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DISCUSSION

SCHECHTER: The first two questions were submitted by a referee: What are the best numerical procedures now available for the analysis of chemical relaxation data of the type presented here? Within what confidence limit can the relaxation decay law be determined and what are some of the main artifacts to be avoided in studies of this type?

PECHT: The analysis includes two major consecutive steps: (*a*) Analysis of the relaxation curves and evaluation of relaxation times and amplitudes (and base lines): Routinely, a sum of six or more relaxation curves has been analyzed. In principle any least-squares fit procedure may be used. We found that the modified algorithm of Marquardt is particularly effective (cf. ref. 6 for the subroutine). This subroutine requires the derivative of the exponential function with respect to the parameters, but the first guesses supplied to the program need not be very accurate. (*b*) Analysis of the concentration dependence of the relaxation times and amplitude: Here the functions are often more complex, and analytical derivations are not always convenient. Therefore

we use a "simplex" nonlinear least-squares algorithm (Powell, ref. 7), which does not require the function derivatives and handles many parameters quite efficiently.

As for the second question, regarding confidence limits for relaxation times and amplitude: τ's separated more than a factor of three can be resolved with good confidence limits. Also if their respective amplitudes are similar in size, better resolution can be achieved. The absolute magnitude of the amplitude should not be lower than 0.01%. Although the base line (final value of the signal) can be found as a parameter by the computer program, having an experimentally reliable value improves the confidence of the values. To examine the possibility of more than two exponents, a separation of a factor of 5–10 between the τ's is required. As stated in the text, the error limits were in the range of ±5–10%.

Artifacts in the measurements mainly arise due to the relatively long relaxation times which often had interference from cooling and convection. To overcome this difficulty, the base lines have been examined by the fitting procedure.

CZERLINSKI: Your data on Gal_3 binding to J-539 protein led you to the cyclic mechanism 3. You find only two relaxation times where three are expected, and you assume that the spectral change of one of the steps is zero. Have you considered the possibility that both bimolecular steps proceed at about the same speed? As error bars are not shown in your figures, I cannot judge what difference between two relaxation times would still permit you to distinguish them as two and determine them with any precision (the amplitude of the *R*-association may be as much as a factor of 4 below that of the *T*-association).

PECHT: Yes, we have considered this possibility. We have tried to fit the observed fast process to a sum of two processes having similar τ's. An important check is the "total amplitude analysis" of the two fast association steps. With the small ligand coupling (at the high hapten excess employed), a sum of two individual amplitude expressions for the T and R association is obtained. Analysis of such terms showed that in this case the R contribution is less than 5%. We cannot exclude a smaller contribution of association with the K state.

CHOCK: Based on your mechanism 3, the results of your analysis show an essentially equal population for T_0 and R_0 and the rates for the isomerization between these two species are significantly slower than the rate of hapten binding. Do you care to comment on the physiological role of this slow isomerization step? In fact your data probably will fit better with your mechanism 1 with an additional step; i.e. $H + T_0 \rightleftharpoons HT \rightleftharpoons HR \rightleftharpoons HR_1$ (similar to your Eq. 9). This is particularly true for your J-539 system.

PECHT: Well, the question is really very relevant and I am grateful that it was raised. I mentioned before that the link between the changes that we see and the actual biological activity of the antibodies has to be established. And that's why I said that it can be interpreted at least in two ways. First, that the conformational changes are confined to the Fab. In this case we just have a case of an induced fit, a way by which the antibody can interact better with the given hapten and prefer it over another one. That would be the minimalistic way to look at it.

A more interesting way to consider our findings is that the structural transition is a trigger for further biological activities. I stressed a moment ago that we can't establish this link at present; furthermore, I should remind the audience that the hapten per se does not trigger the immunological reaction. The haptens should be attached to a macromolecular carrier or, preferably, be polyvalent. That is a very interesting feature of immunochemistry—that small molecules usually do not induce the immune response. So if the hapten induces the actual changes related to triggering of immune phenomena, there are ways of explaining why the conformational equilibrium lies where it does, equally distributed between two states. One way would be to try and fit

it into a mechanism that will amplify the effect by binding a third component. We have not examined that as yet.

The answer to the question whether we tried to add a further step is that we didn't try to fit it to a more complex scheme. The data fit very well to the minimal scheme and we did not feel it necessary to go beyond this. In summary, the really interesting question is to what extent do we expect to see further changes depending on interaction with other components of the immune system, for example, interactions with complement components. In other words, the binding of the hapten is not the end of the process. There are further components that interact with the immunoglobin.

R. P. TAYLOR: My questions relate to the potential generality of your observation. The first is, have you been able to confirm the existence of the slow transition by observing it by stopped-flow methods? And second, has anyone detected the transition in these systems, either by T-jump or stopped flow?

PECHT: Well, to answer your first question, in the case of the galactan-specific antibodies (XRP-24 and J-539), we didn't check it by stopped-flow. We did check it for the 2,4-dinitrophenol (DNP)-specific MOPC-460, and there you definitely see the slow transition by stopped-flow. I should perhaps mention at this point that although we monitored only the intrinsic fluorescence of the antibody for the saccharide-binding antibodies, in the case of MOPC-460, which binds DNP derivatives, we have been monitoring the reaction by following the quenching, or enhancement, of fluorescence of the antibody. We have also studied the changes in transmission due to the formation of the complex of the DNP with the protein; by using an analogue of the DNP, namely a nitrobenzoxadiazol derivative, we could follow it also by quenching of the hapten fluorescence. All three modes of monitoring the reaction gave us essentially the same kinetic behavior. We have also shown the stopped-flow trace for the reaction of protein 460 with nitrobenzoxadiazol alanine and the results are very much in agreement with what the chemical relaxation T-jump analysis gave.

I think I commented already to a certain extent about the generality of the results. More specifically, the question is would we expect multi-step reactions to occur also with normally induced antibodies in contrast to myeloma proteins, which are somewhat suspect, being of tumor origin. The reservation about the immunoglobulins produced by the tumor line has been quite amply proved to be unjustified. These are definitely legitimate representatives of the immunoglobulin antibodies that every animal produces. Still, work has been done also on normally induced antibodies; difficulties emerge with a heterogeneous population of antibodies. In other words an animal challenged with a certain antigen will produce a multitude of different antibodies. They will all recognize a DNP attached to bovine serum albumin, but they will recognize it in rather different ways, and one expression of this will be the rather broad range of binding constants that those antibodies will express against DNP. To expect a clear-cut answer from a broad range of different combining sites is perhaps asking too much. That is the main reason that we have confined ourselves to the homogeneous proteins. There is now a way of circumventing this problem, namely producing homogeneous antibodies from hybrid cells produced from tumor lines and normal antigen-induced cells.

ROMINE: Could you elaborate on what particular immunoglobulins were used in your experiments?

PECHT: Those that I spoke about were all IgAs; however quite similar results were also obtained recently with an IgM (MOPC IONE, specific for nitrosyl oligosaccharides). Unfortunately no measurements on IgG have been done.

ROMINE: IgM is known to bind complement and will itself activate complement in the CH_2 region. Is that not true? Would it not be possible to design experiments using galactan polymers larger than three units to mimic a large macromolecular antigen, as opposed to using the time? That way you can add complement to the system and see if this induces a further relaxation process to check the biological activity?

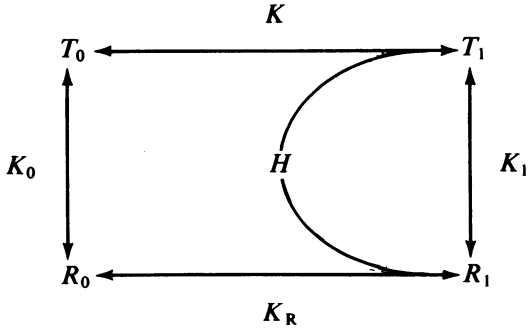
PECHT: The answer is yes, it has to be done.

ROMINE: The other question is what other experiments along these lines might reveal biological activity in relation to the conformational changes involved? This is a rather general question.

PECHT: Well, we can spend the next hour on this, obviously. I think one very interesting line of work, which Dr. Barisas has pursued in the last few years, is examining the reactions of antibodies with divalent haptens and trying to resolve further changes induced by the fact that the hapten is divalent. Porter and his associates have shown that oligomers formed by the interaction of antibodies with divalent haptens can induce complement activation. I refer now to IgG antibodies.

CZERLINSKI: I have two parts to my comment. Part I: Your mechanistic scheme 3 actually has four limiting cases, while you presented only two. I shall employ the terminology of your mechanism: A bimolecular step in mechanism 3 may be coupled to a molecular interconversion either at T_0 or at R_0. One would in both cases observe a decrease of $1/\tau_s$ vs. concentrations and an approach to a plateau level. Furthermore, your bimolecular step could couple to either T_1 or R_1 of your biomolecular interconversion of complexes. These two cases would lead to the same observation: $1/\tau_s$ would increase with increasing concentrations and approach a "saturation"-level. Why did you not discuss the additional two cases?

Part II: You concentrate on cyclic mechanism 3. However, to interpret your data, you assume that one of the steps is not connected with any fluorescence change. This is certainly an acceptable assumption. However, I feel you should also consider the possibility that you do have three relaxation times, with the two fast ones directly connected with the bimolecular steps and closely spaced. I have to ask in this connection how closely could your relaxation times be spaced with the signal-to-noise ratio which you have in your experiments. Could they be spaced within a factor of 2, or less, or a factor of 4? I do not expect that the amplitudes of the two relaxation processes would be the same; they may be different by up to a factor of 4, on the basis of the difference in the equilibria of the two interconversions alone.



LANCET: This general scheme has the following implication: We assume that there are two different conformations for any kind of protein, including our antibody, that bind the hapten

(or the ligand in general) differently, and by that we mean that one has a higher association constant than the other. The idea behind this scheme is that it is symmetric; when we say that T is the low association constant one, and R is the higher association constant (mechanism 1) this is just part of the naming process. When we say, for example, that we have the isomerization on the bound state only (that is mechanism 1 in our paper), we can show that we have implied in it that the second conformation in the sequence is of higher affinity. Actually it has infinite affinity since it does not dissociate at all. We therefore couple the K_T association with the K_1 isomerization. The mechanism with K_R association coupled to K_1 isomerization is just the same, only with affinity R. It's not a different mechanism; it is simply that we use different names. Chemically they are not different. Actually, only two cases out of the four are physically interesting, although in terms of notation there are four. I think this is the answer to the first question.

Now, as to the second question, I would like to refer you to a figure in our ref. 3. These curves are taken again from the very well characterized case of protein 460, but the analysis very much resembles the case of the galactan-binding proteins presented in this conference. The idea is to make a total amplitude analysis for the fast relaxation time. In the general scheme drawn here by Dr. Czerlinski, we expect, because of the cycle, only one slow relaxation time that represents both isomerization steps, and two fast relaxation times. According to what Dr. Czerlinski has proposed, which is very reasonable, we may have one fast relaxation time that represents the two association processes, due to the closeness of the values of the two times. But the amplitudes should show the behavior of a mixture of two different associations characterized by two different association constants. Thus we take the amplitude of the observed fast relaxation and analyze it in terms of concentration dependence, as shown here. Now, for the two association constants, which may be derived by means other than the amplitude analysis, we may expect different amplitude behaviors and we try to fit the observed fast relaxation amplitude to each of those expected behaviors. For protein 460 this is what we expect for what we call T binding, and this is for the R binding, and we see that it fits to the R binding. When we did the same thing for the two proteins presented here we found that the amplitude behavior could be fitted to almost pure T binding behavior; by that I mean that less than 5%, within experimental error, could be ascribed to R binding. Now I wouldn't like to say that there is no residual contribution from the R association. There very probably is, but it could probably not be more than a 5% contribution. Thus we can say that this is essentially pure T binding, and that the R association is not represented in the observed fast association. Therefore, we ascribe a small or zero fluorescence change to this step.

CZERLINKSI: There is one little problem when you assume only one fast relaxation time and thus one amplitude. Two relaxation times are expected to fit your data better, if you trade the assumption of "no signal change" for the assumption of "one more relaxation time." You ought to compare equivalent assumptions. You may use (as I do) the sum of squares of residues in your nonlinear least squares analysis as a criterion.

LANCET: Just a very short answer. One has to remember that here we perform *total* amplitude analysis. Such analysis does not depend at all on kinetics. Total amplitudes are pure thermodynamic magnitudes and we assume that the fast steps are completely uncoupled from the slow ones. We could even not look at all at the kinetics, but just at the magnitude of the effect versus concentration, and get this representation that I have shown, and derive from it the pure T nature of the association.