American Journal of

# PUBLIC HEALTH

Official Monthly Publication of the American Public Health Association, Inc.

Volume 44 May, 1954 Number 5

# Formaldehyde Treatment and Safety Testing of Experimental Poliomyelitis Vaccines

JONAS E. SALK, M.D., F.A.P.H.A.; ULRICH KRECH, M.D.; J. S. YOUNGNER, Sc.D.; MAJOR BYRON L. BENNETT (Ret.); L. J. LEWIS, Ph.D.; and P. L. BAZELEY, M.D.

***What will Journal readers find of use to them in this elaboration of the methods employed in large-scale production of a safe and effective vaccine against poliomyelitis when only a tiny fraction will be technically competent, or equipped, to confirm the validity of the described procedures? The question is rhetorical. The self-evident answer is that all will want the assurance of safety of the vaccine that the details of this report make unquestionable***

✢ The precise details of the methods which have been developed for destroying the infectivity of poliomyelitis virus by formaldehyde in the preparation of an experimental vaccine, and for safety testing, as yet have not been published. It is the purpose of this communication to discuss the principles underlying the procedures being followed in preparing material for more extensive studies than have been carried out thus far; essentially, this represents a discussion and an elaboration of the specifications which have been prepared for processing the vaccine for this purpose. A fuller presentation and documentation of details here referred to will be covered in several reports to be made in the appropriate technical journals.

## Principles of Formaldehyde Treatment for Inactivation

As with other antigens, interaction of formaldehyde with poliomyelitis virus, resulting in destruction of infectivity, occurs in accordance with the laws that govern a first order chemical reaction. This means that if conditions are constant throughout the reaction period, the rate, measured on a logarithmic scale, at which virus infectivity is destroyed, is constant. For this reason it is possible to prepare a poliomyelitis

The authors are associated with the Virus Research Laboratory, School of Medicine, University of Pittsburgh, Pittsburgh, Pa. This work was aided by a grant from the National Foundation for Infantile Paralysis.

vaccine for test that may be said to be free of infectious particles; more than that, it is possible to define the extent of what may be referred to as the "margin of safety" of each batch of such vaccine. Thus the vaccines with which we are concerned are treated in a way that will destroy the infectivity not only of the last virus particle that can be measured, but will destroy the infectivity of many more additional particles that might be postulated to be present and not measurable.

The variables that influence the time required for destruction of infectivity are: (1) concentration of free formaldehyde, (2) the temperature at which the reaction is allowed to take place, (3) the pH of the reaction mixture, and (4) the concentration of virus initially present. It is evident that prediction of the precise time required for the destruction of infectivity of the last virus particle to be so altered depends upon the precise duplication of all four conditions. If any one of the four conditions varies from time-to-time, then the interval required for inactivation will also vary. Until the dynamics of the formaldehyde inactivation procedure for tissue culture fluids containing poliomyelitis virus were worked out to the extent now known, the time required for destruction of infectivity of each of the preparations used in the initial studies in man was established empirically.

Although the influence of the four factors mentioned is well known, they have not always been accorded adequate attention by all who have attempted to use formaldehyde for the preparation of noninfectious vaccines. Careful study of these variables has permitted the establishment of a procedure for handling fluids containing the poliomyelitis virus in a way which will destroy infectivity and yet allow maximal retention of antigenic activity. The objective is to produce material that will have the greatest chance for being effective without sacrificing to any degree whatever the primary prerequisite which is safety from the viewpoint of the absence of infectious virus particles.

## Conditions Selected for Inactivation of Virus by Formaldehyde

Without presenting the details of the experimental basis for arriving at the conditions being used for the preparation of vaccine for large-scale testing, we would like to emphasize that the selection of the particular conditions which were adopted was influenced by considerations not only of safety and of potential effectiveness, but of the practical adaptation of the procedure to mass production. If at all possible, it would be desirable that the procedure should not require special equipment; the conditions should be such that the time required for handling should not be so critical as to be impractical; furthermore, the reaction should be slow enough so that overinactivation would not be excessively deleterious upon antigenic activity; and the kind and amount of safety testing should be reasonable.

The conditions that have been selected have been based upon experience with free formaldehyde in concentration provided by (1) a 1:4,000 formalin solution (this is equivalent to a final concentration of formaldehyde of 0.009 per cent based upon a formaldehyde content of formalin of 37 per cent), (2) temperature of 36°–37° C., and (3) pH 7. If these conditions are kept constant, then the time required for destruction of infectivity might vary, depending upon the concentration of virus in the starting material, i.e., the higher the concentration, the longer will be the exposure time necessary to destroy infectivity. Also, if protein content or amino acid content varies, either of which will bind free formaldehyde, then

the slope of the reaction-time line will change depending upon the direction of the shift in the amount of free formaldehyde—the greater the concentration, the steeper the line.

Thus, the assumption may be made that if in addition to relative constancy of virus titer and composition of the fluid, temperature and pH are kept constant, then inactivation of virus will occur in approximately the same time on repeated occasions and in different laboratories; this, in fact, has been observed. However when dealing with an agent which, if not destroyed, possesses such potential danger as does the poliomyelitis virus, nothing can be left to chance. There is always the possibility of human error, and one cannot always depend upon the predicted constancy of incubation temperature, or of pH, or of the formaldehyde content of the formalin solution, or of the constancy of composition of the medium, or of the virus titer. Slight variations of each, or of all of these conditions, could be compensated for by treatment of the virus for a longer time under the conditions selected; however, overexposure, if arbitrary, and therefore uncertain, might not be sufficient. Understanding the effect of each of the four variables and having at our disposal simple methods for measuring the rate of decline of infectivity during the period of inactivation, it has been possible to devise a means for controlling the inactivation procedure in a way that provides the greatest assurance of safety and at the same time results in maximal retention of potential effectiveness.

Two other considerations that concern safety and effectiveness should be mentioned. The first is related to the possibility that tissue fragments, or isolated cells, or cell debris, might be present in the suspension and that these might contain entrapped or adsorbed virus that might not be as readily accessible to the formaldehyde as are the virus particles that are free in suspension. The elimination of such extraneous material has been accomplished first by clarification of the fluid through a coarse filter and then passage through a bacteria-retaining filter that permits passage of virus without loss of titer. This serves the twofold purpose of eliminating (1) any tissue or cellular particles and (2) any occult bacteria or molds that might be present and are either slow-growing or are suppressed by virtue of the penicillin and streptomycin contained in these fluids. Finally, there are the prescribed tests for bacterial sterility and for safety required by the Laboratory of Biologics Control of the National Institutes of Health which are applied to eliminate the possibility that any living organisms or toxic agents are present. In addition, tests for other agents that might be derived from the monkey, such as "B" virus and the virus of lymphocytic choriomeningitis are also made.

The other safeguard concerns not safety but rather retention of antigenic activity. Since sufficient time has not elapsed to allow a decision in regard to long-term influence of formaldehyde, in the concentration employed, upon the stability of the antigen at ordinary refrigerator temperatures, it seems desirable, for the time being at least, to continue to remove the excess formaldehyde by the addition of sodium bisulfite. In this way the inactivating influence of the formaldehyde upon the virus is brought to an end by its combination with the bisulfite. This may not be the most effective way to handle the poliomyelitis virus antigen, since it has been found, with certain other antigens, that the continued presence of a low concentration of formaldehyde tends to maintain stability rather than to diminish it; however, until this can be studied in relation to the poliomyelitis virus, it is deemed desirable to

continue to neutralize the excess formaldehyde.

## Procedure for Inactivation of Virus and for Safety Testing

The way in which inactivation of the virus is carried out may be summarized briefly as follows: The fluids harvested from virus-infected cultures of monkey kidney tissue are pooled after an appropriate preliminary test for infectious titer has been made. These are then passed through a filter for removal of larger particles that might interfere with bacteriologic filtration and then through the bacteria-retaining filter pad. Either before or after filtration the free $CO_2$ in solution in the culture fluid, as a result of tissue respiration, is removed by transfer from one container to another, under partial vacuum, in a closed system. This is done in order to permit the more reliable adjustment of the pH so that it will remain constant throughout the inactivation procedure. The pH of $CO_2$-free tissue culture fluid is approximately 8.3–8.4 and is adjusted to 7 by the addition of either 0.1 N hydrochloric acid or 0.1 N acetic acid. A sample of this material is then removed for preliminary test to establish the time required for inactivation of the particular batch. This is done by treating it with formalin and then titrating for residual infectivity in samples removed on each successive 24-hour period for the number of days indicated, or at shorter intervals if necessary.

An illustrative example showing the kind of information that is obtained in this preliminary test for establishing time required for inactivation is shown in Figures 1 and 2. Here, it may be seen that a line, drawn as the best fit between the points representing the infectious titers of the daily samples, has a constant slope. If it is assumed that the straight line relationship observed above the abscissa also exists below, then it may be predicted that virus infectivity would no longer be demonstrable at a certain interval beyond the point at which the abscissa is intercepted. For example, from the data shown in Figure 1 it is evident that only one 50 per cent tissue culture infecting dose/0.5 ml. is present at three days after treatment with 1:4,000 formalin and at five and one-half days after treatment with 1:8,000 formalin. This is based upon the results of titrations involving 0.5 ml. inocula into a sufficient number of culture tubes to provide reliable information. If these

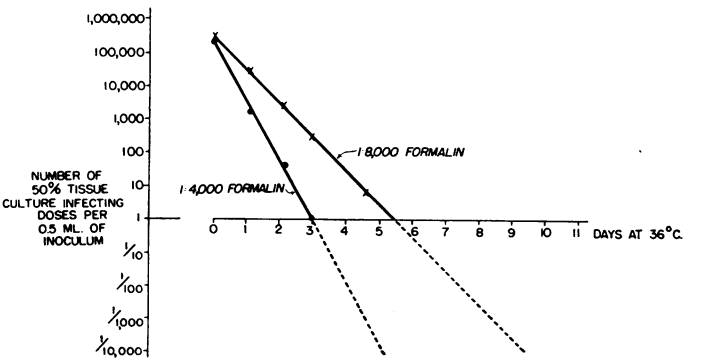


**Figure 1—Rate of Destruction of Infectivity of Poliomyelitis Virus in Tissue Culture Fluid—Treated with Formalin at 36° C. and at pH 7.0**

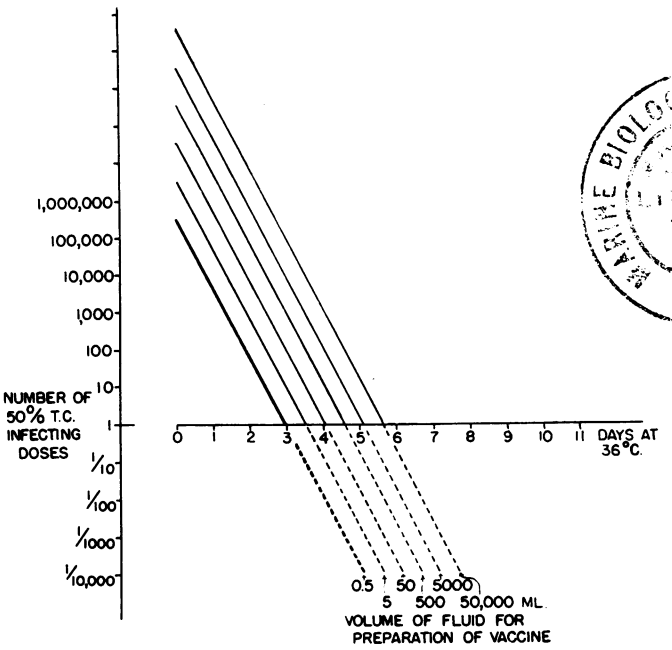


**Figure 2—Influence of Volume of Fluid Treated Upon Time Required for Destruction of Infectivity of Poliomyelitis Virus—by 1:4,000 Formalin at 36° C. and pH 7.0**

results are examined in another way, i.e., in terms of the absolute number of 50 per cent tissue culture infecting doses per volume of a batch, instead of per 0.5 ml., then it becomes apparent (Figure 2) that the time when the number of infectious particles has been reduced to but one 50 per cent infectious unit is influenced by the size of the batch. At the point of interception of the base line, one infectious unit is present in 0.5 ml.; if the size of the batch is 50,000 ml., then there is present, at this time, 100,000 50 per cent infectious units in this volume of material.

In order to destroy all the virus, so that some might not be missed by chance selection of a sample for safety test, the reaction is allowed to proceed beyond the additional time required to destroy all infectious virus in the volume of material being treated. Still further treatment is applied, since the 50 per cent infectious end point is some distance (assumed to be approximately two logarithmic units) from the end point indicating complete absence of infectivity, and since an additional number of infectious particles are assumed to be present to allow for imperfections in the detection-sensitivity of the tissue culture system; with these allowances the inactivation process should be continued to a point where the projected line reaches at least four logarithmic units below the base line, after taking into consideration the volume of fluid involved, as illustrated in Figure 2. It is evident that the length of time required for exposure of a 50,000 ml. batch is greater than that required for a 50 ml. quantity. Under these circumstances the criterion of destruction of infectivity is not merely in terms of the test sample, but in terms.

of the probability of allowing the existence of one live virus particle in any sized batch of material which is to be converted into a vaccine.

In actual practice, the time required for adequate treatment of a 50 liter batch of material is calculated from titration data on 0.5 ml. samples, as shown in Figure 1, and the reaction is allowed to proceed for a total period equal to three times the interval required for interception of the base line. This rule of thumb is in excess of the minimum time required to achieve a degree of inactivation which includes the margin of safety indicated above. The minimum time required for a 50 liter batch depends, in part, on the initial titer, and for different titers, indicating the point of interception of the ordinate, the least amount of time is indicated by the following multiplication factors:

$10^{-5}$ — 2.25 $10^{-6}$ — 2.5 $10^{-7}$ — 2.8

The purpose of the preliminary test just described is to ascertain the time required for complete inactivation under the particular conditions that apply in any given laboratory for any given batch. The large bulk, upon which the preliminary test is made, is then treated for the time indicated by the results of the preliminary test. Titrations of daily samplings for determining the rate of destruction of infectivity are made on the contents of the bulk container during the inactivation process to be certain that the margin of safety calculated was, in fact, obtained. If the principle that applies is followed—namely, that material be treated for the number of days necessary to destroy at least 10,000 more 50 per cent tissue culture infectious doses beyond the point at which the base line is intercepted—then the margin of safety which guarantees absolute safety has been assured.

This procedure should obviate the need for compliance with the suggestion that concentration by ultracentrifugation be employed to exclude the possible presence in a reasonable sample of one living virus particle. The practicable limits for concentration by centrifugation are such that greater assurance of safety is provided by this procedure than would be provided by the centrifugation test. In further support of this view is the fact that a given virus suspension contains a finite number of virus particles; newer methods will determine this number precisely. The last virus particle of the number presumed to be present will be converted to a noninfectious form if the procedure here described is followed correctly.

Final tests for safety, on samples taken from the pool containing a mixture of all three strains, are made by inoculation of roller tube cultures of monkey kidney tissue and by the intracerebral injection of monkeys. The number of cultures employed is 40, each of which is inoculated with 0.5 ml. of formalinized vaccine fluid which has been treated with sodium bisulfite to arrest the action of the formalin and then dialyzed; this involves a test of 20 ml. of fluid representing the trivalent vaccine. In this safety test reliance is not placed entirely upon the appearance of the cells in the cultures inoculated because it has been found, on occasion, that even though cytopathogenic changes are not evident in the first cultures inoculated, subculture will reveal the presence of virus. Such blind subcultures are done twice to be certain that there are at least three serial sets of negative cultures before a fluid is considered free of living virus.

The number of monkeys inoculated intrathalamically is 10 and this involves a test of 10 ml. of a mixture of the three virus types. All monkeys are observed for four weeks and then examined histologically for evidence of nonparalytic polio. Sera of these

animals are tested as a further indication of the presence or absence of a silent infection. In addition, groups of five cynomolgus monkeys are inoculated intramuscularly with 10 ml. divided between four sites to establish safety of vaccine administered by this route. Also, one group of five monkeys is given three doses of 1 ml. each at intervals of one week to establish immunological effectiveness. Blood samples, drawn one week after the third dose, are then titrated for antibody for each virus type. Inoculation by both intracerebral and intramuscular routes are not made in the same animal since the possible immunizing effect of the intramuscular injection may diminish the value of the observation for the effect of the intracerebral inoculation, especially if the incubation period for paralysis induced by the latter is prolonged into the third or fourth week.

While the safety tests are in progress the virus is allowed to remain in contact with the formaldehyde in the cold room (during which time inactivation continues, but at a very slow rate) until all tests indicate that the material contains no infectious virus and possesses an adequate margin of safety. When this has been assured, the excess formaldehyde is neutralized with sodium bisulfite. At the time of the addition of the sodium bisulfite, Thimerosal, N. F. is added as a preservative of sterility. The Thimerosal is added in a concentration not to exceed 1:10,000. After this mercurial compound has been added to the vaccine fluid, tests in tissue culture can no longer be made since this chemical is toxic for the tissue culture and cannot be removed by dialysis.

If for any reason whatever the conditions for inactivation — temperature, pH, or the intended concentration of formalin—were not in the anticipated range for the full period of inactivation, then, depending upon the direction of these deviations, it might be expected that the margin of safety would be inadequate; or, if these deviations are extreme, that living virus could sometimes be present. If this does happen, the particular batch need not be discarded, but may be returned to the incubator for an additional period of time, as indicated by the data on rate of inactivation. All tests would, of course, have to be repeated until they had indicated an adequate margin of safety as well as absolute safety.

## Discussion

Although the procedures here described for virus inactivation and for safety testing of vaccine may seem complicated and to some may seem unnecessary, they do provide a means for knowing when the last virus particle in a particular preparation was converted to the noninfectious form and they provide a means for limiting the amount of overinactivation to guard the antigen against unnecessarily excessive destruction. It should be clear that there is implicit in the method used for inactivation the requirement that infectivity be destroyed. If destruction of infectivity has not taken place, then the process is incomplete and it cannot be said, therefore, that a vaccine has been prepared.

While the investigations dealing with the influence of various factors upon formalin inactivation were in progress, methods for the cultivation of virus became more efficient thereby yielding preparations of higher titer. Whereas the early fluids, of lower titer, could be inactivated at the temperature of melting ice in from seven to 14 days with a 1:250 dilution of formalin, it took approximately three weeks to inactivate the more potent fluids. It was this observation that influenced the change in the conditions adopted for inactivation to the higher temperature of 35°–

37° C. using a lower concentration of formalin (1:4,000 to 1:8,000), requiring approximately one week for inactivation. This change was made for another reason: it now appears that inactivation by the lower concentration of formalin results in better retention of antigenic activity.

Failure in the early phases of these studies to control pH in a constant manner produced curves of inactivation that did not follow the straight line shown in Figure 1; moreover, the slope of the line is influenced by pH. In the earliest studies inactivation was carried out at an uncontrolled pH of approximately 7–7.5; then later it was changed to a fixed pH of 8, and has now been returned to 7, in the region of which slight differences in hydrogen ion concentration are less critical.

The question of reactivation of virus has been raised because the excess formalin in the vaccine is neutralized with sodium bisulfite. In view of the fact that safety tests are done on neutralized and dialyzed samples, it would seem that all of the known safeguards for testing for the occurrence of reactivation of virus are included as part of the procedure for ascertaining the safety of vaccines prepared in the manner described.

In the course of this discussion the assumption is made that there is present in the starting material, before inactivation, a sufficient concentration of virus so that the resulting preparation can be expected to be antigenic. In practice, this is estimated by measuring infectivity titer for tissue culture and only fluids having titers of at least $10^{-5}$, using an 0.1 ml. inoculum, are included in the pool of fluids to be converted into vaccine. Subsequently, the careful control of the inactivation procedure during chemical treatment is designed to protect the antigen that is present initially.

The foregoing provides a guide and merely furnishes presumptive assurance that the resulting preparation will be antigenic. Final assurance is gained from tests for antigenicity in animals and in human subjects. At this early stage of these investigations it is not possible to specify minimal requirements for antigenicity in terms of an animal potency test without having as a point of reference antigenic performance in human subjects. Accordingly, all vaccine prepared in the manner here described is being tested both in small animals and in monkeys and will be tested in children who have no detectable antibody to any of the three immunologic types.

The full extent of the stability of vaccines prepared in this way is not yet known. However, all indications to date are that it is one of the more stabile biological preparations.

---

## Grant to Woman's Medical College

The Woman's Medical College of Pennsylvania, Philadelphia, has received a $30,000 grant from the W. K. Kellogg Foundation to develop a program of teaching and research in preventive medicine. The grant is being paid in annual installments of $10,000. A new Martha Tracy Preventive Medicine Wing is being built to house the department. The director of the department is Katharine R. Boucot, M.D., professor of preventive medicine.