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DEVELOPMENTS IN DIPHTHERIA PROPHYLAXIS

BY

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DEVELOPMENTS IN DIPHTHERIA PROPHYLAXIS

FOREWORD

The work described in the following pages was commenced in 1940. It soon became apparent that the problem most urgently in need of elucidation was to discover the reason for the variation in the physiological activity (*in vivo* potency) of similar and different types of diphtheria prophylactic.

In order to do this it was considered necessary firstly, to learn as precisely as possible the composition of the reagents used, and then to employ pure or highly purified materials. Even when good quality reagents were developed, the actual technique for reliable quantitative animal work had to be evolved. This latter problem proved to be one of first importance, once it was found that the response to an initial inoculation of nearly every form of antigen gave a secondary response effect superimposed on the primary. Therefore, unless allowance was made for this, quite misleading conclusions could be drawn from animal results which, statistically, appeared perfectly sound.

Since it has taken over two years to write this book, and research continues unceasingly, it will be noticed that as new evidence accrued, sometimes a re-orientation of ideas became necessary.

It is felt that a more genuine presentation of the work done demands that the older incorrect ideas should be left in their place, rather than rewrite the book in the light of the most recent findings. The known misconceptions are, of course, indicated by reference to the work which corrected them.

The initial ground-work described in this book, has led to a new interpretation of the mechanism of the physiological activity of adsorbed diphtheria toxoid, when used as a primary stimulus. These new ideas have provided a theoretical basis for further studies on the quantitative aspect of antitoxin production.

October 1948. L. B. H.

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I wish to express my deepest appreciation of the unfailing encouragement in this work given by the late Sir Almroth E. Wright, and to Sir Alexander Fleming; work, which in its early stages seemed to present almost insoluble difficulties and contradictions. Many of these difficulties were, of course, not real, but due to the misuse of words and technical expressions. These latter, although intended only to describe phenomena, contained in their use an interpretation of the phenomena—which was unjustified and often misleading. I owe to Sir Almroth Wright a great debt for his efforts to train me in the correct use of words for describing scientific observations.

Grateful acknowledgments are also due to my former assistants Dr. W. C. Evans, and Dr. N. Walker, and to Mr. G. Clarke for their assistance in this work; and to Dr. R. R. Wilson of our Department of Morbid Anatomy for the histological sections and photomicrographs.

I am also indebted to Sir Percival Hartley, F.R.S., for much help in certain historical details.

The debt due to Dr. Guy Bousfield, is evident from the account of his clinical investigations recorded in this book.

I have to thank the Council of the Wright-Fleming Institute of Microbiology for their assistance in the publication of this book.

INTRODUCTION

The aetiology of the disease of children, which was at one time termed ' croupous ' or ' diphtheritic ' sore throat (Virchow 1847) (1), was elucidated almost simultaneously by Klebs in 1883 (2) and by Löffler in 1884 (3).

The presence of the bacilli in the necrotic mass in the pharynx, and rarely to be found elsewhere, gave rise to speculation especially as at the same time no definite explanation could be put forward for the paralysis of soft palate, neck, etc. or for the more remote pathological conditions in the heart-muscle and adrenal glands, which were so commonly associated with the disease.

Roux and Yersin in a series of papers (1888–1890), (4*a*, *b*, *c*), shewed that microbe-free culture filtrates of Klebs-Löffler bacilli, when injected into guinea-pigs, gave rise to two kinds of pathological condition according to the amount of filtrate or ' diphtheria toxin ' injected. If the dose was such as to cause death of the animal in 3–5 days, postmortem examination generally revealed considerable pleural and peritoneal exudation, often with haemorrhages, necrosis of tissues and markedly haemorrhagic adrenal glands. With smaller doses, however, the animals might live for as long as three weeks, apparently quite well, and then abruptly develop paralysis of the legs and rapidly succumb. On post-mortem examination no gross pathological lesions could be found. Thus Roux and Yersin established the link between the localised infection and remote lesions which are evidently caused by toxins elaborated by the microbes at the seat of infection and distributed in solution throughout the body by the bloodstream.

Late in 1890 von Behring (5) showed that the serum of animals which had received sublethal doses of diphtheria toxin, acquired the capacity of specifically neutralising the poisonous action of toxic culture filtrates ; in other words, that the serum was antitoxic. This important discovery led to the development of antitoxin or serum therapy in children, which began a year later in von Bergmann's clinic in Berlin on Christmas night 1891 (6).

A measure for diphtheria toxin and antitoxin—the ' unit '—was meticulously worked out by Ehrlich (1897) (7), and has since been adopted as the ' standard unit ' throughout the world.[1]

Diphtheria antitoxin—i.e. horse-serum antitoxin—has saved the lives of hundreds of thousands of children ; yet this antitoxin, even in large doses, has not always achieved the results expected and from time to time has failed, particularly in the severe or late stages of the disease. The reasons for these failures soon became clear and in the main were due to the fact that it is almost impossible to ' unfix ' toxin once it has been attached to the tissues. In this connection Ramon, Debre and Uhry (8) shewed that partially detoxified toxin caused delayed paralysis in

[1] Since diphtheria toxin is unstable on storage, the ' unit ' of toxin was found best to be equated to a given weight of dried *antitoxin*—which is stable. For this reason the standard, against which may be measured samples of toxin, toxoid, and antitoxin, is a sample of dried antitoxin, which is periodically dispensed, in solution, by the Licensing Authorities of the different countries accepting that standard.

animals despite the presence of antitoxin which the animals were known to form in the blood as a result of the injection of the mixture. Again many workers have demonstrated the great speed with which the toxin is fixed by the tissues. Thus it has been shewn (9. *a. b.*) that when rabbits were given 10 minimal lethal doses of toxin subcutaneously, and followed by antitoxin intravenously, the minimum amount of antitoxin to effect survival at different time-intervals was as follows :

After	10	Minutes	5 units
,,	20	,,	200 ,,
,,	30	,,	2,000 ,,
,,	60	,,	5,000 ,,
,,	90	,,	no amount.

The significance of these results was the importance of administering antitoxin as early in the disease as possible, a point strongly confirmed by physicians. It follows, therefore, from all the foregoing that if the blood contained antitoxin *before* contracting the disease, potential damage by diphtheria toxin could be completely avoided, even the disease abolished as a clinical entity. The obstacle to this ideal was the impracticability of injecting diphtheria toxin into young children owing to the serious risks involved. For a time a mixture of diphtheria toxin and horse antitoxin—almost neutralised—was employed initially by Theobald Smith (10) for immunising horses and later by von Behring for children (11). A large number of children were thus successfully immunised but the attendant risks in using such a mixture eventually materialised, and led to this procedure being discontinued.

It had long been known that various chemicals such as iodine and formalin reduced or destroyed the toxicity of diphtheria toxin.[1] Moreover, in 1922, Ramon (15 *a*, *b*, *c*) noted that when toxin is mixed with antitoxin it eventually gave rise to a floccular precipitate. This observation was of great practical value to him later when, using antitoxin of known titre, he shewed that of a series of such mixtures the first to flocculate was the *in vivo* equivalent of the other component. Such was the origin of the flocculation technique for assaying toxin, toxoid or antitoxin. It provided a valuable method for measuring the total antigen present in fully or partially detoxified culture filtrates.[2]

Diphtheria toxoid is a safe prophylactic for human use, and Park and coworkers (17) in New York were among the first to use it on a large scale in children with strikingly successful results. They carried out three spaced inoculations.

Now, to test the antitoxic power of the serum of animals either by the flocculation method or by animal assay (using toxin) presents no difficulty as animals are easily bled. But this is not so easy, or practicable, for the test of immunity to diphtheria

[1] Lowenstein, in 1909 (12), and Lowenstein and von Eisler, in 1912 (13), showed that the addition of small quantities of formalin to tetanus toxin, resulted, after four weeks incubation, in a *completely atoxic but specifically antigenic* mixture. In 1923 Ramon (14, *a*, *b*,) successfully extended this to toxigenic culture filtrates of C. diphtheriæ. Lowenstein called his atoxic material ' toxoid ' and Ramon ' anatoxine '.

[2] This valuable finding had been remarkably foreshadowed in a paper by Danysz in 1902 (16), ' Contribution à l'étude des propriétés et de la nature du mélange des toxines avec leur antitoxine ' in which it says ' principle de la précipitation de la ricine par l'antiricine, vitesse de formation du précipité, détermination de l'optimum des proportions dans lesquelles les deux substances mélangées ensemble se fixent et donnent le plus rapidement le précipité le plus volumineux. Dans le tube où les proportions sont égales, le mélange est neutre '.

in children, especially when required to be carried out on a large scale. Prof. Schick, in 1908 (18), attempted to solve the problem in children by intracutaneous injections of very small quantities of diphtheria toxin. No skin reaction followed if the child possessed a sufficient amount of antitoxin but when this was deficient, a reaction, in the form of a red area, 1 to 3 cm. in diameter, appeared at the point of injection in the course of 24–72 hours. The red area usually faded after 7 days, but might leave a darkish area of desquamating skin. This test was eventually standardised and the most stringent regulations applied regarding the toxic properties of the Schick Test Toxin. Children are now Schick tested before and after a course of immunisation, and the measure of immunity conferred is expressed by the Schick conversion rate (S.C.R.) that has been accomplished. In this respect it is a valuable procedure.

The minimal blood level of antitoxin to give a Schick negative result—the dose of toxin being 1/1000 unit injected—is probably in the neighbourhood of 1/250 unit of antitoxin per ml. of serum. (Glenny and Waddington 1929). (19). A reaction to the Schick test dose of toxin occasionally occurs in children, and much more frequently in adolescents and adults which is not due to the absence of sufficient circulating antitoxin and is presumably not caused by the specific antigen, (pseudo-positive reaction), but by some other constituent of the toxin solution derived from the microbe. It is a form of sensitivity which may give rise to untoward reactions in adults when formal-toxoid or A.P.T. is used as a prophylactic, but which can be circumvented by the use of washed toxoid-antitoxin floccules (20).

In 1926, Glenny, Pope, Waddington and Wallace (21) published work on the enhanced antigenic power of diphtheria toxoid in horses, when administered as alum precipitated toxoid (A.P.T.) but this was quite unsuitable for children, owing to severe local reactions. Glenny and Barr (22) shewed later that by careful washing of this precipitate much of its irritant properties were removed and it could, therefore, conveniently be used for children. Since then A.P.T. has become the reagent of choice for the immunisation of children against diphtheria. Alum precipitated toxoid, although considerably more powerful than formal toxoid as an immunising agent, often varies in potency, some preparations being many times more potent than others (23). This may occur even when the preparation contains the same quantity of antigen and is prepared by identical methods. Such obscure variations, affecting the problem of diphtheria prophylaxis, constituted a source of deep concern to the Government, physicians and manufacturers alike. In 1940, the Ministry of Health and the manufacturers agreed on a provisional minimum standard of quantitative and qualitative properties for A.P.T. being adopted for prophylaxis. But the real problem—the lack of accurate knowledge of those factors which governed potency in different samples of A.P.T.—remained. Nor was the exact composition of A.P.T. known in terms of mineral constituents, and the form, or forms, in which the antigen was present in the precipitate.

The need for a reliable and if possible accurately reproducible prophylactic of high potency and stability was felt by all ; a method for the preparation of such a compound was urgently called for ; this latter would serve also as the standard for comparison with the product of other methods.

The work recorded in this book includes the description of a method for preparing

to a precise specification a reproducible diphtheria prophylactic. Also evidence is set out to account for the enhanced potency of A.P.T. over its parent formol-toxoid, which is not in accord with current theory.

It is not claimed that the methods described are the only ones that could be employed to make such a preparation.

The view taken in this investigation was that, for the preparation of the best prophylactic, knowledge of the antibody mechanism in animals is essential. This has received special attention, and the results obtained embody the simultaneous investigation of the immuno-physiology of the guinea-pig, as well as the physical and biochemical problems involved in the preparation of the antigen.

It has been suggested that the use of purified toxoid as the sole antigen for diphtheria prophylaxis is immunologically unsound, as the child is thereby deprived of valuable antibacterial immunity. This argument is correct theoretically, but implies that the non-toxin antigens in modern toxin culture filtrates are useful. Since the strains of C. diphtheriae used to-day for toxin production have been subcultivated selectively for toxin production for over thirty years, it would seem most unwise to rely on them as a source of material for antibacterial immunity. It would be much better to incorporate into the prophylactic a vaccine of recent isolation, for example a virulent Gravis strain shewn to have useful properties.

On the other hand it has recently been shewn by Pappenheimer and Lawrence (95) that the allergic reactions to diphtherial protein may be towards the specific antigen only, towards the non-toxoid protein only, or to both, being most marked in respect of sensitivity towards the non-toxoid protein in Schick positive subjects. The use of highly purified toxoid therefore reduces the risk and severity of reactions when used for large-scale immunisation.

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SECTION 1.0.0.

PROBLEMS INVOLVED IN THE PREPARATION OF DIPHTHERIA PROPHYLACTIC

SECTION 1.1.0.

PREPARATION OF THE ANTIGEN

Section 1.1.1. Toxin Production

Preliminary remarks

In recent years appreciable progress has been made in our knowledge of the nutritional requirements of bacteria. Perhaps the most outstanding advance being the finding that many exacting microbes will grow on simple, chemically defined media. That is, media composed entirely of amino acids, salts, and the appropriate growth factors. These latter ' bacterial vitamins ' are often identical with those required by animals.

The value of investigations carried out in the past using natural amino acids has suffered from the fact that natural amino acid preparations may be contaminated with biologically significant amounts of other amino acids (Fildes) and thus invalidate or make uncertain some of the conclusions reached. For growth factor work, however, acid hydrolysed proteins have—with some measure of reservation, been successfully employed.

An excellent review on the subject of bacterial nutrition has been made by Knight (24) where a detailed account of this type of investigation is made.

One result of the work done on purely synthetic and semi-synthetic media in respect of C. Diphtheriæ has been :

(*a*) the finding of three essential growth factors,
- (i) pimelic acid,
- (ii) nicotinic acid and
- (iii) B-alanine or pantothenic acid ;

(*b*) the complete disproof of the old contention that peptone or proteoses are essential for toxin production.

It has not been until comparatively recently, however, that semi-synthetic media—using acid hydrolysed casein, sugar, salts, and pure growth factors, have reached the stage of being practicable for the successful large-scale production of diphtheria toxin.

The media used up to the time of introduction of efficient acid hydrolysed protein media were usually tryptic digests of some form of meat. And recently these latter have been improved so that compared with earlier methods, far better toxin yields are obtained.

Some figures on this point are as follows :

Date	By	Medium	% N_2	Toxin Titre (Lf.)	Yield, Lf./mgm. Medium N_2
1922	(93) Hartley	Douglas' Digest Broth	0·35–0·4	3–10	1–3
1931	(26) Glenny and Barr	Tryptic digest of Meat	0·35	19–21	6
1939	(27) Linggood and Pope	Weak tryptic digest of Meat, etc.	0·2–0·24	50	25

Since 1931 considerable progress has been made in determining the factors which govern the production of high titre diphtheria toxin.

Two are of particular importance :

(*a*) the most suitable carbohydrate being maltose, (28) and
(*b*) of great importance, the *iron* content of the medium.

This latter was first shewn by Pappenheimer and Johnson in 1936 (29) using digest and synthetic media, and later confirmed by Mueller in 1939 (30) using a semi-synthetic medium. Inorganic iron appears to be essential for growth of C. diphtheriæ. Excessive amounts do not interfere with growth, but there is an optimum concentration at which maximum titres of toxin are obtained. This amount is about 12γ of Fe per 100 ml. of medium, or 60γ $FeSO_4$ $7H_2O$. Twice the optimal amount may reduce the toxin titre by about 33 per cent.

At the present time, the Ministry of Health lays particular emphasis on the quality and titre of crude toxin, intended for conversion to toxoid for human use in the form of alum precipitated toxoid, (A.P.T.). The toxins are required to be of the highest possible quality, and preferably of a titre of not less than Lf. 50. Also, notice is taken of the final product in terms of the purity of the finished A.P.T., when expressed as the amount of nitrogen present per flocculating unit of antigen—Lf./mgm. N_2.

The term ' quality ' applied to toxins may have several meanings. The word ' toxin ' is used commonly to mean ' toxic culture filtrate solution ', whereas the small amount of specific poison only is of major interest, although the remaining constituents of the culture filtrate can play an important part in the behaviour of the specific antigen when processed. Thus the word ' quality ' can apply to the several properties of the specific toxin, or to the solution as a whole. Usually the word is meant to cover such properties of the specific protein as toxicity, flocculation rate and titre, and sometimes antigenicity. The less commonly recognised ' quality ' of a toxic culture is the ratio of toxin to the total nitrogen present. This latter aspect has two facets :

(*a*) the unused medium nitrogen remaining, and
(*b*) the protein nitrogen other than toxin resulting from bacterial metabolism and possibly autolysis.

On this last point, Pope (27) has warned against the use of too rich a medium, for when this is used, the growth of the pellicle is heavier than can be supported by the surface tension of the medium, and it falls or 'curtains'; and he notes that the curtains probably add—by autolysis—to the amount of bacterial substances present in the culture filtrate. The bacillary body-substances may be responsible for the disturbing local reactions produced in subjects over 6–8 years of age, when they are injected with A.P.T. (31).

From these considerations it seemed rational to use a medium composed of the simplest possible components, i.e., one with a minimum of, or even no, nitrogenous molecules similar in size or properties to diphtheria toxin. And this, apart from the risk run when such tryptic digest media are employed, that subjects will be injected with peptone and polypeptide derivatives of the medium to which they are unusually sensitive (32 *a* and *b*).

Mueller in 1939 (30) after several years of brilliant systematic work on the nutritional requirements of C. diphtheriae published his first formula for the preparation of a semi-synthetic medium which yielded high titre toxin. The source of nitrogen was HCl-hydrolysed casein, i.e. amino acids, augmented with crystalline growth factors, salts and maltose, and a titre of Lf. 62 was obtained. This medium was in sharp contrast to any form of digest medium in that the constituents were simple and largely known, and no component was in any way comparable to Diphtheria toxin—a protein whose molecular weight is probably 72,000 (33).

Preparation of semi-synthetic Media for Diphtheria Toxin Production. Historical

In 1936 Pappenheimer and Johnson (34) evolved a medium for the growth of toxigenic diphtheria bacilli in which the source of nitrogen was acid hydrolysed gelatine.

In the same year Pappenheimer (35) obtained a titre of Lf. 20, in an almost completely synthetic medium composed of ten amino acids, mineral salts, maltose, and an extract of liver as a source of growth factors. The total nitrogen content of this medium was 0·087 per cent., which gives a toxin yield of 23 Lf./mgm. medium nitrogen.

In 1937 Pappenheimer, Mueller and Cohen (36) prepared a second, and purely synthetic medium composed of only seven amino acids, but in place of the liver extract crystalline growth factors were employed, namely nicotinic acid, pimelic acid, and B-alanine. In this medium a titre of Lf. 36 was obtained, the iron content being carefully controlled. The total nitrogen content of this medium was 0·082 per cent., giving a toxin yield of 44 Lf./mgm. medium nitrogen. The authors comment on the high titre obtained in respect of the total nitrogen present in the medium.

In 1939 Mueller (30) published his first formula for the production of a semi-synthetic medium using acid hydrolysed casein, mineral salts, crystalline growth factors and maltose. A toxin titre of Lf. 62 was obtained on this medium. The medium contained about 0·27 per cent. nitrogen, and thus gave a toxin yield of about 23 Lf./mgm. nitrogen. The iron content of this medium was rigorously controlled.

Early in 1941 (37) Mueller and Miller shewed that a limiting factor for toxin production in the earlier medium, when the total nitrogen content was 0·27 per cent.,

was the high concentration of NaCl present. The origin of the NaCl was the HCl forming amino-acid hydrochlorides in the HCl hydrolysis concentrates. This HCl cannot be distilled off, and eventually is neutralised by NaOH to become common salt.

An examination of the known amino-acid composition of casein shews that the total NaCl equivalent of the amino-nitrogen contained in these amino-acids is about 54 gms. per 100 gms. of casein, but the amino-acid analysis of casein is as yet incomplete. If all the nitrogen in casein were amino-nitrogen, the NaCl equivalent of 100 gms. of casein would be 65 gms.

As 0·27 per cent. total nitrogen is approximately equivalent to 1·7 per cent. casein, the NaCl equivalent of 1·7 per cent. casein is at least 1·7 per cent. of 54 gms., namely 0·92 per cent. In practice not all the free HCl can be distilled off, and moreover not all the nitrogen in casein has yet been accounted for. These two factors probably account for the concentration of more than 1 per cent. NaCl in the finished medium when 0·27 per cent. nitrogen is present.

Muller *et al.* (second formula) (38) overcame this high salt content in two ways :

(1) by removing most of the amino-acid HCl from the casein hydrolysate with lithage, and subsequently removing the soluble lead chloride with BaS, and :

(2) hydrolysing the casein with a mixture of sulphuric acid and HCl, the latter alone insufficient in amount to combine with all the amino-nitrogen produced. This procedure is stated to require 72 hours hydrolysis, and the removal of the sulphuric acid with $Ba(OH)_2$ is troublesome.

Using either method to avoid the excess NaCl, Mueller obtained a medium containing 0·27 per cent. N_2 and about 0·5 NaCl. Mueller states that 0·5 per cent. NaCl is about optimal for toxin production.

With this medium Mueller obtained the very high toxin titre of Lf. 100. The yield of this medium was therefore 37 Lf./mgm. of medium nitrogen, a considerable improvement.

For some time Mueller's first formula medium was used in these laboratories with consistent results. The only variation employed was to use a slightly lower concentration of casein nitrogen in the medium, namely 0·2 – 0·22 per cent. N_2. A titre of Lf. 62 was obtained, and a considerable quantity of toxoid was prepared from toxin made in this medium.

Experimental

Unless otherwise stated C. diphtheriæ P.W.8, Strain ' Toronto ' was used, and cultures incubated at 34° C. for seven days.

One of the earliest experiments carried out with Mueller's medium, (first formula) was to determine the limiting factor(s) for growth. One factor was shewn to be the source of nitrogen, namely the amino-acid mixture. This was done by adding to samples of a ten day culture filtrate, small quantities of the several components of the medium ; re-inoculating and noting growth. Furthermore, using impure natural amino-acids, the addition of small amounts of glycine, glutamic acid, aspartic acid and leucine, all permitted further growth. An observation of greater significance was that if any of these amino-acids were added to Mueller's medium of low strength