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INACTIVATION OF POLIOVIRUS BY FORMALDEHYDE

Theoretical and Practical Aspects

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SYNOPSIS

Since formaldehyde, either alone or in combination with other inactivating agents, is at present used in the production of all so-called " killed " poliovirus vaccines, a thorough knowledge of the kinetics of the reaction between the chemical agent and the virus, and of the mechanisms involved, is of great practical importance. In this paper the problem is discussed against the background of present knowledge of the structure of the virus and the chemical nature of the action of formaldehyde.

Structure of the Virus

The picture emerging as a result of studies of the ultrastructure of viruses in general is, in the main, a uniform one. Nucleic acid and protein have been identified as essential components of all viruses so far studied. Irrespective of whether the nucleic acid is of the ribose or the desoxyribose type, it seems to be localized to the interior of the virus particle, in the spherical or near-spherical viruses forming a sort of nucleus, covered by a coat of protein. The polioviruses do not seem to form exceptions to this rule. According to the analyses of Schwerdt & Schaffer [11] they contain 22%-30% ribonucleic acid (RNA), the remainder of the particle being presumably made up of protein. Taylor and McCormick [12] have shown the virus to consist of a central part more opaque to an electron beam and mechanically more resistant, surrounded by an electron-transmitting, more plastic and apparently hydrated peripheral layer. The electron optical properties and the size of the central nucleus make its identity with Schwerdt & Schaffer's nucleic acid component highly probable.

The experiments by Gierer & Schramm [4] on the tobacco mosaic virus, now repeated by others on certain animal viruses, indicate clearly that the capacity of inciting infection and virus multiplication resides exclusively in the virus nucleic acid, whereas the antigenic properties of the virus seem to be determined by the protein alone. So far no antibodies reacting with

the nucleic acid have been demonstrable. The free nucleic acid is infective, although proportionately much less so than the intact virus. It is also much less resistant to the action of enzymes and other inactivating agents. It would thus seem that the virus protein serves a double purpose: it affords protection of the labile nucleic acid against external inactivating factors and it provides a mechanism (receptors) whereby the virus is more easily introduced into the host cell.

Nature of Formaldehyde Reactions with Organic Substances

Formaldehyde is a typical tanning-agent and knowledge of its reactions with proteins is therefore of great practical importance. Furthermore, since its introduction by Sorensen as an aid in the quantitative determination of amino acids, the kinetics of formol reactions have become a matter of great theoretical significance. As a result an enormous amount of work has been devoted to studies of the action of formaldehyde on proteins. A review of this field of research is presented in volume 2 of *Advances in Protein Chemistry*.[a]

The picture presented in these studies is extremely complex and, in spite of the amount of work done, is still incomplete. Apparently, amino and imino groups are the most reactive, but in addition a number of other groups, such as peptide linkages, ring structures, sulfhydryl and hydroxyl groups, react more or less easily. Most of these reactions seem to proceed in two or more stages, the initial stage being fully reversible, while secondary, irreversible reactions take place later. Thus, an equilibrium between free and reversibly bound formaldehyde is established initially, to be followed by a gradual slow disappearance of chemically demonstrable formaldehyde. The secondary reactions consist largely in the formation of methylene or polymethylene bridges between two reactive sites. In this way new ring structures may emerge, side chains may be firmly tied together, or bridges between adjacent protein molecules may be formed. The over-all effect is thus a firmer, less flexible, and a denser, less permeable, structure of the proteins which is the basis of the tanning action. By the gradual saturation of highly charged and reactive groups the protein is also rendered less soluble and chemically more inert.

For the chemical identification of the various stages in these gradually proceeding reactions, a number of analytical methods have been devised. The aim has been to find techniques by which a differentiation between free and reversibly bound formaldehyde could be achieved. In this connexion the reversible nature of the reactions presents an obvious difficulty. Any chemical interference with the highly labile system is apt to change the delicate equilibrium, and thus to a certain extent to distort the picture.

[a] Anson, M. L. & Edsall, J. T., ed. (1945) *Advances in protein chemistry*, New York, vol. 2

The so-called " gas diffusion " methods seem to be the least harmful in this respect. At the same time, however, they are the least accurate. In order to avoid these difficulties we have devised a microbiological method of assay of free formaldehyde that seems to be sufficiently accurate and sensitive for the purpose, and which will be described later.

The reactions between formaldehyde and non-proteinaceous organic substances, specifically nucleic acids, has hardly been studied at all. *A priori* it can be assumed that reactions with the nitrogen-containing bases as well as with the sugars of the nucleic acids may be possible. Apparently, however, the affinity of the chemical agent to these sites is comparatively low. Further comprehensive and systematic studies of this question are needed.

Theoretical Aspects of Formaldehyde Inactivation of Viruses

Against the background of what has just been said, it is obvious that formaldehyde inactivation of viruses must be an extremely complicated process. The chemical agent may be expected principally to exert two different types of effect. Destruction of the capacity of multiplication, a true inactivation, must obviously include some irreversible chemical alteration of the nucleic acid. On the other hand, a reaction exclusively or mainly with the protein coat, without virtually rendering the nucleic acid inactive, may well be assumed to have an effect upon the infectivity. Receptor sites may be chemically changed and the virus particle therefore less readily absorbed by the cell; or the firm structure and the chemical inertia of the tanned protein may interfere with the release of the nucleic acid, once the virus has entered the cell.

The last-mentioned type of reaction, proceeding mainly on the surface of the virus particle, may be expected to run the course of a first-order reaction as long as the medium contains an excess of free formaldehyde. It need not necessarily be a first-order reaction, however. In fact, if several chemical groups with different specific inactivation rates exist, functionally interchangeable and unequally distributed over the virus surface as well as between virus particles, the composite reaction rate may slow down as the more easily inactivated groups disappear and, consequently, more resistant ones predominate. Such a mechanism appears less likely, although it cannot be *a priori* excluded.

The first-mentioned effect of formol treatment, a reaction localized to essential sites in the nucleic acid component of the virus, includes of necessity a penetration of the agent through the protein coat into the interior of the virus particle. Theoretically, such a process could assume a first-order type only if the peripheral layer of protein did not in any way interact with the inactivating agent. Such is, of course, not the present situation. The

penetration of formaldehyde into the interior may be visualized in either one or both of two different ways: (*a*) as a diffusion of formaldehyde through the hydrated protein, which would be sterically possible on account of the small size of the formaldehyde molecule, or (*b*) it may proceed step-wise from one reversible reaction to another, amino groups most probably serving as steps of the ladder on which the chemical agent climbs from the surface towards the interior. As secondary reactions take place in the protein, both these routes will be gradually blocked. The growing density of the protein, by bridge and secondary ring formation, will increase the sterical hindrance to free diffusion; by slowly proceeding irreversible reactions the ladder steps of the second type of penetration will be gradually obliterated. Therefore, if a virus particle has escaped inactivation at an early stage of the process, it will grow increasingly resistant to it. It is thus to be expected that inactivation of the nucleic acid will proceed at a continuously decreasing rate. On the assumption that the reaction between formaldehyde and the virus protein is of the first order, the problem of the kinetics of nucleic acid inactivation can be treated mathematically (Gard [2]).

On the assumptions just made, formaldehyde inactivation of viruses in general would take mainly the same course. As few, if any, chemical agents are sufficiently specific to react with the nucleic acid exclusively, leaving the protein intact, the present considerations may be universally applicable to all chemical inactivation of viruses, in which case each chemical agent would probably be characterized by its specific reaction constant. Exceptions to the general rule might be expected in cases where the receptors are indispensable in the mechanism of infection and are at the same time destroyed by formaldehyde. In such cases, and in such cases only, a first-order reaction can be anticipated.

Experimental Results

Salk [9, 10] and later also the United States Technical Committee on Poliomyelitis Vaccine have claimed that formaldehyde inactivation of polioviruses follows the course of a first-order reaction, i.e., that " inactivation data of manufacturers in Australia, Belgium, Canada, Denmark, England, Germany, Israel, South Africa, and the USA show beyond a doubt that the inactivation rate of poliovirus, as established by Salk, is, for practical purposes, sufficiently approximate to a straight line on a semilog plot for the time of inactivation of a pool in which all virus particles are accessible to formaldehyde to be predictable from the slope of the line ".

Results that were not in agreement with this concept of the reaction were obtained in 1954 (Gard [1]) by a Swedish group, who reported observations indicating a reproducible and systematic deviation from the linear course; such observations have since been repeatedly confirmed in system-

atic studies on the kinetics of the reaction (Gard; [2] Wesslén et al.; [14] Lycke, Melén & Wrange; [8] Lycke[7]). The inactivation curve could be adequately described by a comparatively simple empirical formula, which in turn was found to be a very close approximation to the theoretical equation previously mentioned (Gard [2]).

This controversy has not yet been fully resolved. It should first be mentioned that the sweeping statement concerning the experiences of the various manufacturers made by Bodian on behalf of the US Technical Committee on Poliomyelitis Vaccine at the Fourth International Poliomyelitis Conference (Geneva, 1957) can hardly be considered entirely correct. Up to the present time only two groups actively engaged in production have published reports on inactivation (Timm et al.; [13] Haas et al.[5]). Both these reports stress the fact that the inactivation curves deviate systematically from a straight line. Furthermore, Lépine,[6] in reporting the French experiences to the Third Symposium of the European Association against Poliomyelitis in Zurich, could not accept the official American version. Finally, according to personal communications by Nagler, by Goffe and by Goldblum, the observations made at the Connaught Laboratories, the Burroughs Wellcome Laboratories, and in Israel have failed to confirm Salk's results. It may be added that the data of Haas et al.[5] are excellently fitted by the empirical formula developed to describe the Swedish observations [3] and the same appears to hold also for the Burroughs Wellcome data.

The explanation of the discrepancies in results is most probably to be found in some technical differences in experimental conditions or in methods of assay of remaining activity. All those who claim to have found deviations from a first-order reaction seem to have kept tissue cultures used for assay of virus activity for at least two weeks, and the Swedish workers, at least, have in addition confirmed all crucial results by subculturing and typing. According to a recent personal communication by Salk this was not done in the tests on which his claims and those of the US Technical Committee were based. Instead, the final readings in all titrations were taken on the sixth or seventh day after inoculation. This is an extremely important difference, which probably provides the clue to the riddle. Swedish observations, as yet unpublished, have shown that the time needed for production of cytopathogenic changes increases progressively with the period of formol treatment of the virus. At the survival level of 10^{-6} to 10^{-7} it is not uncommon to find no trace of degeneration of the cultures after 7 days and almost 100% after 14 days—a finding which is never observed in cultures inoculated with limiting dilutions of untreated virus. This phenomenon may be considered to be an expression of the action of formaldehyde on the surface of the virus particle as previously discussed. At least it indicates that changes may occur in the virus apart from outright inactivation. Readings taken on the sixth day may well measure the residual,

virtually intact virus, and thus, according to the above reasoning, follow a course that coincides with or approximates to that of a first-order reaction.

It goes without saying that the surface reactions, as presumably recorded by Salk, although of a certain theoretical interest, are nevertheless of very limited practical significance, except from a technical point of view. In particular, it seems hardly advisable to devise a procedure for vaccine production on the basis of observations referring to the surface reactions only.

To sum up, available evidence appears to indicate that the virus—as expected from theoretical considerations—is affected by exposure to formaldehyde in at least two respects: (*a*) what might be termed the avidity of the virus is diminished, presumably as a result of chemical changes in the virus protein: (*b*) an increasing fraction of the virus particles is inactivated in the true sense of the word. While in an individual virus particle the former effect may develop gradually, the latter has to be considered as an " all or none " phenomenon.

Formaldehyde Concentration

Obviously there is not much point in talking about a type or a rate of reaction unless the reaction conditions are defined and constant. In the present system such factors as pH, temperature and ionic composition are easily controlled. The content of organic matter, however, is less easily standardized.

The presence in the tissue-culture fluid of amino acids, protein-degradation products and cell debris introduces specific problems. Non-viral matter of this nature reacts with formaldehyde, reversibly as well as irreversibly, thus interfering with the inactivation of the virus. The reversible reactions, without actually depriving the system of reactive formaldehyde, might nevertheless be expected to affect the rate of inactivation by lowering the concentration of free formaldehyde in the mixture. This would, however, mainly serve to stabilize the system, reversibly bound formaldehyde forming a reservoir from which possible losses could be replaced and the equilibrium thus maintained. On the other hand, the effect upon the principal course of the reaction would be negligible, i.e., the reaction formula would be the same, although the numerical values of its constants might be changed. The irreversible reactions would consume the formaldehyde. As such processes proceeded, gradual deviations from the theoretical course would have to be expected.

The difficulties encountered by commercial producers in obtaining consistently uniform results were interpreted by Salk and the US Technical Committee as evidence of the presence in the tissue-culture material of aggregates of virus particles or the coating of virus particles by debris and other material derived from the cell, affording a protection against the action

of formaldehyde and thus causing a deviation from the presumed linear course of the reaction. As a remedy, interposed filtrations were recommended and corresponding amendments were introduced into the Minimum Requirements. This procedure is at present employed by most producers, not only in the USA, but elsewhere. Depending upon the type of filters used, this technique seems to yield preparations which more consistently pass the prescribed safety tests, but at a price: in the filtration process a considerable fraction of the antigenically active material is lost. Types 3, 1, and 2 are affected in that order.

It should be emphasized that no experimental or other significant evidence of the validity of the above interpretation of the observations has so far been presented. It may now be considered an established fact that inactivation is not a first-order reaction and that its course for that reason alone must deviate from linearity. That aggregate formation or other types of inhomogeneity are not responsible for this deviation has been convincingly shown by Lycke.[7] The inability of the commercial producers to achieve satisfactory results when following the original Salk procedure, which prescribed a holding-time of only seven days, can easily be explained on this basis alone. The expected survival ratio at this time is of the order of 10^{-11}. With an initial activity of $10^{7.5}$ ID_{50}/ml, the expected content of active virus would thus be approximately one ID_{50} per 3 litres and thus well within the range of demonstrability with the present safety tests. The positive results obtained after holding-times of up to 14 days (37°C, 1:4000 formalin) can, however, hardly be accounted for in this way.

Since in our laboratories it was on some occasions observed that inactivation, after having followed the theoretical course down to a survival ratio of about 10^{-8}, was slowing down significantly and since, according to the argument presented above, such a phenomenon might be expected, if a loss of formaldehyde occurred, attempts were made to study the possible variations in the concentration of free formaldehyde in the course of the inactivation process. The various chemical methods of analysis of " free " formaldehyde tested all indicated a decrease in the concentration as the reaction proceeded. The numerical values obtained differed according to the methods used, and the reproducibility in duplicate tests was not quite satisfactory. Attempts were therefore made to develop a microbiological method of assay, more sensitive than the chemical methods and by which the particular quality on which information was desired could be assayed, namely, the capacity to inactivate viruses. Bacteriophage, easily and accurately assayable by plaque count, seemed to be an ideal test object.

A *Staphylococcus* phage giving plaques of a uniform size was chosen for the purpose. In inactivation experiments it was found to conform to the pattern set by the polioviruses. During the first hours, however, to a survival ratio of about 10^{-3}, the course of inactivation approximated sufficiently to a straight line to make this interval suitable for assay purposes.

It was found that the rate of inactivation in this interval was proportional to the concentration of formaldehyde added, and the method of assay finally adopted was therefore as follows.

To 19 parts of the test material, one part of a concentrated and partially purified phage was added. The reaction mixture was adjusted to pH 7 and kept at 37°C for 3 hours. After addition of bisulfite, tenfold dilutions were prepared and counts of surviving phage were made. Of each dilution 12 drops of 0.01 ml were administered by drop pipettes to *Staphylococcus*-seeded plates, which were incubated for 4 hours at 37°C and overnight at room temperature. Plaque counts were recorded for each drop separately and the variation within each dilution was checked. As controls, phage plus tissue-culture fluid alone and phage plus tissue-culture fluid with added formaldehyde were included. The relative formaldehyde concentration was recorded as the log difference between phage control and test sample. Comments: (*a*) The presence of antibiotics in the tissue-culture fluid makes dilutions of less than 10^{-4} unsuitable or unreliable for counting; hence the use of concentrated phage preparations. (*b*) In order not to upset the formaldehyde equilibrium the volume of phage as well as the amount of organic matter added should be proportionately insignificant; hence the use of purified material. (*c*) The most accurate results are obtained with counts of 20-50 plaques per drop. Half-log steps of dilution may be included for achievement of this purpose. Under such conditions the standard deviation was found to be 0.03 log units.

With the aid of this technique the variation in formaldehyde activity was studied. A slow decrease in the course of inactivation was regularly observed. At 25°C and 0.006 M formaldehyde, this was first demonstrable about the fifth day, slowly increasing to a maximum reached after two to three weeks and amounting to 15%-30% of the original activity. At 37°C and 0.003 M formaldehyde (the Salk technique), changes appeared earlier; a significant decrease was observed on the third day, the final level was reached on the eighth day and the loss amounted to up to 45%.

These observations seem to provide a satisfactory explanation of the irregularities met with in routine production. At least, the Stockholm findings can be fully explained on this basis. The effect of filtration also fits into the picture. This procedure may be expected to affect the results in two ways: (*a*) by retention of virus, active as well as inactivated, as previously mentioned; (*b*) by removing from the reaction mixture tissue-debris and possibly precipitated non-viral organic matter and absorbing molecularly dispersed amino acids and proteins, and thus decreasing the reduction in formaldehyde activity that would otherwise be expected later in the process. The procedure, outlined in the US Minimum Requirements and now adopted by the majority of producers,[a] includes two filtrations,

[a] At least two manufacturers outside the USA use sintered-glass filters instead of filters of the Seitz-pad type prescribed in the Minimum Requirements, reportedly with satisfactory results.

one before the addition of formaldehyde and the other after the inactivation curve has reached the " base line ". This technique seems to have been efficient in reducing the proportion of vaccine batches failing to pass the safety tests. On the other hand, filtration is associated with considerable losses in antigenicity. Thus, the problem is no longer that of the safety of the vaccines, but of their potency.

Since the inconsistencies in results of inactivation seem to arise in variations in the amount of formaldehyde consumed in the process and the rate at which its concentration is reduced, the most rational way to overcome the difficulties appears to be through continuous control and maintenance of a constant level of formaldehyde activity by supplementary addition of the agent as need arises. The need for an intermediate filtration is thus eliminated. This procedure has been tried in our laboratories in several small-scale experiments as well as in a limited number of full-scale vaccine batches (each batch 6×15 litres). Since its introduction, no deviations from the theoretical course of inactivation have been demonstrable. Thus, complications due to possible physical inhomogeneity of the virus, such as aggregation or coating, have not been encountered. Although the preliminary results are promising, with regard to consistency of inactivation as well as the immunogenic capacity of the product, further experience is needed before a final judgement can be passed.

RÉSUMÉ

Les virus poliomyélitiques destinés à la préparation de vaccins dits « tués » sont inactivés par la formaldéhyde seule ou associée à d'autres inactivants. Le problème de l'inactivation est envisagé dans cet article, à la lumière des connaissances sur la constitution des virus et de la cinétique de leurs réactions avec la formaldéhyde. L'acide nucléique — dans le cas particulier l'acide ribonucléique — formant le centre de la particule de virus est porteur des propriétés infectieuses et reproductrices. Une enveloppe de protéines l'entoure, qui semble le protéger contre les facteurs inactivants, et porter des « récepteurs » facilitant l'accès du virus à la cellule-hôte. L'acide nucléique libre est infectant, mais beaucoup moins que le virus intact. La formaldéhyde est un agent tannant et ses réactions avec les protéines ont un intérêt général. D'autre part, la cinétique de la réaction entre la formaldéhyde et les protéines — utilisée dans la détermination quantitative des acides aminés — a fait l'objet de nombreuses études. La plupart des réactions d'inactivation semblent s'effectuer en deux étapes au moins; au premier stade, elles sont entièrement réversibles, au second, elles deviennent irréversibles. Au début, un équilibre s'établit entre la formaldéhyde libre et la formaldéhyde réversiblement fixée; ensuite, la formaldéhyde cesse d'être chimiquement décelable. L'effet final est une modification de la structure des protéines, rendues moins solubles et chimiquement plus inertes.

Salk, et plus tard la Commission des Etats-Unis pour l'étude du vaccin poliomyélitique, ont affirmé que l'inactivation du virus par la formaldéhyde était une réaction du premier degré et que le taux d'inactivation, pour les besoins de la pratique, se rapprochait assez d'une droite sur l'échelle semi-logarithmique, pour que le temps d'inactivation d'un mélange de virus dont toutes les particules sont en contact avec la formaldéhyde, puisse être déterminé d'après la pente de la courbe.

Un groupe d'expérimentateurs, parmi lesquels l'auteur de cette étude, ont obtenu en 1954 des résultats différents et observé systématiquement des déviations de cette droite. Depuis lors, plusieurs rapports ont été publiés dans le même sens, par d'autres chercheurs. L'auteur estime que ces résultats divergents peuvent s'expliquer en partie par la diversité des méthodes appliquées pour évaluer l'activité résiduelle des lots de vaccins inactivés. Alors que les chercheurs américains lisent les résultats 6-7 jours après l'inoculation de la culture de tissu, les chercheurs suédois ont montré que le temps nécessaire à l'apparition des manifestations cytopathogéniques augmente progressivement avec la durée du traitement par la formaldéhyde. Au niveau de survie 10^{-6} à 10^{-7}, il n'est pas rare en effet qu'une culture inoculée ne présente aucune trace de dégénérescence après 7 jours, mais qu'elle soit atteinte à environ 100% après 14 jours. Ce fait ne se produit pas avec des dilutions limites de cultures de virus non traité.

Les résultats inconstants obtenus par les fabricants de vaccin ont été expliqués par Salk et la Commission américaine par la présence dans le matériel d'agrégats de particules et de débris cellulaires enrobant les virus et empêchant l'action de la formaldéhyde, ce qui provoquerait une déviation de la linéarité. (Pour y obvier, ils ont prescrit la filtration du matériel). Aucune preuve expérimentale cependant n'est venue confirmer l'hypothèse selon laquelle l'hétérogénéité serait la cause de la déviation. Il a été démontré au contraire qu'elle ne l'est pas. On peut considérer comme un fait établi que l'inactivation n'est pas une réaction du premier degré et que, par conséquent, elle ne peut être représentée par une droite.

L'auteur a cherché à étudier les variations éventuelles de la concentration en formaldéhyde libre au cours du processus d'inactivation, afin d'expliquer le fait suivant, observé dans ses laboratoires: l'inactivation, après avoir suivi le cours prévu par la théorie jusqu'au taux de survie de 10^{-8}, se ralentit de façon significative, ce qui semble indiquer une perte en formaldéhyde. Pour résoudre cette question, l'auteur a mis au point une méthode microbiologique — qu'il décrit dans cet article — permettant de suivre l'inactivation du virus par la formaldéhyde de façon plus précise que par les méthodes chimiques. Cette méthode consiste à soumettre à l'action de la formaldéhyde des phages staphylococciques, qui présentent de nombreuses analogies avec le virus poliomyélitique. Les résultats indiquent de façon constante que le processus d'inactivation diminue graduellement. A 37° C et à une concentration de formaldéhyde de 0,003 M (technique de Salk) le ralentissement est sensible dès le 3e jour, et le niveau final (correspondant à une perte allant jusqu'à 45%) est atteint le 8e jour. Ces faits peuvent expliquer les résultats irréguliers observés dans la production courante du vaccin, ainsi que l'amélioration des résultats des tests d'innocuité à la suite de la filtration. Celle-ci, en éliminant les substances susceptibles de fixer la formaldéhyde, diminue les pertes et prolonge l'action inactivante. Les résultats inconstants de l'inactivation paraissent donc dus à la variation de la quantité de formaldéhyde consommée et de la vitesse à laquelle sa concentration diminue. Il doit être possible, par conséquent, d'y remédier en ajoutant de la formaldéhyde au fur et à mesure des besoins, afin de maintenir une concentration constante. Des essais, expérimentaux puis semi-industriels, ont justifié cette hypothèse: la courbe d'inactivation — exprimée par une formule empirique simple — n'a jamais présenté de déviation et l'hétérogénéité n'a nullement affecté les résultats. Cette méthode paraît prometteuse, tant au point de vue de la régularité de l'inactivation que de l'antigénicité du vaccin. Les expériences doivent être cependant poursuivies, avant qu'elle soit pleinement agréée.

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