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REPORT ON AN INFORMAL CONSULTATION ON THE POTENCY
 ASSAY OF DIPHTHERIA AND TETANUS TOXOIDS

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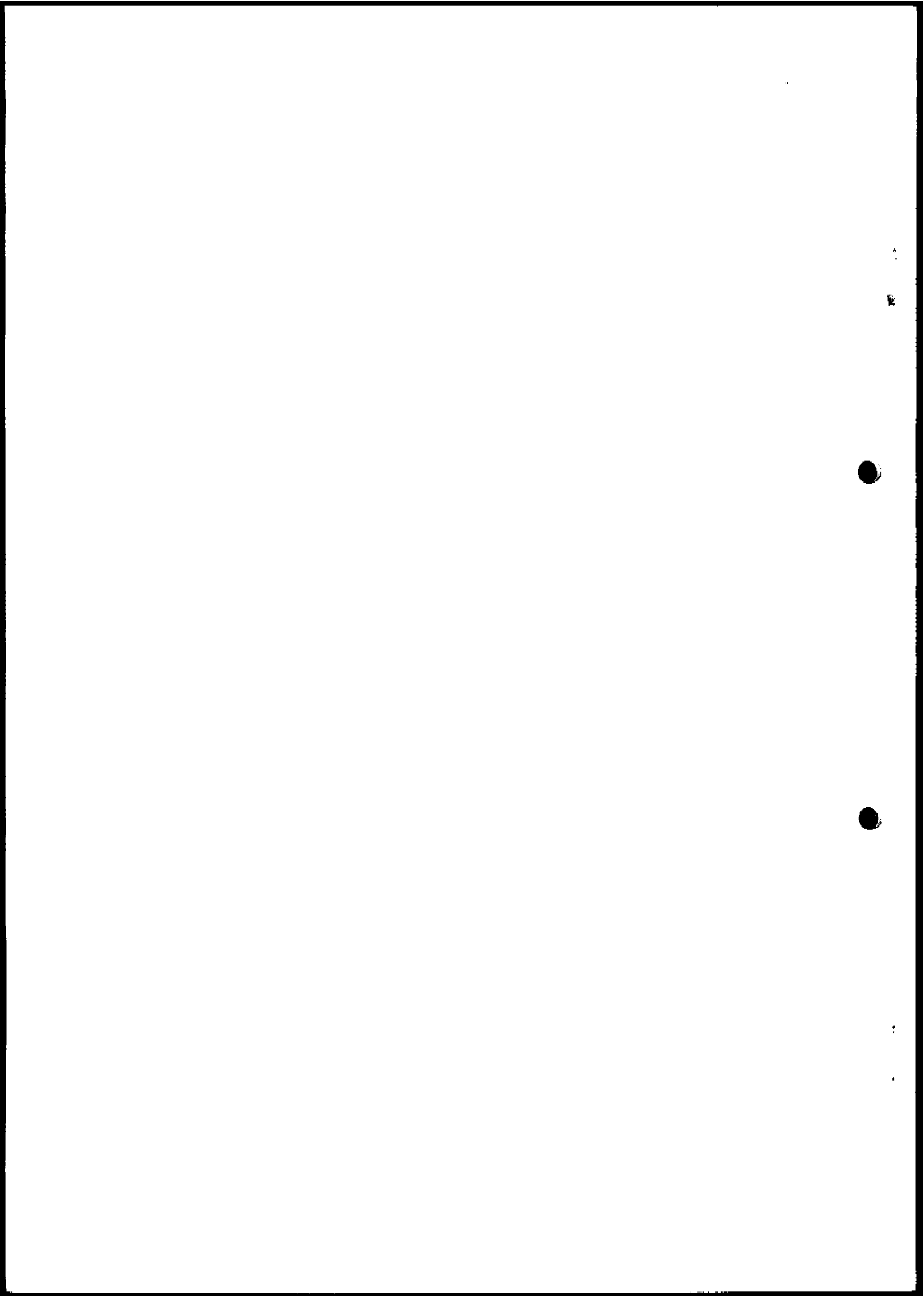
Geneva, 12-14 December 1983

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1. INTRODUCTION

A WHO informal consultation on the assay of potency of diphtheria and tetanus toxoids occurring either as a single adsorbed component or in a combined vaccine took place in Geneva from 12-14 December 1983. The meeting was opened on behalf of the Director-General by Dr F.T. Perkins, Chief, Biologicals who noted that it was important to try to arrive at a test that was less demanding on animals, less costly and yet gave an assurance that the vaccine would protect children against the two diseases. He wished to take this opportunity of thanking the scientists who had given so much of their time to work on these toxoids. A list of participants is shown in Annex I.

For many years the potency assay of diphtheria and tetanus toxoids was achieved by relatively simple methods that varied from one country to another. Some of these methods have relied upon the ability of the toxoids to stimulate the formation of arbitrary levels of antitoxin in the sera of experimental animals; others on assays of doubtful statistical validity were based on the results of challenge with the appropriate toxin. Opportunities to improve on this situation occurred in the late 60's and early 70's and the WHO introduced into its Requirements for Diphtheria and Tetanus Toxoids statistically acceptable assays in which three dilutions of a test preparation are compared with three dilutions of a reference preparation, calibrated in international units (IU), known as a 3+3 assay on the basis of lethal, paralytic or intradermal challenge as appropriate. Assays in this form became routine in those countries in which they were a statutory requirement but were regarded with reserve in those in which they were not.

By the early years of the 80's much information on the application of the 3+3 type of assay had been acquired in many countries and much experience had been gained, but it was becoming apparent that the assays had certain inherent disadvantages. Non-parallelism of dose-response due to dissimilarities between standard preparations and test vaccines, difficulties in estimating the ED₅₀, concern about the use of animals in such large numbers, and the very marked rise in the cost of animals and their husbandry were among these. Furthermore, it was becoming clear that the more elaborate methods of assay were difficult for laboratories other than those with extensive animal premises and computational facilities, and that they did not lead to more effective prevention of either diphtheria or tetanus. It was thus that a situation developed in which there were two basic but opposing approaches to the potency control of toxoids:

1. The older procedure of immunize and then challenge or assay of antibody and thereafter acceptance or rejection of a batch on the basis of an arbitrary number of survivors or an arbitrary mean antitoxin titre, or
2. The newer procedure of a 3+3 (or larger) assay with pass or fail requirement based upon an immunogenicity estimated in I.U.

The older procedure has an excellent record of ensuring vaccine efficacy, is relatively simple to undertake, is not inordinately expensive in either time or animals, but it is statistically indefensible. The newer procedure has a shorter record as a control procedure, is more difficult to perform, is very expensive in animals, requires computational ability but is statistically valid. The problem that arises, therefore, is whether a very arbitrary but readily achieved procedure is preferable to a theoretically sound but costly one.

Although the immunogenicity of a vaccine in an experimental host may not be paralleled exactly by immunogenicity in humans there is no doubt whatsoever that preparations that are protective in animals also protect humans. Thus in the course of a vaccine manufacturing programme a number of at least five consecutive batches of a vaccine shall be shown by means of a 3+3 assay to have adequate immunizing potency in terms of I.U. This demonstration provides both evidence of production consistency of potent vaccine as well as an ability to carry out consistent quality control. One batch of the tested vaccine held at 2-5°C and re-calibrated or reviewed at intervals of two years might then be reserved for use as a "manufacturer's working standard".

In such a procedure the need might well be no more than a demonstration that a test vaccine induced no less antitoxin in a group of animals or protected no fewer animals from challenge than an appropriate dilution of the manufacturer's working standard vaccine.

Only in the event of the five batches of DPT having potencies of three times the required level, 90 I.U. per human dose in the case of diphtheria vaccine and 120 I.U.* per human dose in the case of tetanus vaccine may a simpler test be used. Such a test should be adapted to the facilities of the organization undertaking it and not linked indissolubly to any particular method of measuring neutralizing antibodies or challenge, although for humane reasons lethal challenge should be avoided.

2. PROBLEMS IN THE ESTABLISHMENT OF THE INTERNATIONAL STANDARDS FOR DIPHtheria AND TETANUS ADSORBED TOXOIDS

The biological standardization of diphtheria and tetanus toxoid vaccines have always caused heavy debate, and most of the details are still unresolved.

The theoretical basis for biological potency assays as outlined (e.g. by Nils Jerne) has never been complied with since the compositions of these vaccines have never been so well controlled that, for example, the fundamental claim for dilution assays could be fulfilled. Early in the history of toxoid vaccine production it was shown that some impurities, that one strived to remove, had a significant influence on the biological activity of the vaccines. Furthermore different adjuvants have been used by different manufacturers. Some use vaccines precipitated by aluminium phosphate, some use toxoid adsorbed onto aluminium hydroxide gels, and some use calcium phosphate. Some toxoid vaccines are commonly combined with other vaccines, e.g. pertussis vaccine, which in itself exerts an adjuvant effect.

The last addition to the list of problems is the observation that even toxoids of comparable degree of purification and containing similar adjuvants are heterogeneous with regard to components affecting the potency assay results.

These differences between vaccines might show up in different ways. Some come to light by different slopes of dose-response lines and some by giving different relative potencies between preparations when different assay systems are used.

In the collaborative study preceding the establishment of the second international standard it was observed that the relative potency of the two preparations depended on the species of animals used for the potency assays. Similar observations were made by several investigators.

Such observations suggest that differences exist between strains of animals and also between different toxoid products which might otherwise be thought to be fairly comparable with regard to composition. The availability of good quality animals is essential for any bioassay. Moreover the use of large numbers of guinea pigs is not possible in many countries and even the supply of mice of good quality is often a severe constraint.

This situation therefore mitigates against recommending a single potency assay but it is important and essential to have some sort of biological potency assay of such vaccines. Both the toxoid components and the adjuvant components of the vaccines are too ill defined to be described and controlled by simple means.

It is anticipated that by means of modern techniques it may eventually be possible to obtain a characterization of the toxoids sufficient to reduce the need for a full scale potency assay. This stage has however not yet been achieved and therefore it is important to continue to define reasonable Requirements that ensure safe and effective vaccines.

* In the case of a vaccine without a pertussis component the potencies of the diphtheria component must be at least 75 I.U. per human dose, of the tetanus component at least 100 I.U. per human dose.

3. THE NEED FOR AN INTERNATIONAL STANDARD FOR DPT (ADSORBED)

The need for an international standard for the potency assay of each of the three components of combined (diphtheria, pertussis and tetanus (DPT))vaccine containing an adjuvant was discussed. The majority of these vaccines are given in combined forms and there are ample data from several authors to demonstrate that there is interaction when the three components are mixed. In view of the observations that the potencies of the components may be different when assayed in guinea pigs and mice against monovalent standards, the group agreed that it would be important to have a more appropriate standard. Of particular relevance was the need to have the potency of the tetanus assayed in mice because this is the animal in most common use for the test.

In a lengthy discussion of the need for, and the tests to be used in, the calibration of such a standard in an international collaborative study the following points were agreed:

3.1 The vaccine: This should be as close as possible in the contents of its components to a vaccine commonly used and should be freeze dried in ampoules.

Dr Böszörményi offered to provide a batch of vaccine already dried in Hungary and containing 100 Lf tetanus, 300 Lf diphtheria, 40 I.U. pertussis and 33.5 mg of aluminium phosphate per ampoule of ten human doses. This vaccine was freeze dried, the ampoules filled with dry nitrogen and sealed by the fusion of glass. The moisture content was less than 2%.

There were about 4000 ampoules, 500 of which would be reserved for clinical evaluation and testing in an international collaborative assay as a proposed international standard for DPT (Ads.).

3.2 The animals: The tests for the potency of the T and P components are traditionally done in mice and these tests will be retained. Some countries are already using mice for the potency testing of diphtheria toxoid by measurement of specific antitoxin.

A single injection will be given and the mice challenged 14-17 days later for pertussis. Further groups of mice will be challenged or bled after suitable intervals for estimation of response to the diphtheria and tetanus components.

Mice give a wider range of responses to tetanus toxoid and different strains of mice may also give different results; for this reason the Second International Standard for Tetanus Vaccine, Adsorbed, was assigned a potency by comparison with the first International Standard by potency assays in guinea pigs. Nevertheless, it is important now to assign a potency for the tetanus component in the proposed DPT (Ads.) standard in mice.

3.3 Measurement of responses. There are two ways in which the responses to vaccine may be measured: antibody response or protection against a lethal challenge.

- (a) Diphtheria antitoxin will be determined by the antibody level in cell cultures sensitive to diphtheria toxin. Details of such a suitable test for VERO cells will be distributed for the agreement of all participants in an international collaborative assay. In addition, individual laboratories may also employ the method that they use routinely in order that comparisons may be made. Many laboratories may wish to use the method suggested in the WHO Requirements (WHO Technical Report Series No. 638, 1979, p.49).
- (b) Pertussis protection will be measured by the intracerebral challenge test as suggested in the WHO Requirements (WHO Technical Report Series No. 638, 1979, p.69).
- (c) Tetanus will be measured by a challenge test of the mice.

Some laboratories may wish to measure the antibody response by the inoculation of toxin-antitoxin mixtures in mice. Details of the collaborative study will be distributed for the agreement of all participants.

3.4 Reference preparations: the reference preparations to be used for the comparison of the assay will be:

- (i) the Second International Standard for Diphtheria Toxoid, Adsorbed;
- (ii) the Second International Standard for Pertussis Vaccine;
- (iii) the Second International Standard for Tetanus Toxoid, Adsorbed;
- (iv) another tetanus toxoid, adsorbed, that has been subjected to an international collaborative assay.

It was suggested that a mixture of the first three standards might be made but there were several reasons why this should not be done, not the least of which was the different adjuvant content in the different standards. The Second International Standard for Tetanus was adsorbed on aluminium hydroxide which may not give the same adjuvant effect as aluminium phosphate.

In a determined attempt towards a more meaningful control of potency of DPT (Ads.) vaccine this international collaborative assay should be completed as soon as possible. The proposed tests to be investigated are shown in Annex II.

4. FUTURE TESTS FOR THE ESTIMATION OF ANTITOXIN LEVELS IN SERA

For the prevention of toxin mediated diseases such as tetanus and diphtheria the presence of antitoxin following immunization is considered essential. Antitoxin may be detected in vivo by direct challenge of animals with a suitable toxin or by an assay of sera obtained from animals or man. Classically antitoxins have been measured by toxin neutralization assay by mixing serum with test levels of toxin and injecting the mixture into animals. For tetanus the lethal or paralytic effects of the toxin are prevented by antitoxin; for diphtheria the lethal or skin necrotizing effects are prevented by antitoxin. Suitable in vitro toxin neutralization assays of sera from immunized animals, including mice, have been developed in cell cultures for the evaluation of diphtheria antitoxin but no suitable cell culture is available for the routine in vitro assay of tetanus antitoxin.

Several other in vitro methods for the titration of tetanus and diphtheria antibodies have been described. These include haemagglutination, radioimmuno-assay (RIA), a variety of different types of immunodiffusion assays and more recently enzyme-linked immunosorbent assay (ELISA). These assays measure both toxin neutralizing and other non-neutralizing antibodies. At the present time the degree of correlation of levels of antibodies measured in these in vitro assays with antibodies detected in toxin neutralization assays is not considered acceptable for the measurement of antibodies in sera obtained from animals being utilized in evaluation of the potency of tetanus or diphtheria toxoids.

As highly defined antigens bearing the epitopes necessary for the induction of neutralizing antitoxin are developed for use in immunization it may be possible to develop more acceptable in vitro assays must be capable of detecting sites that recognize toxin neutralizing antibodies. The use of various modified binding assays such as ELISA unique monoclonal antibodies capable of interaction with appropriate recognition sites or the development of new cell cultures or broken cell preparations for the evaluation of toxins may be expected in the future. The definition of the mechanism of action of tetanus toxin can be expected to facilitate the development of in vitro methods for the assay of this toxin and the findings may be appropriate for the further characterization of other toxins.

5. CHANGES PROPOSED FOR THE CURRENT REQUIREMENTS

In the light of the discussions of this consultation a number of changes were proposed to the WHO Requirements for Diphtheria, Pertussis and Tetanus and Combined Vaccines (WHO Technical Report Series, No. 638, 1979) and will be placed before the Expert Committee on Biological Standardization (June 1984). It must be emphasized that there was no attempt to review all the Requirements contained in this document. A full review and reformulation of the Requirements will be made when data on the collaborative assay proposed at this meeting have been analyzed

and agreement has been reached on new potency assay tests should these prove feasible.

A summary of the tests that should be investigated is shown in Annex II.

6. RECOMMENDATIONS

The group agreed that many data on potency assays were available and an analysis of these might give important leads to a test of the future for each of the toxoids.

- i) The data on individual responses of guinea pigs used in the assay of diphtheria toxoid should be assembled and statistically analyzed.
- ii) An international collaborative assay of potency of each of the three components of a DPT (Ads.) vaccine should be carried out as soon as possible.
- iii) The responsiveness of strains of mice used in such potency assays especially with respect to the coefficient b of the response curve should be investigated.
- iv) The use of specifically characterized monoclonal antibodies both for the identification of antigens and the development of suitable in vitro quantitative assays of antitoxin should be encouraged.
- v) The investigation of a mouse model for the estimation of potency of the diphtheria toxoid component of vaccines should be studied.
- vi) The substitution of graded response assays as opposed to quantal assays in order to retain precision using a smaller number of animals should be pursued.
- vii) Suitable confirmatory test assays should be investigated.
- viii) Efforts to identify the mechanism of action of tetanus toxin should be encouraged in order to arrive at a meaningful in vitro assay for tetanus antitoxin.

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SUMMARY OF METHODS TO BE INVESTIGATED IN A COLLABORATIVE ASSAY
OF DPT (ADS.) STANDARD

(a)	<u>Study methods</u>	Species of animal	Route of injection	Interval between vaccination and evaluation (days)	Method of Evaluation	Standard used
	Diphtheria	mice	sub-cutaneous	35	Titration of sera in VERO cells or rabbit skin	2nd International Standard for Diphtheria (Ads.)
	Tetanus	mice	sub-cutaneous	28	Challenge sub-cutaneous with toxin	2nd International Standard for Tetanus (Ads.) and another International preparation
	Pertussis	mice	intra-peritoneal	14	Challenge intracerebrally with B.pertussis	2nd International Standard for Pertussis Vaccine
(b)	<u>Optical methods</u>					
	Diphtheria	guinea pigs	sub-cutaneous	28	Subcutaneous challenge or intradermal challenge or multiple intradermal challenge	2nd International Standard for Diphtheria (Ads.)
	Tetanus	guinea pigs	sub-cutaneous	28	Subcutaneous challenge or titration of sera in mice	2nd International Standard for Tetanus Toxoid (Ads.) and another International preparation