Micro-Complement Fixation: A Quantitative Estimator of Protein Evolution[1]

Richard D. Maxson and Linda R. Maxson*,†*

*Department of Genetics and Development and †Department of Ecology, Ethology, and Evolution, University of Illinois at Urbana-Champaign

The quantitative immunological technique of micro-complement fixation (MC'F) has been routinely used during the past decade to assess evolutionary relationships among living vertebrate species. The large data base that has been generated, along with the excellent correlations between immunologically measured genetic distances and paleontologically derived estimates of divergence times, have formed the basis for the albumin molecular clock. Immunological distance (ID) involves a logarithmic transformation of experimentally measured antibody concentrations. The justification for this transformation has rested entirely on empirical correlations. Consequently, several other transformations have been proposed as giving better fits to particular data sets. We derive, from first principles, the relationship between ID and the amino acid sequence replacements (AAR) between compared albumins. ID is shown to be a linear estimator of AAR. This ID-AAR relationship is based on a proposed process of antibody assortment and exclusion. We present experimental data confirming that such an antibody assortment-exclusion process occurs in MC'F. This process can explain both the high sensitivity and the quantitative phylogenetic nature of the MC'F assay. The assortment-exclusion process also predicts a divergence limit beyond which MC'F data no longer provide robust phylogenetic data.

Introduction

The immunological technique of micro-complement fixation (MC'F) is routinely used to obtain the immunological distance (ID) between diverse vertebrate species (e.g., Wilson et al. 1977; Maxson 1984). The MC'F assay has largely been viewed as a mysterious black box: antigens go in and IDs come out. The phylogenetic usefulness of this ID measure rests on (1) the excellent correlation between ID and amino acid sequence differentiation (Benjamin et al. 1984), (2) the correlation between ID and other measures of molecular evolution (Prager and Wilson 1971; Champion et al. 1975; Sarich 1977; Maxson and Maxson 1979), (3) the correlation between ID and paleontologically derived time estimates (see Wilson et al. 1977 for a review), (4) the relative uniformity of molecular evolution as contrasted with erratic rates of morphological evolution (Wilson 1976; Wilson et al. 1977), and (5) the ease with which extensive data comparing diverse species can be rapidly collected.

Shortly after the introduction of MC'F as a phylogenetic probe (Sarich and Wilson 1966, 1967), it became customary to use logarithmic transformations of experimentally

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Address for correspondence and reprints: Dr. L. R. Maxson, The University of Illinois, 515 Morrill Hall, 505 South Goodwin, Urbana, Illinois 61801.

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measured indices of dissimilarity[2] (Sarich and Wilson 1967; Sarich 1968; Wilson and Sarich 1969). This particular transformation was selected because empirical studies suggested that an exponential relationship exists between the amount of time since speciation and the amount of change in the related albumins as measured by MC′F analyses. Other transformations have been proposed that closely fit the exponential curve over limited ranges but increasingly diverge over more extended ranges (Read and Lestrel 1970, 1972; Uzzell and Pilbeam 1971; Read 1975). The issue of which transformation is correct has never been resolved because it depends on understanding the underlying mechanism responsible for the observed correlations between (experimental) MC′F data and (causal) amino acid sequence replacements (AAR). Statistical "best-fit" criteria for particular data sets, or more general correlations over all available data, are not appropriate if there is a specific mechanism involved.

We provide data that support a particular model that we call antibody assortment-exclusion. We describe the model and test it using a specially modified experimental procedure that can directly measure excluded antibody. We derive the expected theoretical relationship between ID and AAR based on this model. Our results predict a linear relationship between ID and AAR with a protein-dependent constant of proportionality that depends linearly on the number of antigenic determinants on the compared proteins.

Material and Methods

All MC′F data were obtained using conditions described by Champion et al. (1974) and modified by Maxson et al. (1979). The antiserum-production protocol has been described in earlier studies (Maxson et al. 1979), and all antisera were determined to be directed solely to albumin. In particular, great care was taken in preparing the antisera to assure maximum antibody affinity and diversity and in matching the peaks of heterologous and homologous reactions in the *same* experimental setup (fig. 1).

Data for figure 2 were obtained using 17 specific antisera to serum albumins of diverse amphibians used in previous phylogenetic studies (Maxson et al. 1977, 1979, 1981; Maxson 1981*a*, 1981*b*). An evolutionary framework for these data is provided by the empirical correlations between ID and paleontological data that indicate the log of R is proportional to divergence time[2] (Wilson et al. 1977; Thorpe 1982). For albumin, each doubling of R represents ~16 Myr of interspecies divergence. Using this interpretation, the data points of figure 2 can be seen to cover a vast range of molecular and organismal evolution. For example, the smallest ID in figure 2 is that between eastern and western populations of the grey treefrog *Hyla chrysoscelis* with a probable divergence 3–4 Myr ago in the Pliocene (Maxson et al. 1977). The most divergent species pair shown, with an antigen ratio of 2.5 and an antibody ratio of 30, is that of the toads *Bufo blombergi* and *B. regularis* with a probable divergence 80 Myr ago during the late Mesozoic (Maxson 1981*a*).

Data reduction for figure 2 differs somewhat from standard protocol. Each point requires three complement fixation curves, one for the homologous reaction (using the antigen to which the antiserum is prepared, species A), a second for the heterologous reaction (using antigen from species B), and a third curve using antiserum to species B with species B as the homologous antigen. By using all three reactions, the relative

2. Index of dissimilarity is seldom used. It was defined as the ratio of the concentration of antiserum used in the heterologous reaction (using the divergent protein) to the concentration of antiserum used in the homologous reaction (using the antibody-specific protein), when the amount of complement fixation is identical. We call this ratio R. Immunological distance is (arbitrarily) defined as ID = 100 log R.

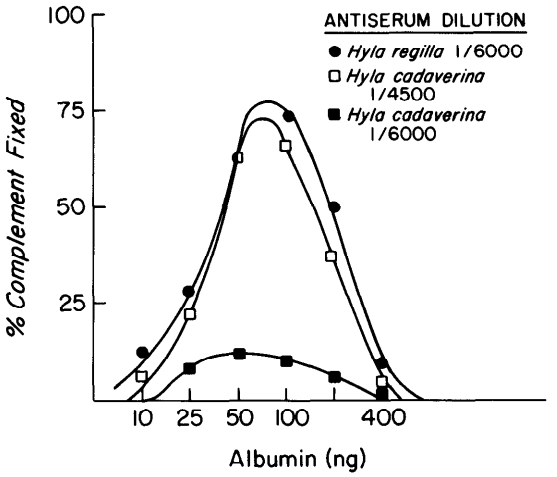

FIG. 1.—Micro-complement fixation (MC'F) curves. The antiserum is always to *Hyla regilla* albumin at a relative concentration of 1/6,000 (●, ■) and 1/4,500 (□). The antigen is either that of *H. regilla* (●) or of *H. cadaverina* (■, □). Since the heterologous curve (□) at a 1/4,500 dilution fixes complement equivalent to the homologous curve at 1/6,000 (●), the immunological distance between these two species is 100 log(6,000/4,500) = 12.5.

concentrations of antigen used in both the homologous and heterologous reactions can be calculated. Normally, only antiserum concentrations are used to monitor antigen divergence and the absolute antigen concentrations are not calculated, serving only to locate the curve peak.

The data in figure 3 use a special serial dilution schedule that provides two additional data points between each standard data point. This allows a more precise

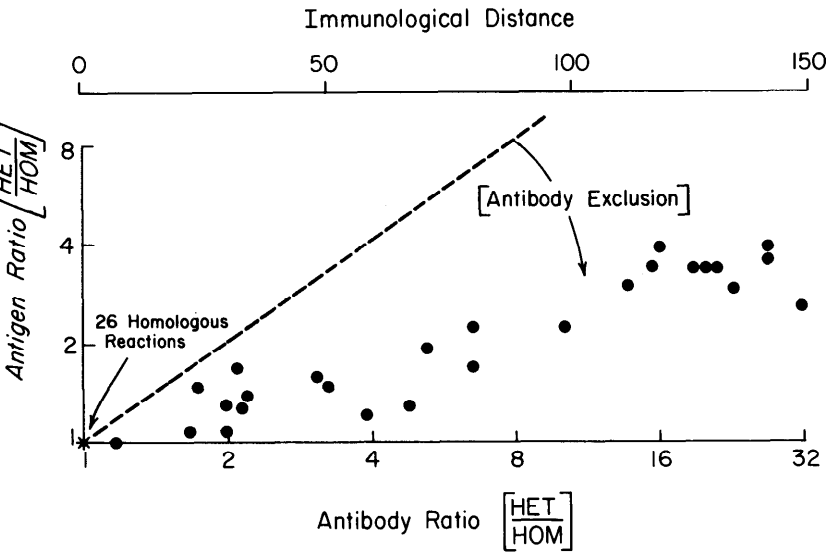

FIG. 2.—Plot of relative antigen concentrations vs. relative antibody ratio for 26 determinations of amphibian immunological distances. Data are from comparisons among 13 species of toads of the genus *Bufo* (Maxson 1984), 2 species of *Plethodon* (Maxson et al. 1979), and 2 species of *Hyla* (Maxson et al. 1977).

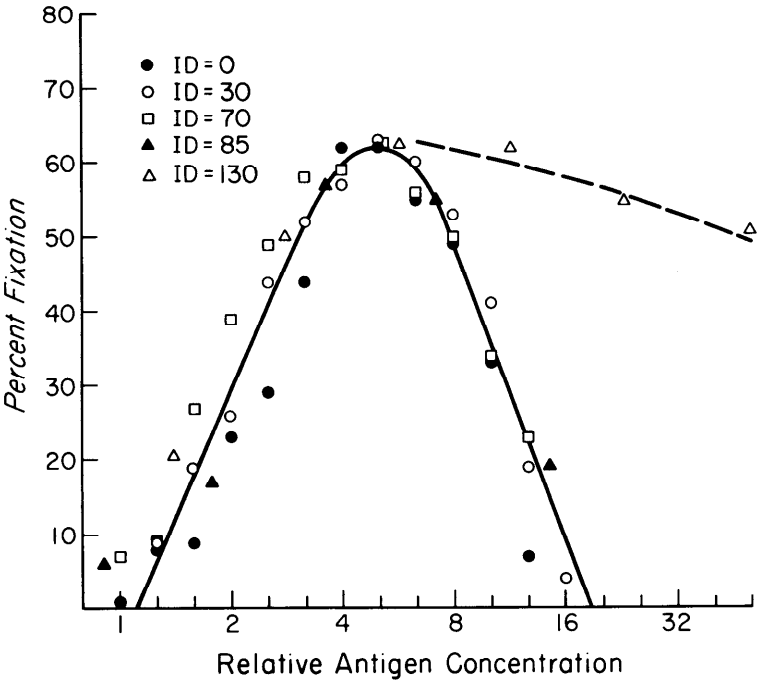

FIG. 3.—Superimposed micro-complement fixation curves at five different immunological distances. Antiserum to the albumin of the salamander, *Plethodon vehiculum,* was run with plasma from the following species as antigen: *P. vehiculum* (●), *P. elongatus* (○), and *P. cinereus* (■) (Maxson et al. 1979). Antiserum to the toad *Bufo blombergi* was run with plasma from *B. bufo* (▲) and *B. melanostictus* (△) (Maxson 1984). For most reactions, additional data points were obtained between the standard points to better delineate the curves. Actual antigen concentrations may differ between different species, since the curves have been moved horizontally to superimpose the peaks. Such curve congruency as seen with the salamander data and *B. bufo* is common with albumin. However, at large immunological distances, some antisera no longer produce such congruent curves and a broadening of the "peak" occurs, as with *B. melanostictus* (△).

comparison of the MC'F curve shapes at different IDs. The MC'F protocol used in figure 4 is modified by using variable mixtures of two antigens for the heterologous comparison. Also, the time at which the two antigens were added is varied as described below in Results and Discussion. This procedure allows a direct test of the assortment-exclusion hypothesis.

Results and Discussion

The Assortment-Exclusion Hypothesis

Two reactions are run in order to obtain an ID. The same antiserum is used with two different antigens, one homologous (to which the antiserum is specific) and one heterologous (divergent). When an appropriately increased antiserum concentration is used for the heterologous reaction, the same amount of complement can be "fixed" (i.e., bound to antibody that is itself tightly bound to the antigen) as is fixed in the homologous reaction. One hundred times the log (base 10) of the ratio of the two antiserum concentrations then gives the ID. For example, using antiserum to *Hyla regilla* albumin with *H. regilla* serum, the MC'F reaction illustrated in figure 1 is observed. Repeating the experiment but substituting the serum of a related species, *H. cadaverina,* we see virtually no complement fixation. By increasing the concentration

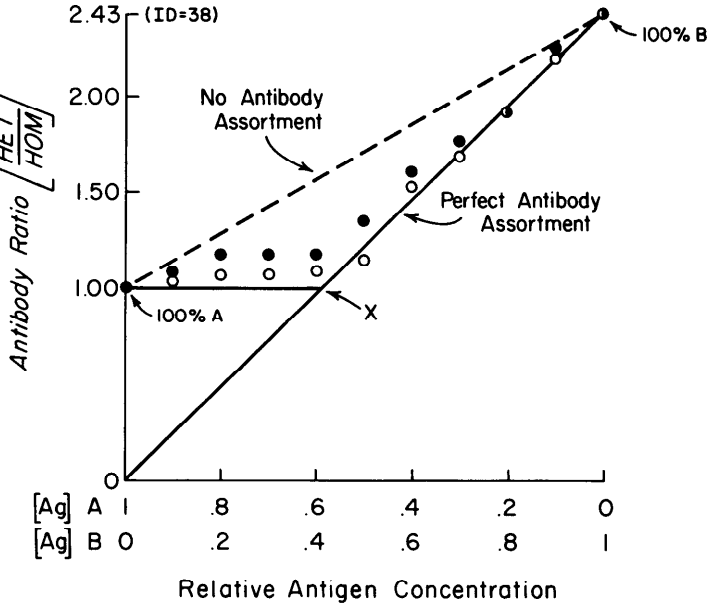

FIG. 4.—Plot of antibody ratio when varying proportions of two antigens (with a measured immunological distance of 38) are used. Antiserum is to albumin of the toad *Bufo boreas* (species A). The heterologous species B is *B. holdridgei*. Black dots are immunological distances from standard micro-complement fixation (MC'F) experiments using indicated proportions of antigens A and B. Circles are experiments in which the protocol was altered so that antigen A was added to the reaction 3 h after antigen B. (Total incubation time was 21 h at 0 C). The dashed line represents anticipated R from direct averaging. The solid line represents the theoretical curve with antibody assortment-exclusion. At point X, antibody assortment should theoretically be complete and antibody exclusion should begin.

of anti–*H. regilla* antiserum $1.\bar{3}$ fold, we can obtain the same amount of complement fixation. The ID is calculated as 100 log $1.\bar{3}$ = 12.5. Note that while there has been virtually no change in the concentration of *H. cadaverina* antigen (the location of the peak on the horizontal axis), the antiserum concentration has been boosted by a factor of $1.\bar{3}$. This curious asymmetry between antigen and antiserum (while the amount of complement binding to bound antibody remains constant) suggests a testable molecular mechanism for the "black box" of MC'F.

Assume that there are more antigenic sites (determinants) on an antigen (perhaps 30 total) than are required in the complement-binding reaction (perhaps three at the peak of the curve). For each antigen molecule there will be considerable freedom as to which sites bind antibody (since only three of the 30 are used). If antibody is not present in excess, different albumin molecules must complex using *different* subsets of antibodies until all the (high-affinity) antibodies are bound. Avoiding excess antibody is an important attribute of the MC'F protocol relative to other immunological techniques. This diversity of binding among identical antigen molecules (caused by the large number of sites relative to the number of *bound* sites at peak complement fixation) is the key for predicting what will happen if a few of the antigenic sites are altered by AAR. As heterologous albumins diverge in sequence (relative to the homologous albumin), more and more antigenic sites will lose their high antibody-binding affinities. Consequently the antibodies to these sites will be unable to fix complement. These altered sites and the antibodies associated with them will no longer take part in the

complement fixation process. Because there are more antigenic sites than are actually used for MC'F, all of the antigen can still be bound simply by using the remaining unaltered sites. Experimentally, just enough additional antiserum is added to the reaction to maintain the identical amount of fixed complement (which serves as a sensitive measure of tightly bound antibodies). Only that fraction of additional antibodies that are specific to sites that have *not* had an amino acid replacement will fix complement. The antigen concentration will tend to remain the same (if full equilibrium is achieved), since only the altered sites and their associated antibodies (and not the antigen molecules) are excluded from the MC'F process.

We call this process assortment-exclusion and propose that in the MC'F reaction the antigenic sites are *assorted* into altered and unaltered sites and that the antibodies to the altered sites are *excluded* from the MC'F reaction. In our view, the increase in antiserum does not compensate for the decreased binding affinity of the antigenic sites by increasing the overall concentrations of the reaction components—as occurs, for example, in conventional precipitin cross-reactions. Altered sites are simply replaced by increased usage of unaltered sites, while the functional antigen-antibody-complement complexes remain essentially the same.[2]

The basic requirements for an experimentally observable assortment-exclusion process are (1) a large number of antigenic sites, (2) an antibody-reaction protocol that uses only a fraction of these sites on any single antigen molecule, (3) sufficient molar dilution of antigen and antiserum so that binding (as measured by complement) is sensitive to single AAR, and (4) a reaction protocol that can sensitively and repeatably determine an equivalency point where neither antibody nor antigen is present in excess. Each of these requirements is separately addressed in the next section.

Indirect Evidence of Assortment-Exclusion

Antigen and Antibody Valence

The valence contrast between divalent complement-binding antibodies (IgG) and multivalent antigens is well documented. Recent work with monoclonal antibodies suggests that serum albumin has 25–33 distinct antigenic sites (Benjamin et al. 1984). The complement-binding reaction in MC'F requires the simultaneous cross-linking of multivalent antigen molecules with several divalent antibody molecules. This triggers the conformational changes within the antibody that cause complement to bind (Osler 1976; Levine 1978). Only two to four antigenic sites per antigen molecule participate at the peak of the MC'F reaction curve. The reasons for this are unknown, but may in part be the result of the large size of the antibodies relative to the antigen and the low concentration of the reaction components (10^{-9} M).[3]

Apparent Antibody Exclusion

We quantify the observed asymmetry between antigen and antiserum by monitoring the antigen concentrations in experiments involving 17 amphibian antisera and

3. At the low concentrations used in MC'F, there is no precipitation of the antibody-antigen-complement complexes. This means that the MC'F complexes do *not* form extended lattice structures. It seems likely that complexes of $Ag_2Ab_iC_i$ are forming where i = 2–4, even when the experimental antibody to complement ratios vary with ID. When i is too small, there may not be enough stress generated within the complexes to cause the conformational changes in the antibody molecules that trigger complement binding. When i is too large there may be packing constraints that prevent antibody binding. Whatever the actual reasons, the inhibition of complement binding in regions of either antigen or antibody excess is well confirmed experimentally by the sharpness of the complement fixation peak (figs. 1, 3).

summarized in figure 2. There are 26 unplotted homologous reactions at the origin of the graph (ID = 0). While the antiserum concentrations are determined within normal pipetting errors of a few percent, the antigen concentrations are being interpolated between doubling antigen dilutions. The probable error about each point is, therefore, strongly ellipsoidal, and the observed scatter in figure 2 is almost entirely along the ordinate.

The dotted line in figure 2 represents antigen and antiserum ratios identical to those present in the homologous reaction. In the absence of antibody exclusion, and with the degree of antibody and complement binding dependent on the *concentrations* of the reacting components, all points should fall along this line. The log scales tend to obscure the striking asymmetry in the data. Even at IDs approaching 150, requiring up to a 32-fold increase in antiserum (fig. 2), only a threefold increase in antigen is required. It is apparent why only the antiserum concentration is monitored, since the antigen concentration is not a sensitive indicator of antigen divergence. The critical observation is that the amount of bound complement is identical in all these reactions. Since complement binds directly to antibody (provided the antibody is itself tightly bound to antigen), all the antiserum cannot possibly be participating in the complement fixation process. As antigenic sites diverge, some antibody exclusion must be occurring. We postulate that exclusion occurs because of lowered binding affinity of altered antigenic sites.

Sensitivity of MC'F to Single AAR

For the postulated assortment-exclusion process to occur, single AAR must affect an antigenic site sufficiently to cause the antibodies specific to that site to be excluded (with respect to binding complement). Such sensitivity of the MC'F assay has been experimentally shown in studies of single amino acid mutants of alkaline phosphatase in *E. coli* (Cocks and Wilson 1969) and in studies of single amino acid variants of human hemoglobins (Reichlin 1972, 1974). There are no a priori reasons to expect such results to be protein dependent. It is likely that this sensitivity is critically dependent on the low concentrations of antigen and antiserum at peak fixation, which allow only those interactions having very high affinities.

Antibody exclusion, as postulated, depends on conformational changes in the antigenic sites caused by AAR in the diverging albumins. Whether such AAR are directly within the antigenic site, or only sufficiently close to affect the site by a more general conformational change, makes little difference. Available evidence suggests that both types of effects play a role in antigenic site specificity (White et al. 1978; Jolles et al. 1979; Hornbeck and Wilson 1984). A small fraction of AAR do not affect antibody binding and thus will not be detectable by MC'F. This poses little problem in using MC'F to generate phylogenetic data—other than marginally reducing the sensitivity of the assay (and introducing an additional constant since *measured* AAR can be less than *actual* AAR).

MC'F Interaction Topology and Curve Congruency

The postulated assortment-exclusion process preserves the complement-antigen-antibody interaction ratios (the topology of the reaction complexes) even when the ratio of antiserum to antigen dramatically increases in cross-reactions (fig. 2). We suggest that complement fixation curves are sensitive indicators of the actual interaction topology at the molecular level.[3] These curves essentially span the stability parameters

of the complement-binding process, from maximum stability at peak fixation to zero stability and no complement binding in regions of antigen or antibody excess. Figure 3 illustrates high curve congruency over a wide range of IDs. Such congruency would not be expected at different IDs unless the molecular complexes of antigen-antibody-complement have *functionally* identical antibody-antigen ratios despite *apparent* difference in the antiserum ratios.

Because of the obvious peak in the curve with respect to antigen concentration, and the sensitivity of peak heights with respect to antiserum concentration, the ratio of antibody to antigen at each data point is well defined experimentally (figs. 1, 3). In contrast, radioimmunoassay (Lowenstein 1980; Klein 1982) requires only a single antibody molecule to bind to each antigen molecule to register as cross-reactivity, and an excess of antiserum is always used. With excess antibody, a single antigenic site can dominate the cross-reaction, giving a poor indication of total antigenic divergence. Moreover, at high antibody concentrations even low-affinity interactions can occur, such as those at antigenic sites that have been partially altered by AAR. This distinction between our proposed MC'F assortment-exclusion process and radioimmunoassay is the distinction between *counting* the number of altered antigenic sites and *measuring* the residual affinities of the least-altered antigenic sites.

Figure 3 also illustrates that the heterologous MC'F curve can change shape at sufficiently high IDs (at an ID of 130, R is 20). While we have argued that curve congruency provides an indirect argument for topologically equivalent molecular complexes, a change in curve shape is direct evidence of a significant change in the complement-binding reaction. All antisera we have tested lose their sharp peaks at sufficiently high IDs. We believe this to be evidence of a divergence limit for using MC'F data as a phylogenetic probe—and can predict why and where this limit will occur based on the assortment-exclusion hypothesis.

A Direct Test of Antibody Assortment-Exclusion

We have shown that MC'F analyses of serum albumin satisfy the four basic requirements for the occurrence and measurement of assortment-exclusion. A direct test of this hypothesis is to titrate the excluded antibody to see whether it is present in the predicted amount. This is difficult experimentally because the reaction complex of antibody-antigen-complement remains in solution at the low concentrations used in MC'F. We have devised an experiment that uses the MC'F reaction itself to titrate the excluded antibody at the same time that the cross-reaction is occurring. A mixture of homologous and heterologous antigens is used, to allow the homologous antigen to react with and titrate any antibody excluded by the heterologous antigen.

In these multiple-antigen experiments we compare two species (toads of the genus *Bufo*) using antiserum to albumin of species A (fig. 4). The ID between species A and B is 38 (representing ~23 Myr of divergence; Wilson et al. 1977). The varying proportions of the homologous and heterologous antigens are indicated on the abscissa. The homologous reaction is equivalent to 100% A, and 100% B gives the normal heterologous reaction.

The dashed line (fig. 4) represents the expected results with simple averaging, if no assortment and exclusion were occurring. The line along which the data points would fall if there were perfect antibody assortment and exclusion is also indicated. With assortment, no additional antibody would be needed for small percentages of heterologous antigen. The assorting of antibodies between the two antigens would be

sufficient to allow all of the antibodies to bind to unaltered antigenic sites. Then at a critical point, there would not be sufficient antigen A to titrate all of the excluded antibodies. More and more antibody would be needed, in a linear fashion, until a complete heterologous reaction is obtained. Point X is where antibodies begin to be excluded instead of just being assorted between the two antigens. At this point antibodies will react with antigen A *only* at those sites that differ between antigen A and antigen B. Along the portion of the curve with zero slope, the antibody that is excluded from antigen B is being completely titrated by the homologous antigen A.

We performed two sets of experiments with slightly different protocols. Solid points are from MC'F experiments using varying mixtures of the two antigens as the "antigen" in otherwise standard MC'F experiments. Open points are reactions where we first added the indicated proportion of the heterologous antigen B, waited 3 h (standard incubation time is 21 h), and then added the indicated proportion of antigen A. This time delay permits antigen B, the less reactive antigen, to begin reacting with the antiserum without any competition from antigen A. Consequently, antigen A reacts preferentially with those antibody molecules *not* reacting with antigen B, i.e., with the excluded antibody. The data in figure 4 show that this time delay allows the reaction to closely approach the predicted response curve for perfect antibody assortment-exclusion.

These results demonstrate that much of the excess antibody that is normally added to cross-reactions does not take part in the MC'F reaction. No additional antibody is needed when the mixture is as much as 40% of antigen B, despite the known divergence, because the antibodies excluded from reacting with antigen B are still free to react with antigen A. It is reasonable that full equilibrium is not reached in the fractionation process when both antigens are added simultaneously. In this case, some of antigen A probably reacts by using sites that antigen B must use to achieve complete assortment—and therefore full equilibrium is not reached.

Because of the assortment process, a mixture of two antigens does not give an average ID. A second divergent antigen, present in small amounts, produces no detectable difference at all. We have previously observed this unusual result within the tetraploid *H. versicolor,* without understanding the underlying cause for the nonlinearity when two different albumins are present (Maxson et al. 1977).

Theoretical Derivation of the ID-AAR Transform

The assortment-exclusion process is unusual in predicting a *quantitative* relationship between ID and AAR, even when ID might seem to depend only *qualitatively* on the various binding affinities. The quantitative relationship results because antibody exclusion is an all-or-none process on a site-by-site basis and, therefore, can be *counted.*

The amount of antibody exclusion is measured experimentally by the reciprocal of the antibody ratio, 1/R. This is the fraction of antibody specific to those antigenic sites that have *not* been sufficiently altered by AAR to cause preferential antibody exclusion. Thus $1 - (1/R)$ is the fraction of antigenic sites that have sustained AAR. For example, a necessary 10-fold increase in antiserum to achieve identical peak fixation (an R of 10 or an ID of 100), implies that only 1/10 of the antibodies are participating in the complement-binding process. Put differently, 90% of the antigenic sites in the heterologous albumin have sufficient conformational changes to exclude antibodies and prevent complement fixation.

The number of changed or unchanged antigenic sites can also be expressed theoretically in terms of the Poisson distribution and the average number of (detectable) AAR per antigenic site, AAR/S, where S is the total number of antigenic sites. Use of the Poisson distribution assumes a random model for the distribution of replacements among the various antigenic sites and corrects for sites that have more than a single AAR. This correction is necessary because once an antigenic site excludes the related antibodies, further replacements at that site will go undetected by MC′F analysis. The expected fraction of unchanged sites is $e^{-AAR/S}$ and the expected fraction of changed sites, i.e., those with one *or more* replacements, is one minus this quantity.[4]

By setting the experimentally measured and theoretically estimated fractions of changed (or unchanged) sites equal to each other, the relationship between ID and AAR follows. The algebra is outlined in the Appendix. This relationship, within a region where antibody assortment-exclusion predominates, is $AAR = (S/43)\ ID$.

That the relationship between ID and AAR is linearly dependent on the number of antigenic sites is not surprising. Reichlin (1972), for example, used the percent inactivated (i.e., excluded) antibody to single amino acid mutants of human hemoglobins to estimate the number of immunogenic sites. The log-exponential relationship follows from extending the basic logic to multiple differences within numerous immunological sites. The original heuristic log transformation of Sarich and Wilson (1967), set within a controversial framework concerning the descent of man, appears to be confirmed. Other proposed correlations (Reed and Lestrel 1970, 1972; Uzzell and Pilbeam 1971; Read 1975) are not supported by our work.

Additional Considerations

Estimating versus Sequencing

It is apparent that we are discussing a procedure for *estimating* AAR when it is possible to infer AAR from amino acid differences obtained directly by protein or DNA sequencing. An estimation procedure needs defending in an era when sequencing has become commonplace. The phylogenetic tree of extant species is extremely dense, with well over a million speciation nodes. Because of this, successful phylogenetic reconstruction of life's diversity depends primarily on the *operational* aspects of a phylogenetic probe. MC′F, in terms of time, effort, cost, and conservation of often rare biological material, is operational to a degree that protein sequencing and direct nucleic acid sequencing are not (at this time). The efficiency of MC′F as a quantitative estimator of divergence within a large and rapidly evolving protein (Minghetti et al. 1985) is the key to having an operational probe for surveying many of the interesting features of the phylogenetic tree. It is always possible to carry out more precise sequencing work to better resolve some particular portion of the tree that MC′F may highlight as being particularly interesting but cannot resolve. Ongoing research on the human-chimp-gorilla divergence (initiated by MC′F studies of Sarich and Wilson 1966, 1967) is an instance of this (Ferris et al. 1981; Brown et al. 1982; Sibley and Ahlquist 1984; Templeton 1985).

4. This mathematical simplicity can be misleading. The distribution for the actual *physical* differences among the various sites requires first a Poisson correction to convert AAR to the total number of differences (AAD) and then the binomial distribution to estimate how these differences are distributed among the various sites. However, since MC′F is *directly* measuring the number of *unhit* sites, the full distribution for how the differences are distributed among the various sites is not needed. In other words, the sites that are "unhit" are not affected by "multihit" phenomena, so using AAR/S in the Poisson distribution gives the correct estimate of the number of unhit sites.

Measuring Antigenic Determinant Diversity

The ID-AAR relationship suggests that MC'F data may allow estimates of S on various proteins. The spectrum of divergent proteins provided by the evolutionary process can be used to probe the antigenic diversity of a specific antiserum. This contrasts with other immunological procedures that often use short-term (low-diversity) antisera, fragmented proteins, and hapten-inhibition analysis to estimate antibody diversity (Habeeb and Atassi 1976; Peters et al. 1977).

Actual sequences are necessary to estimate S using the ID-AAR relationship. Albumin has been difficult to sequence because of its size, and no sequences yet exist between species that are phylogenetically close enough to obtain good ID values. The smallest repeatable ID measurement (at or near the experimental noise level) is 1 or 2 ID units. If this is presumed to correspond to the loss of a single antigenic site, the resulting S is equal to 1/R and ranges from 20 to 50, a rather poorly determined value.

It has been customary to claim for albumin, by a somewhat circuitous argument, that 1 ID is approximately equal to one amino acid difference (Maxson and Wilson 1974). This is equivalent to claiming that S/43 (table 1) = ~1. There is a correction that must be applied, since the AAR used in the ID-AAR relationship represents only immunologically detectable replacements. Turning the argument around and using S = 30 as an estimate of the number of antigenic sites on albumin (Benjamin et al. 1984), the ID-AAR relationship implies that 70% of the differences, i.e., 30/43, are detectable immunologically.

When ID was plotted against the percent amino acid difference, the same slope was obtained for different-sized proteins although no justification was given for using the percent difference rather than absolute sequence difference (Champion et al. 1975; Prager et al. 1978). This correlation is predicted by the ID-AAR relationship if the number of sites is directly proportional to the molecular weights of the proteins. For these data, the mean "area" per antigenic site (which is different from the number of amino acids in the actual antigenic sites) is in the range of 12–16 amino acids.

Event Horizon for MC'F Analyses

The assortment-exclusion hypothesis implies the existence of a divergence limit dependent on the number of sites and beyond which the ID-AAR functional relationship breaks down. This limit occurs when only two or three sites remain unaltered, since no additional assortment-exclusion can then take place. Because measurable complement fixation can often be detected at IDs in excess of 200, the presence of such a horizon has not been anticipated. But just because measurements can be made does not imply that the data are of phylogenetic value. Once there are no more sites to exclude, the data will instead depend on the residual binding affinities of the least-changed antigenic sites. A high antibody concentration and an increased concentration of antigen can compensate for the loss of complement-binding efficiency. When this happens, the *quantitative* relationship between ID and AAR that is based on the assortment-exclusion process will deceptively change to a *qualitative* relationship that is not necessarily related to AAR in any predictable manner.

Since this limit critically depends on S, it is far more limiting for small proteins having few antigenic sites than for a protein the size of serum albumin (~580 amino acids long; Benjamin et al. 1984). For S = 30, the horizon is approximately at an ID of 100 (i.e., 100 log 30/3 = 100). For a small protein in which S = 3, the horizon is only a single mutation away—and the data will have little to offer in a phylogenetic

context, even though a range of ID values may still be measured. We suggest that broad fixation curves (fig. 3) and a marked increase in the amount of antigen are good indicators of having exceeded the limits of the assortment-exclusion process.

It is interesting to compare and contrast this limit with the problem of "saturation" within electrophoretic-based genetic distances that much exceed unity (Maxson and Maxson 1979). Both horizons occur because of the impossibility of counting beyond the first detectable "event." For electrophoretic distances, entire proteins are monitored for a detectable mobility change. For ID values, individual antigenic sites are monitored for a detectable conformational change. In a protein with 30 antigenic sites, the evolutionary rate of each site will be $\sim 1/30$ the evolutionary rate of the entire protein. The intrinsic limit for MC'F should therefore be ~ 30 times more distant (and statistically comparable to using 30 variable proteins in an electrophoretic survey). The actual divergence *time* contrast between the two methods is more complicated and would depend on the differing evolutionary rates of the various proteins.

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APPENDIX

Derivation of Relationship between ID and AAR within Antigenic Sites

R is the ratio of heterologous to homologous antiserum concentrations, and S is the number of antigenic sites. Log is base 10 and ln is base *e*. (AAR as used here may exclude some AAR to the degree that portions of the protein are not open to immunological review. See fn. 4 concerning use of the Poisson correction in line 2.)

$$1/R = \text{Fraction of unchanged sites} \quad \text{(measured)}$$

$$e^{-AAR/S} = \text{Fraction of unchanged sites} \quad \text{(theoretical)}$$

Therefore, $1/R = e^{-AAR/S}$, or $\ln R = AAR/S$, and because $ID = 100 \log R = 43 \ln R$, thus $AAR = (S/43)\, ID$.

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