RECEPTOR BIOCHEMISTRY AND METHODOLOGY, VOLUME 10

Series Editors: J. Craig Venter and Len C. Harrison

TARGET-SIZE ANALYSIS OF MEMBRANE PROTEINS

Editors
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Alan R. Liss, Inc., New York

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ALAN R. LISS, INC., NEW YORK

Address all Inquiries to the Publisher
Alan R. Liss, Inc., 41 East 11th Street, New York, NY 10003

Printed in the United States of America

Library of Congress Cataloging-in-Publication Data
Target-size analysis of membrane proteins.

(Receptor biochemistry and methodology; v. 10)
Includes bibliographies and index.
1. Membrane proteins—Analysis. 2. Membrane proteins—Effect of radiation on. I. Venter, J. Craig II. Jung, Chan Y. III. Series.
QP552.M44T37 1987 574.87'5 87-16858
ISBN 0-8451-3709-3

TARGET-SIZE ANALYSIS OF MEMBRANE PROTEINS

RECEPTOR BIOCHEMISTRY AND METHODOLOGY

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Target-Size Analysis of Membrane Proteins
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Preface

The field of biomembranes represents one of the most active and exciting fields in current biomedical research. Membrane-associated proteins occupy a central importance in this regard, subserving structural and functional entities for many crucial cellular activities such as mass, energy and information transfer across cell membranes. The ultimate understanding of these membrane-associated protein activities may be approached directly only through studying the structure of these proteins within the framework of the membrane organization. Undoubtedly, such an approach requires specialized noninvasive techniques that are not readily expected from most of existing biochemical methodology. Radiation target size analysis represents one such noninvasive technique.

Radiation target analysis as an idea is not new. It was introduced in the early 1920s, and represents one of more vivid examples wherein physical and mathematical principles are successfully applied to help understand biological problems. It originally helped biologists to understand quantitative aspects of the actions of radiations on living cells. The classical monograph by D.E. Lea, first published in 1946, summarizes these early developments. Continued work by many biophysicists that followed during the fifties and sixties has established radiation target analysis as a potent experimental tool in estimating molecular sizes. The technique is found to be truly unique in that sizes of functional biomacromolecules in their native environment such as membranes can be studied in the presence of other proteins, bypassing all the experimental artifacts secondary to the use of detergents for protein solubilization and purification.

Because of its unique quality, radiation target size analysis has recently become increasingly popular among investigators in biomembrane fields. The radiation target size of a number of membrane-associated enzymes, receptors and transporters have been studied, providing valuable insight into their structure within the membrane organization. With this surge of interest, radiation target size analysis has been the subject of several recent review articles. None of these articles, however, provide biochemists with the much needed understanding of basic principles underlying the technique, or specific procedures of application to each of many diverse biological systems. The objective of this volume is to provide such a comprehensive treatise of the subject. The volume begins with the two chapters discussing the basic aspects of radiation target analysis, written by two eminent biophysicists known for their contributions to the development of the analysis of radiation inactivation. These are followed by the chapters written by noted biochemists who have actively used this method, discussing first-hand experience as to how the method may be applied to specific questions in biomembrane fields. These chapters detail experimental procedures and data analyses, and discuss both the usefulness and the limitation of the technique as it is applied to individual systems.

J. Craig Venter
Chan Y. Jung

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Physical Principles of Radiation Inactivation

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A BACKWARD GLANCE: SOME HISTORICAL POINTS

From the very first days, when the examination of matter by the new techniques of electrical discharges and the study of radioactivity began to be used, the nature of the interaction of radiation with matter developed importance. The existence of a finite and measurable range to alpha particles was of great value in the early interpretation of the interaction of alpha particles with matter. In particular, the extension of the understanding of the reason for that finite range to hydrogen nuclei provided one of the most important lines of evidence that a nuclear reaction had been achieved in 1919. It is not surprising that Bohr, after his monumental paper on the structure of the hydrogen atom, should turn his attention to the way energy is lost by a fast charged particle in traversing matter. It was apparent that just as the hydrogen atom had specific levels of energy in which an electron could be found, so the action of a fast charged particle in traversing matter was to lose energy in discrete amounts. These losses of energy could readily be far greater than the energy of chemical binding.

So, from the very earliest times, in the modern era of atomic physics and chemistry, the potency of the interaction between radiation and matter was realized. This was soon made quantitative by two means. The first was the direct visualization of ionization in the Wilson cloud chamber with the added ability of delaying the expansion to permit diffusion of ions, a technique that showed that primary and secondary ionizations were often so close together that they formed a cluster; and the second, the direct measurement of the energy necessary to create an ion pair, found to be about 33 V. Both of these gave convincing evidence that the energy releases in the absorption of fast charged particles were considerable and definitely greater than the energy of chemical bonding. Moreover, the energy releases were separated by considerable distances in which no action took place.

As soon as attention turned to the effect of radiations on living things, a startling fact intruded itself. The amount of energy needed to kill a living being was astonishingly small. To kill a human being by ionizing radiation takes one-tenth as much energy as is absorbed by drinking a small cup of hot coffee. This extreme efficiency further manifested itself in studies on small living things and these find-

ings quickly led to what is called today the "target " theory. This theory is interesting as a very good example of the simultaneous development of scientific concept in different places. Within a period of a few years, four different origins of this idea occurred in four different nations. In Germany, Dessauer [1922,1923] realized that the separated large releases of energy would produce local intense heating and suggested the "point-heat" idea. This required a sensitive target. In England, Crowther [1927], impressed by the studies of Strangeways and Oakley [1923] on the effect of soft X-rays on tissue cells, proposed both a simple and a multi-hit target hypothesis. In France, Holweck [1928, 1929,1930], and Holweck and Lacassagne [1929] proposed very much the same thing. In their own words: "Everything acts as if the microbial body contains a certain proportion of 'sensitive substance' in which it is sufficient to alter, for example by ionization, a small number of molecules to cause the death of the bacterium" [Holweck and Lacassagne, 1929]. In the United States, Condon and Terrill [1927], impressed by the studies of Wood [1924,1925] and Packard [1926,1927], proposed the target theory for single-hit, multi-hit and multi-target cases. They made some estimate of the target size but were frustrated by inadequate dosimetry. The early work was also made more difficult by the uncertainty about the effects of X-rays of different wavelengths.

In the following decade, several advances took place that widened the scope of the studies and started the target hypothesis into the realm of actual molecules. One advance was in equipment for irradiation. It is most interesting to read about the actual means of irradiation used by the pioneers who built their own machines, which clearly shows the extent to which self-sufficiency has been lost in scientific research. In any event, more potent means of irradiation became available so that better studies of microorganisms and even on viruses could be started. Also, the production of mutations by radiation was observed and this impressed Schrodinger [1947], building on the ideas of Gowen, Timofeef-Ressovsky, and Delbruck, who gave careful consideration to the quantitative nature of the production of mutations by X-rays, pointing out that with the accurate dosimetry then available, an estimate of the volume that had to be involved with a mutation could be made. He estimated "ten atomic distances cubed." As he puts it, "the energy for overcoming the threshold (for mutation) must obviously be furnished by that explosion-like process, ionization or excitation."

In the early 1940s, D.E. Lea and collaborators, working with a home-made X-ray machine delivering considerable radiation, were able to study the inactivation, by radiation, of ribonuclease and myosin. They found plausibly exponential inactivation and, by using the target theory, were able to deduce target molecular weights of 30,000 for ribonuclease and 470,000 for myosin. These values are so reasonable, especially in view of the difficulties of dose measurement, that these experiments can be taken as starting the extension of target theory into the study of the size of protein and other important biological molecules. Not long after this work, two important reviews of radiation target analysis appeared. They were developed separately and have some differences, but they still present much the same story. They are by Lea [1947] and Timofeef-Ressovsky and Zimmer [1947].

THE MOLECULAR ASPECT OF RADIATION INACTIVATION

It is helpful to continue, for a little, the historical account of further studies. For many reasons, including the explosion of two atomic bombs, it became necessary to have a better idea of the action of ionizing radiation on living systems. As soon as this objective was pursued by new groups of workers, one outstanding fact attracted major attention. This was the effect of ionizing radiation on water, already carefully studied by Fricke [1934], producing reactive species of varying life-

times and certainly capable of chemical alteration of biological molecules. It was argued that about 80% of all living things is water and so the most probable reason for the action of ionizing radiation on cells would be via the effect on water. Since this introduces migrating chemically active agents, the concept of a target became drastically altered. No change in the belief of the presence of critical sensitive targets was introduced: The change was in the manner by which they could be altered. It was felt that the normal chemistry of active radicals would be encountered, rather than the "explosionlike" process mentioned by Schrodinger. This naturally led to the search for agents that could influence radiation action, and such were found: sulfhydryl compounds for protection and dissolved oxygen for potentiation. The usefulness of the target theory for the study of radiation action, on the one hand, and to determine molecular weights, on the other, receded. Other types of study, for example, a thorough investigation into the radiation chemistry of water, took their place. There were exceptions to this, and the researches that continued into the statistical character of radiation inactivation were fruitful. It is these that now require some description.

The Radiation Inactivation of Viruses

The first observation of the loss of virus activity in relation to X-ray dose was made by Gowen and Price [1936] on tobacco mosaic virus. They observed a single exponential inactivation and commented on the relation between this virus and a gene. In the years that intervened before World War II, several studies on viruses were made, and these are reviewed by Lea. We can select one for discussion. Wollman, Holweck, and Luria [1940], in France, almost at the last moment before disaster to the nation, studied radiation action on bacteriophage C16. They concluded that the kinetics of inactivation is simple exponential; that the rate of delivery of X-rays is immaterial; and that the killing, by alpha particles, for a given dose, is seven times less efficient than by X-rays. All these points are significant and led them to the conclusion that the virus acted as a single entity with a radius around 250 Å.

Inevitably, the next studies on viruses had to aim at clarifying the different effects of direct ionization and action via the medium. On the one hand, Lea and Salaman [1942] succeeded in retaining the infectivity of vaccinia virus in the dry state and were able to examine the sensitivity to X-rays and polonium alpha particles. Their target analysis led them to conclude that the virus had a number of sensitive subunits, 110 in their later estimate, and this is the first recognition that a virus has some structure. A series of experiments by Watson [1950,1952] showed the different effects of irradiation of T2 bacteriophage in broth and in synthetic medium and clearly implicated the effects due to the radiation action on the medium as related to the process of attachment, that is, the surface of the phage. Watson also found that for the sensitivity of development of infection, the target size of T2 phage was approximately 1/20 of the size expected from the amount of DNA, a finding that is approximately found in all the double-stranded DNA phages.

From the point of view of target analysis the first definitely analyzable data were obtained by the author and F. Forro [1951]. Here, T1 bacteriophage was bombarded by deuterons of controlled energy and also by X-rays, the work being done in the dry state. It was found that as the density of ionization by deuterons was increased, the target area of the virus increased proportionally. This precludes the possibility of a single target of considerable thickness and led the authors to conclude that there were either, as suggested for vaccinia, a number of smaller targets, or else a thin structure they characterized as "true virus." Since this antedates the Hershey-Chase experiments and since it was accompanied by the following words: "It is then supposed that the head is essentially inert material which leaves the virus after entry into the host . . .", we include, as Figure 1, this

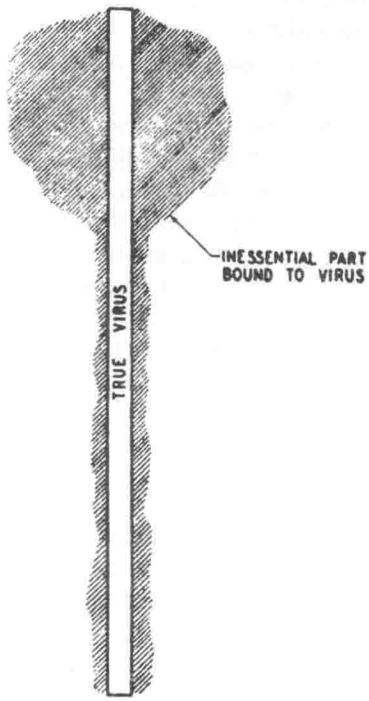

Fig. 1. *Reproduction of a diagram in a paper by the author and F. Forro giving one way to interpret the results of inactivation of T1 phage in the dry state by several kinds of ionizing radiations. This appeared in 1951. The Hershey-Chase experiment appeared in 1952.*

early analysis, by target methods, of the structure of a virus.

This work, particularly that of Watson, convinced many molecular biologists that the conditions under which target theory was applicable were too stringent for normal study and also that the necessary bombarding arrangements were too elaborate and physical in character. They therefore turned away from this technique and, even though, on occasion, the findings of target analysis were relevant, they were never noticed, or, if noticed, not mentioned.

In addition to this decision to seek more standard genetic and biochemical methods of study, a second factor operated: the factor of repair of damaged DNA. The most interesting aspect of a virus to study, at least initially, is its infectivity and since most cells have a mechanism that utilizes the opposite strand to provide the information to permit the removal of damage and replacement in the correct way, damage to only one strand of DNA is not expressed as a loss of infectivity. Thus the early work, and in particular that of Watson, was pointing the way to the existence of DNA repair, even before the structure of DNA was known. Target analysis of RNA and single-stranded DNA gave very much closer estimates of molecular size.

Some Results of Target Analysis on Viruses

These require some knowledge of radiation statistics and below we give a brief statement of the applicable material.

As illustration of the application of target analysis to virus studies we give two examples. The first is for T1 bacteriophage and the second for tobacco mosaic virus. In the first case a considerable number of properties have been studied and an intercomparison is possible. It is, however, a double-stranded DNA virus and so host cell modification of the damaged DNA is possible and hence the target size for infectivity does not correspond to the DNA mass measured biochemically. In the second case, only two properties have been studied, but since the virus is an RNA virus, the observation of infectivity permits the deduction of a target size that is in accord with the biochemically observed molecule.*

*Radiation Statistics. In solid material one primary ionization (p.i.) average 60 electron volts (e.v.).

One rad is 100 ergs/g of absorber, which is 1.04×10^{12} p.i. per gram of solid.

If a sensitive target has a molecular weight M and is inactivated by 1 p.i., then if the average number of p.i. per target is x, the probability of escape is e^{-x} and if n/n_o is the ratio of survivors to original number $n/n_o = e^{-x}$. The actual mass of the target is $M/6.02 \times 10^{23}$ g and for a radiation dose of D rads there are 1.04×10^{13} D p.i. per gram. So, $x = 1.04 \times 10^{12} DM/6.02 \times 10^{23} = DM/5.8 \times 10^{11}$.

When $x = 1$, $n/n_o = e^{-1} = 0.37$. The dose to cause this reduction is denoted D_{37}. We thus get a commonly used relation: $D_{37} \times M = 5.8 \times 10^{11}$.

Essentially, the tabulated values are derived from this relation.

TABLE I. Examples of Target Analysis Applied to Viruses

Virus	Property	Target molecular weight		Reference
T1 phage	Infectivity	1.5×10^6	(whole DNA 4.2×10^7)	Pollard [1959]
	Crossreactivation of genetic markers	0.9×10^6		Till and Pollard [1958]
	Development of protein	0.66×10^6		Whitmore and Pollard [1958]
	Bacterial killing	5×10^5	Fluke and Pollard [1958]	
	Ability to lyse the host	5.5×10^5		Pollard and Woodyatt, see Pollard [1973]
	Ability to elicit antibodies in rabbits	2×10^5		Pollard and Helen Yeisley, see Yeisley [1966]
	Attachment to host	2×10^5		Pollard and J.K. Setlow [1956]
	Ability to combine with antibody	1.5×10^4		Pollard and J.K. Setlow [1954]
TMV	Infectivity	2.5×10^6		Pollard [1959]
	Antiserum combination	3×10^4		Pollard [1959]

Some comments on the figures given in Table I are in order. First, the ability to renovate the double-stranded phage after considerable radiation is reflected in the low target molecular weight for the first five items, all of which require the irradiated DNA to develop in the cell, meaning that the repair processes also act upon it. The relative values are significant, however, and it can be seen that the target for cross-reactivation and for making the protein that will combine with antibody together add up to the size of the target for infectivity. Also, the targets for bacterial killing and for causing the cell to lyse are nearly the same, suggesting that the killing is associated with cell lysis. It is also interesting that the target size for the ability to elicit antibodies and the ability to attach give the same molecular weight. Since the assay for the neutralization of infectivity by antibody attachment almost certainly involves preventing attachment, it is not surprising that the two are the same. Finally, the ability of the virus to combine with antiserum seems to be a different matter from the prevention of attachment and suggests that a considerable coating of antibody can occur without preventing the phage from infecting. The protein involved with this combination is evidently much smaller and probably represents the unit that is assembled to make the head.

In the case of the tobacco mosaic virus, only two target molecular weights are available. That for infectivity agrees quite well with the biochemical value and this is mainly for the reason that TMV is an RNA virus and so is single-stranded and not capable of being renovated by reference to the opposite strand. Thus the radiation damage is promptly expressed in the loss of function. The combination between the RNA and the protein that encases it does have some influence on the response to radiation and this is seen if more densely ionizing particles are used to cause

the inactivation. The target for the combination with specific antiserum is small and, again, is related to the size of the units that assemble to make the capsid around the nucleic acid.

Studies of Radiation Effects on Proteins

The bombardment of living things by X-rays was readily conceded to be a potent means of inactivation. The question arose as to the nature of the sensitive material. In the early days, one important component of living cells was seen to be protein. Accordingly, it was of interest to know the nature of radiation action on protein. Knowledge about this was advanced markedly by the work of Lea, Smith, Holmes, and Markham [1944], a very interesting collaborative group, who studied the effect of X-rays on ribonuclease and myosin. Mention has been made of their work and the very surprising finding that these protein molecules responded to radiation as though the whole molecule was rendered inactive by one ionization anywhere within the molecule, permitting target analysis to derive the molecular weight.

This original work was not followed up for a while until a careful examination of the concept of the use of the effect of primary ionization on biological molecular structures was undertaken by members of the Yale University biophysics group.

The suggestion that this kind of work be undertaken was made during a seminar by Forro, and the first study was made with the same bombarding arrangement used for the study on T1 bacteriophage. The two enzymes studied were pepsin and trypsin. The inactivation followed the exponential relationship and the target size was determined in two ways. The first involved the measurement of the sensitive area for bombardment by deuterons of different energy and hence different linear ion density. The sensitive area increases with increasing ion density and by extrapolating, a maximum can be estimated. If r is the radius of a molecule assumed to be spherical, the extrapolated area is πr^2 and r can be found. However the increase in sensitive cross section (S) should follow the relation $S = 1 - e^{-x}$, where x is the average number of primary ionizations within the thickness (4/3)r of the molecule. This is an independent way to measure the thickness. In addition to these studies, the effect of fast electrons was also observed. This gives a direct measure of the sensitive mass. For trypsin, the three studies led to an estimated molecular weight of 32,000, which compared with the value given by Northrop, Kunitz, and Herriott [1948] of 36,000. Deuteron bombardment of pepsin led to the same kind of agreement: target analysis 39,000 versus 36,000 by biochemical studies (Pollard et al., 1951a).

These initial studies suggested that further work might establish the validity of using target analysis to estimate the molecular weight and possibly the size and shape of protein molecules. A considerable series of studies was made, largely summarized in an article by the author, Guild, Setlow, and Hutchinson [1955] and more briefly by the author [1959]. Among these studies were observations on the effect of low-voltage electrons, which showed that the effect observed on the protein was due to ionization and not to excitation to the same degree [Hutchinson, 1954]. From this considerable body of work, two major conclusions were reached. The first is illustrated by a plot of the "radiation molecular weight" versus the otherwise measured molecular weight for a number of molecules. It is shown in Figure 2. Clearly, the radiation target analysis is not misleading. The second major conclusion was that there is an effect of temperature on the radiation sensitivity of protein molecules. This becomes very marked as the temperature is raised toward the level at which thermal inactivation independently of radiation effects is to be expected. Reduction of the temperature causes a diminution in sensitivity of not quite so marked an amount. This temperature effect is discussed later in this volume by Dr. Fluke.

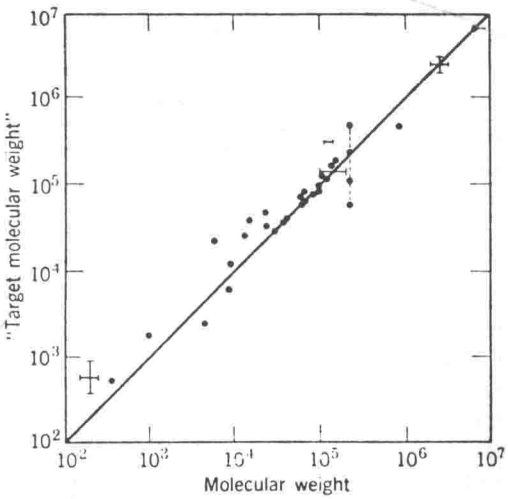

Fig. 2. *A plot comparing the observed molecular weight with the target molecular weight for a wide variety of bombarded substances. This graph has been prepared by Guild and shows the plausibility of the idea that a primary ionization anywhere within the molecule will cause the loss of biological activity. There are exceptions and quite clearly there can be means of influence which will alter the sensitivity of the target, but by and large there is a good relationship.*

Radiation Effects on Nucleic Acids

Early work, in the same area as the above, was undertaken on nucleic acids. At that time these were not understood as to function and one aspect of the action of nucleic acids, that of bacterial transformation, though still in a very imperfect technically understood state, was looked at by Fluke, Drew, and the author. The result was a great surprise and must be considered as an early success of radiation target analysis. The "transforming principle" was found to be inactivated, both by deuterons and fast electrons by remarkably small doses, indicating a large target and hence a large molecule. No consistent biochemically determined value for the molecular weight of transforming principle at that time existed and the figure of 6,500,000 deduced by target analysis seemed to be large. Subsequent studies by Guild, in which the effect of radiation on the ability to transform specific genetic markers was studied, indicate that this large figure is probably to be associated with the entry of the DNA into the cell and not with any individual marker. If individual markers are studied, the target size is found to be much less, around 350,000 [Guild, 1963]. If a different and extreme measure of function is taken, that of the ability to be digested by deoxyribonuclease, the figure found by Smith was 4,000. These various target sizes are to be reconciled by the realization that the DNA molecule is large and if some function requires it to be intact, then the radiation target is large, unless some repair mechanism is at work. If, however, only a fraction is needed, then the radiation target size reflects that fact.

Radiation experiments on RNA are, in general, much easier to interpret. Irradiation of plant viruses gives radiation targets that are very close to the required size for the RNA content of the virus.

We can now turn to a second important consideration: the sensitive character of biological function.

The Sensitive Character of Biological Function

Perhaps the most striking illustration of the great sensitivity of biological function is in the great importance that attaches to the knowledge of the exact sequence of molecules of DNA and RNA. A major effort is being mounted to determine as many of these sequences as possible and it would clearly be a matter of indifference if the exact sequence were only somewhat related to the function of the molecule. Because the exact sequence is so important, the action of ionizing radiation in introducing a break, or even a base modification in the chain of a large molecule of nucleic acid, is bound to have drastic consequences. Therefore, it is safe to assert that unless the biological system has a repair mechanism, the effect of ionizing radiation on a nucleic acid molecule will be to remove its functionality. If this is readily measured, then