoctoral fellowship awarded to V.M.S. by the NIMH, by an NSF grant to A.C.W., and an NIH grant to S. L. Washburn. We thank S. L. Washburn in particular for his helpful discussions and K. Reinheimer for technical assistance. We also thank N. Arnheim for assistance in the preparation of this paper.

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